Investigating the Role of Stereochemistry in the Activity of Anticancer Acylfulvenes: Synthesis, Reductase-Mediated Bioactivation, and Cellular Toxicity

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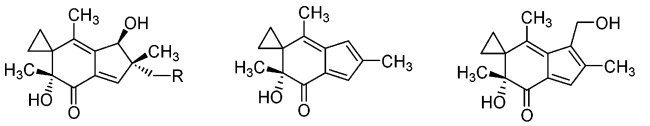
Acylfulvenes comprise a family of semisynthetic natural product derivatives with potent antitumor activities. Previous studies indicated that acylfulvenes are bioactivated by NADPH-dependent alkenal/one reductase (AOR), presumably generating intermediates with the capacity to alkylate cellular targets, such as DNA, proteins, and glutathione. This process is thought to induce apoptosis, and the chemical and biochemical pathways involved are topics of current investigation. In this study, four acylfulvene analogues were synthesized: (−)-acylfulvene, (+)-acylfulvene, (−)-(hydroxymethyl)acylfulvene, and (+)-(hydroxymethyl)-acylfulvene. These compounds were synthesized by a chiral-resolution method, described for the first time in this report, and by asymmetric synthesis using a method formally demonstrated previously. Cell toxicity studies indicate a positive correlation between AOR level and acylfulvene sensitivity. The absolute configuration of acylfulvene analogues has a significant influence on cytotoxicity. (−)-(Hydroxymethyl)-acylfulvene is 25 times more potent than (+)-(hydroxymethyl)acylfulvene in cells transfected with an AOR overexpression vector. Based on kinetic parameters, the rates of AOR-mediated activation are more strongly dependent on acylfulvene substitution than on absolute stereochemistry. These data support the role of AOR-mediated metabolism and indicate the involvement of other stereochemically dictated pathways, such as transport and biomolecule binding, in contributing to the cytotoxicity of acylfulvenes.

Introduction

DNA-alkylating agents are among the most effective classes of drugs for the treatment of cancer.[1] Due to their low selectivity and high toxicity, extensive research has focused on the development of antitumor agents with increased selectivity and decreased toxicity to normal cells. Illudins are mushroom-derived sesquiterpene natural products. Illudin S (**1**) and M (**2**) (Chart 1) are toxic to a number of tumor cells, but have low therapeutic indices as antitumor compounds.[2−4] Acylfulvenes, semisynthetic derivatives of illudin S, are selectively cytotoxic to certain tumor cells and thus are promising chemotherapeutic agents.[5−10] One member of this family, (−)-(hydroxymethyl)-acyfulvene (**4**, (−)-HMAF; Chart 1), is currently in phase II clinical trials for a number of cancers.[3;6;10−12] Acylfulvenes are alkylating agents that covalently bind to biological macromolecules, including DNA and protein.[13;14] It was observed that 60−70% of HMAF that was taken up by cells binds to macromolecules.[8;15] The selective toxicity profile of acylfulvenes may originate from energy-dependent selective uptake mechanisms, from differences in metabolic bioactivation to reactive species, and from differences in DNA and protein alkylation.[13;16]

Illudins and acylfulvenes can alkylate cellular targets by different chemical pathways (Scheme 1): Direct nucleophilic addition of cellular nucleophiles by conjugate addition to the 8-position, or enzymatic reduction of the carbon−carbon double bond of the α,β-unsaturated ketone, leading to reactive intermediate **5**. Both pathways may lead to the formation of adducts of cellular macromolecules and could be involved in the induction of apoptosis.[13;14;17]

Chart 1. Structures of Natural Illudins and Semisynthetic Derivatives, Acylfulvenes



1 Illudin S (R = OH)
2 Illudin M (R = H)
3 (-)-Acylfulvene (AF)
4 (-)-Hydroxymthlacylfulvene (HMAF)

(−)-HMAF undergoes NADPH-dependent metabolism to generate **6**, and this process is catalyzed by rat liver cytosol as well as recombinant rat alkenal/one oxidoreductase (AOR).[18] AOR reduces a variety of α,β-unsaturated aldehydes and ketones including cytotoxic and carcinogenic byproducts of lipid peroxidation.[18−20] Its cellular localization, cofactor preference and inhibition profile are identical to the acylfulvene-reducing enzyme that was partially characterized from rat liver cytosol.[13;14] In addition, a positive correlation was observed between (−)-HMAF sensitivity and levels of AOR expression in tumor cells.[18]

