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Chemical and enzymatic reductive activation of acylfulvene to isomeric cytotoxic reactive intermediates

Kathryn E. Pietsch[†], **James F. Neels**[‡],

Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, Minnesota 55455

Xiang Yu[§], **Jiachang Gong**[‡], and

Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, Minneapolis, Minnesota 55455

Shana J. Sturla[*,†]

[†]Institute of Food, Nutrition and Health, Laboratory of Food and Nutrition Toxicology, ETH Zurich, 8092 Zurich, Switzerland [§]Cancer Cell and Biology Branch, National Cancer Institute, National Institute of Health, Bethesda, MD 20814

Abstract

Acylfulvenes, a class of semisynthetic analogues of the sesquiterpene natural product illudin S, are cytotoxic towards 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 illustrates 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 analog 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.

[*]sturlas@ethz.ch .
[‡]Current address: Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 08543

Supporting Information Available. 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.

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[1]), are converted to reactive chemical species by preferential enzymatic activation in target cells.[1, 2] AFs are semi-synthetic derivatives of the natural products illudin M and S (Chart 1), sesquiterpenes produced by the mushroom *Omphalotus olearius*.[3, 4, 5, 6, 7, 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, 16, 17, 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 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 analog 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-

[1]AFs, acylfulvenes; PTGR1, prostaglandin reductase 1; AOR, alkenal/one oxidoreductase; HMAF, hydroxymethylacylfulvene; AF, acylfulvene; 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; 4,4′-d_2-NADPH, NADPD; NTH, neutral thermal hydrolysis.

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), sodium borodeuteride ($NaBD_4$) (90–95% purity) were purchased from Cambridge Isotope Labs, 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. Pyridine was dried with an 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 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. ^1H NMR spectra for both compounds matched the published spectra. On the basis of ^1H NMR the isotopic purity of NADPD was >90%. The reactivity of the labeled co-factor was confirmed by reaction with benzylidene acetone.[21]

Instrumentation and apparatus

1D ^1H 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. 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 × 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; activation time, 30 msec. 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, 7 points). Samples were analyzed in duplicate and data reported is the average of duplicate measurements.

(−)-Acylfulvene

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 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), 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 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 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.[2] The reaction was quenched by adding 3 mL 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, Quebec City, Quebec, Canada) 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: **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.

[2]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 minutes). 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.

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 THF. The reaction was allowed to stir for 24 h. **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 THF. The mixture was allowed to stir for 19 h. **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 acetone-H_2O (1:1 v/v). Zinc dust (160 mg, 2.5 mmol) and 0.75 mL 10% aqueous H_2SO_4 (v/v) was 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) were 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 waterbath 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 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 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) were 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 waterbath 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) were combined and diluted to a final volume of 624 μL with H_2O. The reactions were incubated in a 37 °C waterbath, 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 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 MeOH and analyzed by HPLC-ESI-MS/MS as described above.

Plasmid construction

Human PTGR1 (hPTGR1) was 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. PCR product was cleaned and ligated into pBlueScript between *Eco*RI and *Xho*I sites. DNA insert was confirmed by sequencing and subcloned into episomal vector pCEP4.

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 hour 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 Corporation, 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 pre-acquisition delay experiment, taking 4 scans every 5 minutes for 12 hours. 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), 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, i.e. 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), 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.

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. Amongst 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, ^{13}C NMR in Figure S3), 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 1, panel C). 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). The ^{1}H NMR doublet at δ 0.95 ppm, associated with the C4 proton of **5** (Figure 1, panel C), collapses to a singlet in the case of the deuterated analogue **6** (Figure 1, panel D). 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 1, panel A). In **6** the q_{AB} signal corresponding to the same protons is also at δ 3.13–3.36 ppm (Figure 1, panel B). 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 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, i.e. **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 minutes for 12 hours. Chemical shifts were referenced 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 displays representative data. The observed rate constant k_{obs} was calculated on the basis of the well-distinguished methyl groups highlighted in Figure S6B. Figure S6C 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 timescale similar to AF toxicity and DNA reactivity. Further, 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 thirty days, at which time no changes could be observed in the spectrum.