Acylfulvenes are obtained by semisynthesis from isolated natural products and have (*R*)-configurations at the 2-position. (−)-Acylfulvene (**3**, (−)-AF; Chart 1) and (−)-HMAF are readily obtained by treatment of illudin S with dilute H_2SO_4, in the absence or presence of formaldehyde, respectively.[6] Semisynthesis methods limit investigations of structure−activity relationships including study of the influence of absolute stereochemistry on biological effects. A method for the asymmetric synthesis of acylfulvenes using a Sharpless asymmetric dihydroxylation reaction and the allenic Pauson−Khand type

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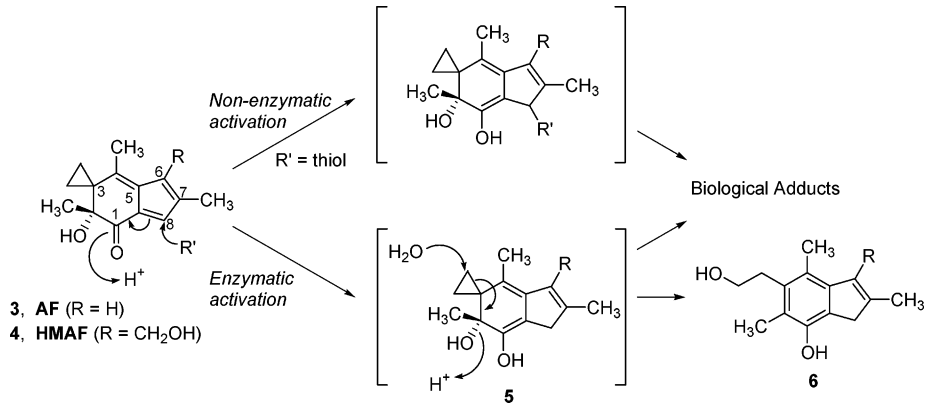
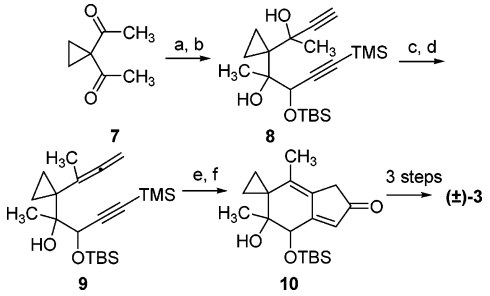
Scheme 1. Chemical Routes for Acylfulvene Metabolism and Adduct Formation



Scheme 2. Brummond Method for the Racemic Synthesis of Acylfulvenes[a] [21]



[a] Reagents and conditions: (a) *t*-BuLi, TBS ether of 3-trimethylsilylpropyn-1-ol, THF, −78 °C, 60%; (b) $CeCl_3$, ethynylmagnesium bromide, THF, 0 °C, 95%; (c) Ac_2O, DMAP, Et_3N; (d) $[CuH(PPh_3)]_6$, toluene, H_2O, 55% for two steps; (e) K_2CO_3, MeOH, H_2O, 96%; (f) $Mo(CO)_6$, DMSO, toluene, 72%.

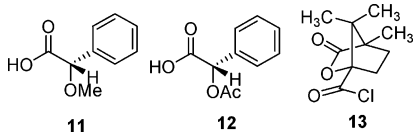
reaction was reported by Brummond and co-workers.[21] Additionally, the total synthesis of either enantiomer of acylfulvenes was accomplished recently by McMorris and co-workers utilizing the Padwa carbonyl ylide 1,3-dipolar cycloaddition methodology and enantiomerically pure starting materal.[22] In MV 522 adenocarcinoma cells, the synthetic (−)-acylfulvenes were 5−6 times more potent than the corresponding (+)-isomers.[22] The detailed mechanism of how stereochemistry influences toxicity is not known.

In our investigations of the mechanism of action of acylfulvenes, we aim to understand the chemical origins of selective cytotoxicity, including the relationship of absolute stereochemistry to biological activity, and the role of bioactivation. We report here two synthetic approaches to obtaining both enantiomers of acylfulvenes. Using compounds obtained by total synthesis, we have examined the influence of stereochemistry on the cytotoxicity of acylfulvenes to human embryonic kidney cells engineered to overexpress AOR. Furthermore, using purified AOR, we disclose the first report of configuration-based differences in the kinetics of AF and HMAF reductive activation.

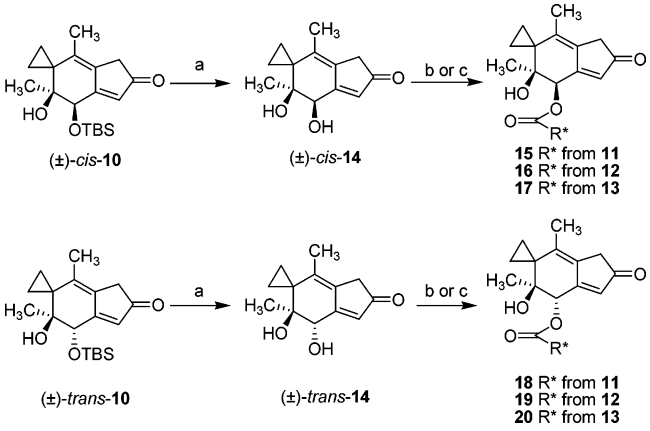
Results and Discussion

Chemistry. To obtain both enantiomers of acylfulvene, we explored two preparative approaches: chiral resolution and asymmetric synthesis. Brummond and co-workers have reported a concise synthesis of (±)-acylfulvenes (Scheme 2) in which the TBS-protected diol **10** is an intermediate.[23] We chose to investigate the chiral resolution of this substrate (**10**) because of its chemical stability and convenient functionality. Racemic **10** was synthesized in eight steps starting from 1,1-diacetylcyclopropane **7**.[23] The secondary alcohol was deprotected and

Chart 2. Structures of Chiral Derivatizing Agents Used for the Resolution of Acylfulvene Synthetic Intermediates



Scheme 3. Synthesis of Diastereomeric Esters Used for the Chiral Resolution of Acylfulvene Synthetic Intermediates[a]



[a] Reagents and conditions: (a) TBAF, THF, 95%; (b) respective chiral acid, HBTU, DMAP, THF, 86%; (c) camphanic acid chloride, DMAP, THF, 88%.

derivatized with an enantiomerically pure chiral acid or acid chloride. Three chiral reagents, (+)-methoxyphenylacetic acid **11**, (−)-*O*-acetylmandelic acid **12**, and (−)-camphanic acid chloride **13** (Chart 2), were chosen as derivatizing agents and their resolving efficiencies were compared. Thus, *cis*- and *trans*-**10** were treated with TBAF and independently afforded free racemic diols *cis*- and *trans*-**14**. The diols were coupled with **11**, **12** or **13** to afford diastereomeric esters (**15**−**20**) in 70−90% yields (Scheme 3). The resulting diastereomeric esters **15**, **16**, **17**, and **18** were separated by reversed phase HPLC (Figure 1, isocratic elution, 1:1 MeOH:H_2O). Balancing various factors including degree of separation, total time needed for collection, long-term chemical stability of the compounds and cost of the chiral derivative, **11** was the derivatizing agent of choice for obtaining diastereomerically pure material.

Diastereomeric esters **15** and **18** were resolved by preparative HPLC. ^{1}H NMR analysis indicated high optical purities of the resulting esters (ee > 98%). The chiral esters were hydrolyzed in the presence of one equivalent of NaOH and the resulting free secondary hydroxyl was reacted with TBS-Cl to give the corresponding enantiomerically pure **10**. A method for direct

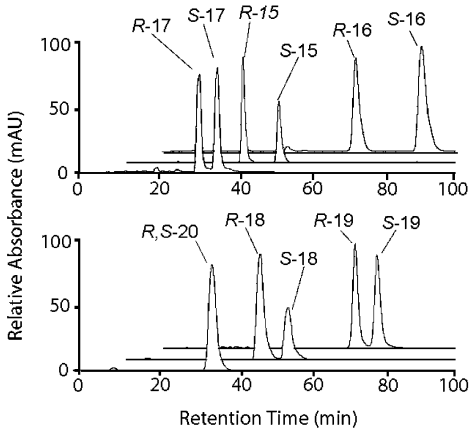
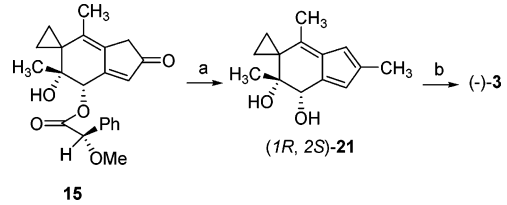


Figure 1. Overlay of HPLC chromatograms of the diastereomeric ester derivatives. The top trace displays *cis*-ester derivatives **15**−**17** and the bottom trace displays trans-ester derivatives **18**−**20**. Absolute stereochemistry assignments for (*R*)-**15** and (*S*)-**15** were determined after preparative separation of the compounds, hydrolysis of the ester, and measurement of optical rotations. The (*R*)- and (*S*)-assignments for **16**, **17**, **18**, and **19** were not determined and are indicated as a convention based on the results from compound **15**.