Reactions of 5 with nucleosides and ctDNA

In order 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-deoxyadenosine (3-AF-dAdo) and 7-AF-deoxyguanosine (7-AF-dGuo), formed by AF in the presence of PTGR1 and NADPH, are thermally unstable and depurinate during neutral thermal hydrolysis[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. **5** was reacted with dAdo, dGuo, or ctDNA in aqueous solution (pH 7) at 37 °C for 24 hours. 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 methanol 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, 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 were confirmed by co-injection 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 3A, panel i) and *m/z* 152 (Figure 3A, panel ii), 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 doesn't 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 and 3D) match 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 4A, panels i and 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, 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. 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

In order 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 pre-activated 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 hours. 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, Figure S7) 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 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 test cells. Thus, the 3-fold difference is relatively small 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, i.e. cells are similarly sensitive to the compound even with large differences in enzyme levels (Table 1). These data, together with 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, 32, 33, 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. Further, 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 $NaBH_4$.

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, i.e. 1,3-diaxial to one another (Figure S8A). 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). 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.

Since 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 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 in order 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 non-covalently associating with DNA, possibly intercalating within the duplex, prior to alkylation. Analogous non-covalent pre-associations have been suggested for a number of alkylating agents, such as aflatoxin-B,[39] CC-1065,[40] 1 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 suggests 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, compared to the 20- to 100-fold-differences of drug potencies for AF or 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Boger DL, Garbaccio RM. Shape-dependent catalysis: insights into the source of catalysis for the CC-1065 and duocarmycin DNA alkylation reaction. Acc. Chem. Res. 1999; 32:1043–1052.
2. Lin AJ, Cosby LA, Shansky CW, Sartorelli AC. Potential bioreductive alkylating agents. 1. Benzoquinone derivatives. J. Med. Chem. 1972; 15:1247–1252. [PubMed: 4635968]
3. Anchel M, Hervey A, Robbins WJ. Production of illudin M and of a fourth crystalline compound by Clitocybe Illudens. Proc. Natl. Acad. Sci. U.S.A. 1952; 38:927–928. [PubMed: 16589202]
4. McMorris TC, Anchel M. Fungal metabolites. The structures of the novel sesquiterpenoids illudin-S and -M. J. Am. Chem. Soc. 1965; 87:1594–1600. [PubMed: 14302677]