Scheme 4. Representative Synthesis of an AF from Direct Reaction of MeLi with a Diastereomerically Pure Ester[a]

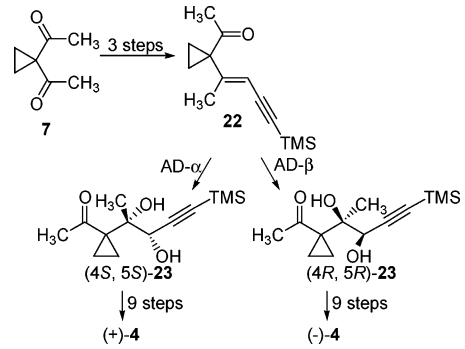


[a] Reagents and conditions: (a) $CeCl_3$, MeLi, THF, 90%; (b) IBX, DMSO, 88%.

conversion of the resolved esters **15** and **18** to the corresponding optically pure diol **21** was developed (Scheme 4) to circumvent the alternative inefficient sequence of hydrolysis and protection. Thus, esters **15** or **18** were treated with 10 equiv MeLi, providing **21** in 90% yield. To assign the absolute stereochemistry of the tertiary alcohol in the resolved ester, **21** was oxidized with IBX and the optical rotation of the corresponding acylfulvene was measured. Initially, cis-and trans-isomers of intermediates were handled separately because we observed large differences in the reactivity of *cis*- and *trans*-**21** toward Dess−Martin oxidation. However, both diols were converted to acylfulvene by IBX, the Dess−Martin precursor, in DMSO in 90% yield. [24;25]

To determine the optimal method of obtaining enantiomerically pure acylfulvenes, we completed the asymmetric synthesis formally demonstrated by Brummond and co-workers (Scheme 5). Following published procedures, *E*-enyne **22** was obtained in three steps from **7**. Enyne **22** was subjected to Sharpless asymmetric dihydroxylation conditions to generate (4*S*,5*S*) and (4*R*,5*R*)-**23** using (DHQ)PYR or (DHQD)PYR as chiral catalysts, respectively. Each enantiomer of **23** was advanced to HMAF utilizing the same conditions as for the racemic synthesis. To determine the optical purity of the TBS-protected diol **10** produced by this method, it was treated with TBAF, and the resulting free alcohol was coupled with chiral acid **11**. NMR and HPLC analysis of the resulting diastereomeric esters were consistent with the presence of a single isomer (ee% > 95%). The completion of the synthesis validated the effectiveness of Brummond's method for the asymmetric synthesis of either enantiomer of HMAF. Considering the time-consuming nature of the HPLC separation, we currently find that in our

Scheme 5. Asymmetric Synthesis of Acylfulvenes Using the Method Reported by Brummond and Co-workers[a]



[a] AD-α represents an asymmetric dihydroxylation reaction utilizing the following reagents: $(DHQ)_2PYR$, $K_2OsO_2(OH)_4$, $K_3Fe(CN)_6$, K_2CO_3, $CH_3SO_2NH_2$. AD-β utilizes analogous conditions, replacing the DHQ ligand with DHQD. DHQ, dihydroquinine; DHQD, dihydroquinidine.

laboratory, the asymmetric synthesis pathway is a more viable route and substrates for the biological studies described here were prepared in this manner.

Biochemistry

McMorris and co-workers reported that the sensitivity of MV 522 adenocarcinoma cells to acylfulvenes was dependent on the compound's absolute stereochemistry.[22] It was observed that the IC_{50} values of (−)-acylfulvenes are 5−6 times lower than those of the corresponding (+)-isomers after 2 or 48 h.[22] These differences in toxicities may originate from differences in cellular uptake, enzyme-mediated metabolic bioactivation, or subsequent interaction with biological systems. To investigate the potential role of metabolism, we measured the sensitivity of cells engineered to express either low or high AOR levels and determined kinetic parameters for AOR-catalyzed NADPH-mediated reductive bioactivation of the analogues prepared using the synthetic routes described above.