5. McMorris TC, Kelner MJ, Wang W, Diaz MA, Estes LA, Taetle R. Acylfulvenes, a new class of potent antitumor agents. Experientia. 1996; 52:75–80. [PubMed: 8575564]
6. McMorris TC, Kelner MJ, Wang W, Yu J, Estes LA, Taetle R. (Hydroxymethyl)acylfulvene: an illudin derivative with superior antitumor properties. J. Nat. Prod. 1996; 59:896–899. [PubMed: 8864242]
7. McMorris TC. Discovery and development of sesquiterpenoid derived hydroxymethylacylfulvene: a new anticancer drug. Bioorg. Med. Chem. 1999; 7:881–886. [PubMed: 10400341]
8. Schobert R, Knauer S, Seibt S, Biersack B. Anticancer active illudins: recent developments of a potent alkylating compound class. Curr. Med. Chem. 2011; 18:790–807. [PubMed: 21182482]
9. Kelner MJ, McMorris TC, Taetle R. Preclinical evaluation of illudins as anticancer agents: basis for selective cytotoxicity. J. Natl. Cancer Inst. 1990; 82:1562–1565. [PubMed: 2402017]
10. Kelner MJ, McMorris TC, Beck WT, Zamora JM, Taetle R. Preclinical evaluation of illudins as anticancer agents. Cancer Res. 1987; 47:3186–3189. [PubMed: 3472654]
11. Woynarowska BA, Woynarowski JM, Herzig MC, Roberts K, Higdon AL, MacDonald JR. Differential cytotoxicity and induction of apoptosis in tumor and normal cells by hydroxymethylacylfulvene (HMAF). Biochem. Pharmacol. 2000; 59:1217–1226. [PubMed: 10736422]
12. MacDonald JR, Muscoplat CC, Dexter DL, Mangold GL, Chen SF, Kelner MJ, McMorris TC, Von Hoff DD. Preclinical antitumor activity of 6-hydroxymethylacylfulvene, a semisynthetic derivative of the mushroom toxin illudin S. Cancer Res. 1997; 57:279–283. [PubMed: 9000568]
13. Gong J, Neels JF, Yu X, Kensler TW, Peterson LA, Sturla SJ. Investigating the role of stereochemistry in the activity of anticancer acylfulvenes: synthesis, reductase-mediated bioactivation, and cellular toxicity. J. Med. Chem. 2006; 49:2593–2599. [PubMed: 16610802]
14. Dick RA, Yu X, Kensler TW. NADPH alkenal/one oxidoreductase activity determines sensitivity of cancer cells to the chemotherapeutic alkylating agent irofulven. Clin. Cancer Res. 2004; 10:1492–1499. [PubMed: 14977853]
15. Kelner MJ, McMorris TC, Montoya MA, Estes L, Uglik SF, Rutherford M, Samson KM, Bagnell RD, Taetle R. Characterization of acylfulvene histiospecific toxicity in human tumor cell lines. Cancer Chemother. Pharmacol. 1998; 41:237–242. [PubMed: 9443641]
16. Kelner MJ, McMorris TC, Montoya MA, Estes L, Uglik SF, Rutherford M, Samson KM, Bagnell RD, Taetle R. Characterization of MGI 114 (HMAF) histiospecific toxicity in human tumor cell lines. Cancer Chemother. Pharmacol. 1999; 44:235–240. [PubMed: 10453725]
17. Herzig MCS, Arnett B, MacDonald JR, Woynarowski JM. Drug uptake and cellular targets of hydroxymethylacylfulvene (HMAF). Biochem. Pharmacol. 1999; 58:217–225. [PubMed: 10423161]
18. Woynarowski JM, Napier C, Koester SK, Chen S-F, Troyer D, Chapman W, MacDonald JR. Effects on DNA integrity and apoptosis induction by a novel antitumor sesquiterpene drug, 6-hydroxymethylacylfulvene (HMAF, MGI 114). Biochem. Pharmacol. 1997; 54:1181–1193. [PubMed: 9416969]
19. McMorris TC, Elayadi AN, Yu J, Hu Y, Kelner MJ. Metabolism of antitumor hydroxymethylacylfulvene by rat liver cytosol. Drug Metab. Dispos. 1999; 27:983–985. [PubMed: 10460795]
20. McMorris TC, Elayadi AN, Yu J, Kelner MJ. Metabolism of antitumor acylfulvene by rat liver cytosol. Biochem. Pharmacol. 1999; 57:83–88. [PubMed: 9920288]
21. Dick RA, Kwak MK, Sutter TR, Kensler TW. 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. 2001; 276:40803–40810. [PubMed: 11524419]
22. Neels JF, Gong J, Yu X, Sturla SJ. Quantitative correlation of drug bioactivation and deoxyadenosine alkylation by acylfulvene. Chem. Res. Toxicol. 2007; 20:1513–1519. [PubMed: 17900171]
23. Gong J, Vaidyanathan VG, Yu X, Kensler TW, Peterson LA, Sturla SJ. Depurinating acylfulvene-DNA adducts: characterizing cellular chemical reactions of a selective antitumor agent. J. Am. Chem. Soc. 2007; 129:2101–2111. [PubMed: 17256933]

24. Ichinose K, Kodera M, Leeper FJ, Battersby AR. 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. 1999; 8:879–887.
25. Evans DA, Fu GC. Conjugate reduction of α,β-unsaturated carbonyl compounds by catecholborane. J. Org. Chem. 1990; 55:5678–5680.
26. Mahoney WS, Brestensky DM, Stryker JM. Selective hydride-mediated conjugate reduction of α,β-unsaturated carbonyl compounds using $[(Ph_3P)CuH]_6$. J. Am. Chem. Soc. 1988; 110:291–293.
27. Jackson WR, Zurqiyah A. The occurrence of 1,2- or 1,4-addition in the reduction of some α,β-unsaturated ketones with metal hydrides. J. Chem. Soc. 1965:5280–5287.
28. Hays PA. Proton Nuclear Magnetic Resonance Spectroscopy (NMR) Methods for determining the purity of reference drug standards and illicit forensic drug seizures. J Forensic Sci. 2005; 50:1342. [PubMed: 16382828]
29. Anthoney DA, Twelves CJ. DNA: still a target worth aiming at? A review of new DNA-interactive agents. Am. J. PharmacoGenomics. 2001; 1:67–81. [PubMed: 12173316]
30. Lin AJ, Sartorelli AC. 2,3-Dimethyl-5,6-bis(methylene)-1,4-benzoquinone. The active intermediate of bioreductive alkylating agents. J. Org. Chem. 1973; 38:813–815.
31. Zein N, Kohn H. Covalent binding of mitomycin C to nucleosides under reductive conditions. J. Am. Chem. Soc. 1987; 109:1576–1577.
32. Tomasz M, Chowdary D, Lipman R, Shimotakahara S, Veiro D, Walker V, Verdine GL. 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. 1986; 83:6702–6706. [PubMed: 3018744]
33. Iyer VN, Szybalski W. Mitomycins and porfiromycin: chemical mechanism of activation and cross-linking of DNA. Science. 1964; 145:55–58. [PubMed: 14162693]
34. Tomasz M, Lipman R. Alkylation reactions of mitomycin C at acid pH. J. Am. Chem. Soc. 1979; 101:6063–6067.
35. Erker G, Nolte R, Aul R, Wilker S, Krueger C, Noe R. 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. 1991; 113:7594–7602.
36. Sweeney NJ, Mendoza O, Müller-Bunz H, Pampillón C, Rehmann F-JK, Strohfeldt K, Tacke M. Novel benzyl substituted titanocene anti-cancer drugs. J. Organomet. Chem. 2005; 690:4537–4544.
37. Breydo L, Zang H, Mitra K, Gates KS. Thiol-independent DNA alkylation by leinamycin. J. Am. Chem. Soc. 2001; 123:2060–2061. [PubMed: 11456831]
38. Chatterji T, Kizil M, Keerthi K, Chowdhury G, Pospísil T, Gates KS. Small molecules that mimic the thiol-triggered alkylating properties seen in the natural product leinamycin. J. Am. Chem. Soc. 2003; 125:4996–4997. [PubMed: 12708847]
39. Gopalakrishnan S, Byrd S, Stone MP, Harris TM. 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. Biochem. 1989; 28:726–734. [PubMed: 2496751]
40. Boger DL, Invergo BJ, Coleman RS, Zarrinmayeh H, Kitos PA, Thompson SC, Leong T, McLaughlin LW. 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. 1990; 73:29–52. [PubMed: 2406033]
41. Geacintov NE, Yoshida H, Ibanez V, Harvey RG. Non-covalent intercalative binding of 7,8-dihydroxy-9,10-epoxybenzo(a)pyrene to DNA. Biochem. Biophys. Res. Commun. 1981; 100:1569–1577. [PubMed: 6794569]
42. Guza R, Kotandeniya D, Murphy K, Dissanayake T, Lin C, Giambasu GM, Lad RR, Wojciechowski F, Amin S, Sturla SJ, Hudson RHE, York DM, Jankowiak R, Jones R, Tretyakova NY. 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. 2011; 39:3988–4006. [PubMed: 21245046]

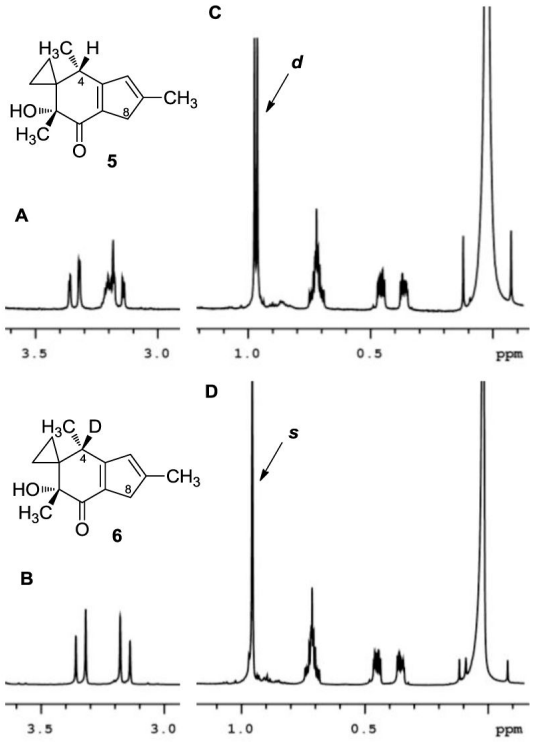


Figure 1.

NMR spectra for **5** (panels A, C) and **6** (panels B, 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**.

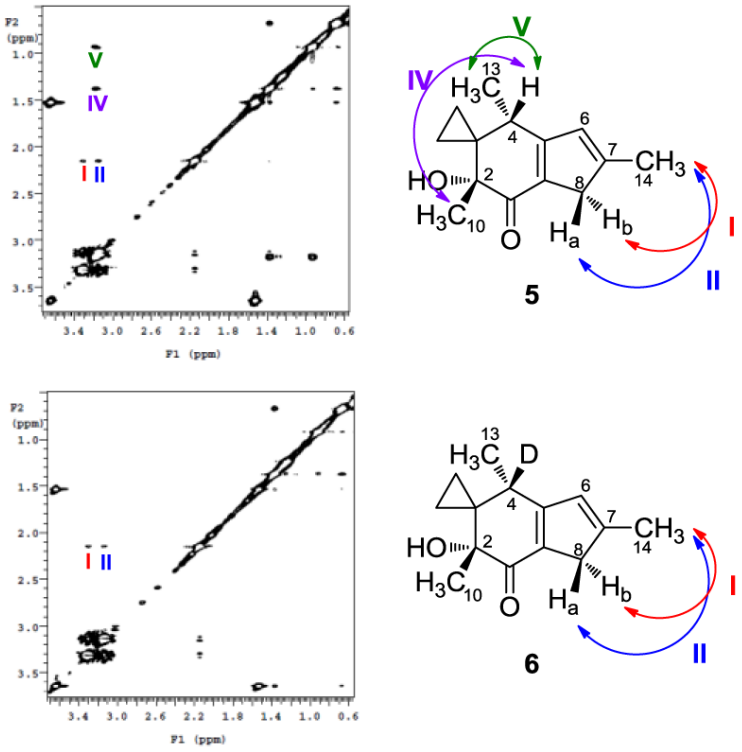


Figure 2.
NOE interactions in **5** (top) and **6** (bottom).

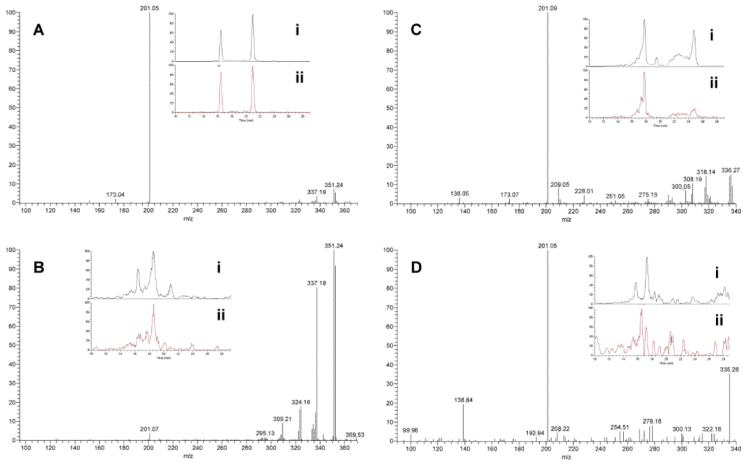


Figure 3.

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).

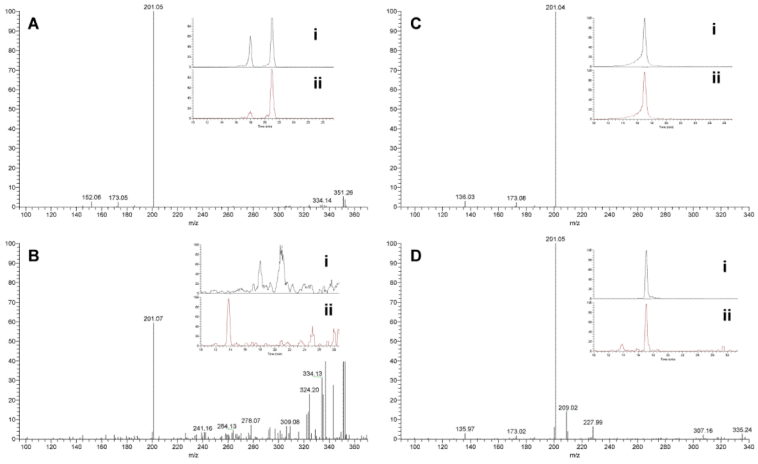
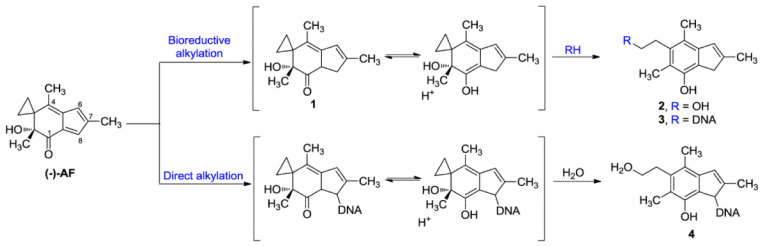
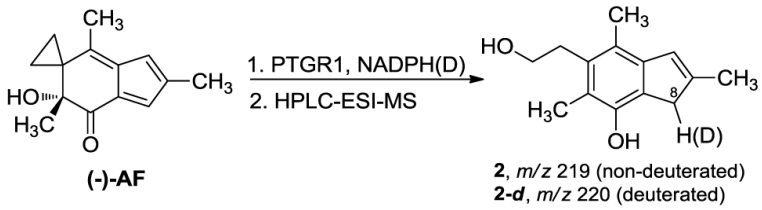


Figure 4.

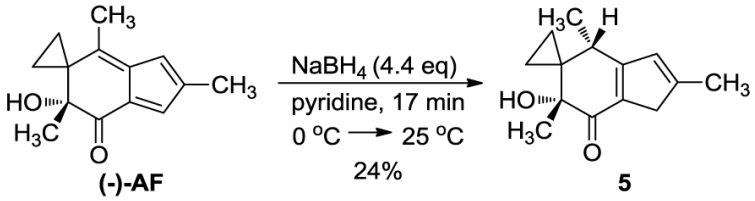
HPLC-MS^2 analysis of ctDNA reactions with **5** and bioactivated AF**. A)** MS^2 *m/z* 352 → 201 mass spectra for the reaction of bioactivated AF with ctDNA. Extracted ion chromatographs for MS^2 *m/z* 352 → 201 (i) and MS^2 *m/z* 352 → 152 (ii). **B)** MS^2 *m/z* 352 → 201 mass spectra for the reaction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



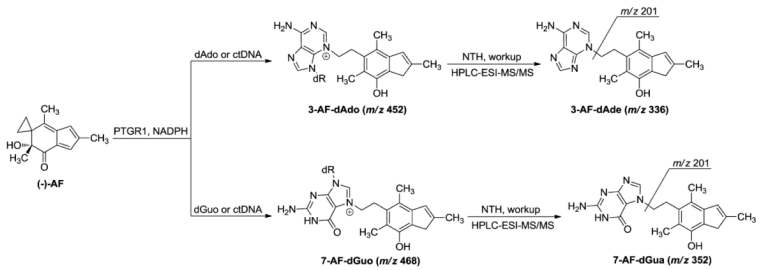
Scheme 1.
Proposed pathways for the formation of AF adducts via direct alkylation and bioreductive alkylation.



Scheme 2.
Strategy for probing the AF-bioactivation mechanism using HPLC-ESI-MS.

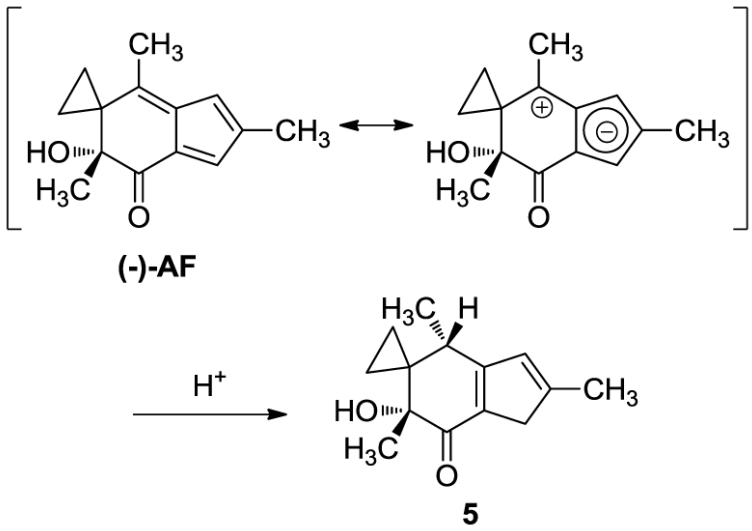


Scheme 3.
Chemical reduction of AF with $NaBH_4$.

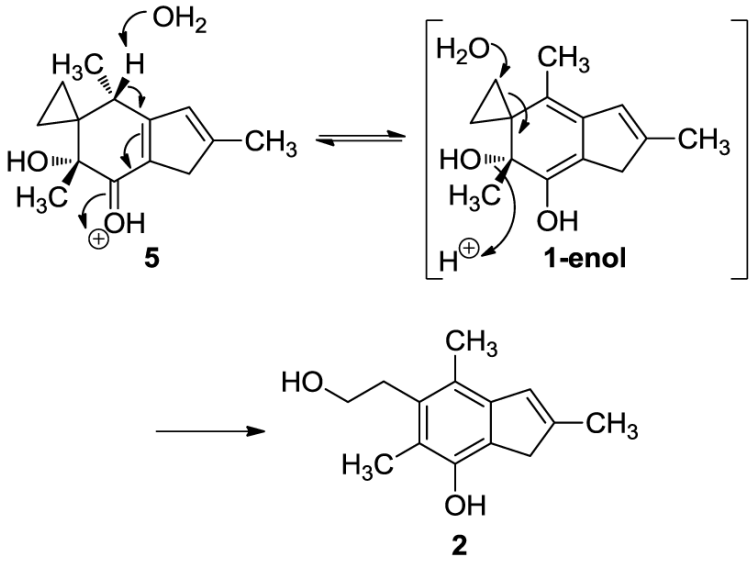


Scheme 4.

Reaction of bioactivated AF with purine bases or ctDNA. The DNA adducts and resulting fragments are identified on the basis of known *m/z* values.



Scheme 5.
Reduction of AF to yield **5** and instructive dipolar resonance precursor structure.



Scheme 6.
Proposed mechanism for conversion of **5** to **2** in aqueous acid.

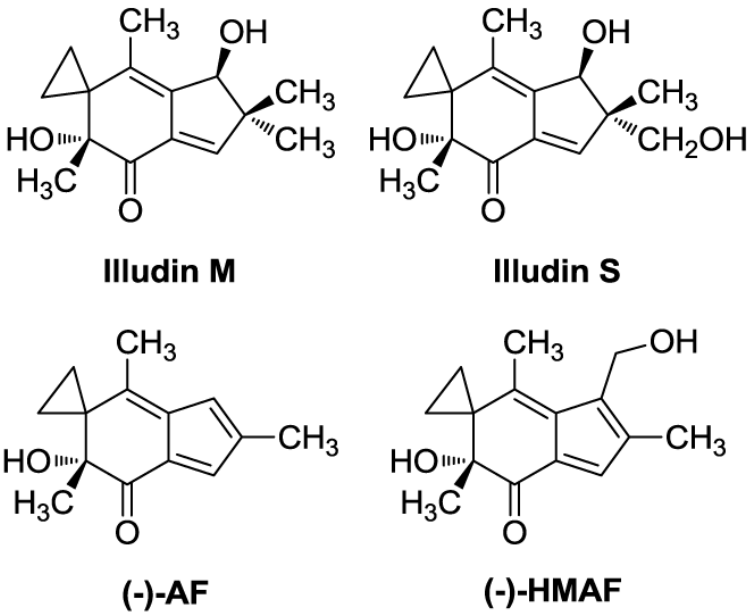


Chart 1.
Natural product illudins and semi-synthetic analogues acylfulvene (AF) and hydroxymethylacylfulvene (HMAF).

Table 1

IC_{50} values for AF and **5** in HEK-293T cells transfected with a blank (control) or hPTGR1 overexpressing vector.

compound	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