Human embryonic kidney cells (293 cell line) were transiently transfected either with an episomal rAOR overexpression vector (pCEP4-rAOR) or control vector (pCEP4).[18] Following hygromycin selection, AOR transfected cells produced a 7−8-fold higher expression of AOR than control cells. AOR levels were determined spectrophotometrically.[18] The cytotoxicity of each compound to the AOR-transfected cells was assessed by measuring cell viability via MTT assay 24 h posttreatment. Our observations echoed the previous report that enhanced AOR activity greatly sensitized cells to HMAF toxicity.[18] The toxicity of each compound tested in this study was 15−20-fold higher in AOR transfected cells compared to control cells. The IC_{50} values of control cells toward (−)-AF and (−)-HMAF were 1.4 and 1.2 μM, respectively. These cells were significantly more resistant to the (+)-enantiomers, such that IC_{50} values were not determined because of the exceedingly high levels of compound required. Relative sensitivities of the cells to the four agents tested are indicated in Figure 2. Consistent with the findings of McMorris and co-workers,[22] (−)-configuration agents demonstrated significantly increased toxicity compared to the (+)-counterparts. (−) −HMAF and (−)-AF are the two most potent compounds, with relative IC_{50} values of 55 and 95 nM, respectively. More than 25-fold and 50-fold higher concentrations of (+)-HMAF and (+)-AF, or 1420 and 5000 nM, respectively, were required to achieve the same cytotoxicity as the corresponding (−)-enantiomers in AOR-transfected 293 cells.

We measured kinetic parameters for AOR-catalyzed metabolism of each compound to determine if stereochemistry at the

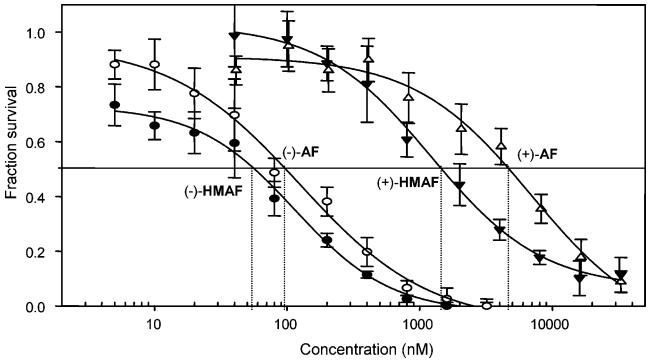


Figure 2. AOR transfected 293 cells were challenged for 24 h with the indicated compounds. Cell viability was measured by MTT assay in 96-well plates. Each point represents the mean of eight determinations, and bars indicate standard deviation.

Table 1. Kinetic Parameters for Acylfulvene Metabolism by AOR[a]

substrate	K_m (μM)	V_{max} (μM/min)	V_{max}/K_m (min^{-1})
(+)-AF	465	1.7	3.7
(−)-AF	538	5.0	9.3
(+)-HMAF	243	4.5	18.5
(−)-HMAF	213	8.3	39.0

[a] Values represent averages determined from three runs with errors within 10%.

2-position affects the rate of this process. Enzymatic activation of acylfulvenes results in loss of conjugation in the five-membered ring, cyclopropyl ring-opening, and aromatization. In the UV−vis spectra, the absorptions at 330 and 420 nm disappear. Due to the interference of NADPH at 330 nm, the change of the absorbance at 420 nm was monitored spectrophotometrically to determine kinetic parameters for AOR-catalyzed reduction of the α,β-unsaturated ketones. HPLC analysis was also performed on these incubation mixtures to confirm that **6** (Nu = OH, R′ = H, Scheme 1) was the major metabolite. The UV change and metabolite formation required both AOR and NADPH. The identity of **6** was confirmed by coelution with a synthetic standard,[26] as well as by LC-MS analysis.

Kinetic parameters for the metabolism of synthetic acylfulvenes by AOR are shown in Table 1. Both HMAF enantiomers display higher K_m values than AF. These results are consistent with earlier AOR studies in which a K_m value of 145 μM for (−)-HMAF was determined by monitoring the appearance of the hydroxyl metabolite **6**.[18]

Despite the strong correlation between cell sensitivity and AOR expression levels, cytotoxicities do not correlate directly with bioactivation parameters. For example, the AOR activation parameters positively correlate with (−)-HMAF as the most active analogue and (+)-AF as the least active, but there is only a 10-fold difference in V_{max}/K_m, while there is a 100-fold enhancement in cytotoxicity for (−)-HMAF vs (+)-AF. Interestingly, (+)-HMAF is activated about twice as efficiently as (−)-AF, but is about 40-fold less potent in the cell-based assay. The nonlinear relationship between kinetic parameters and cell sensitivity suggests that additional biochemical factors are important in dictating cell sensitivity to these drugs. Cellular accumulation, i.e., selective uptake or efflux of a drug, is an important mechanism that accounts for differences in cytotoxicity, is implicated in drug resistance, and has been the subject of extensive investigation.[27−29] In the case of acylfulvenes, relative cellular accumulations have been compared in relatively sensitive and insensitive cells. In that study, a 263-fold increase was observed in accumulation of the drug in MV522 (sensitive) cells vs 8392 B-cells (insensitive).[9] Additionally, our observations may be related to downstream effects such as the binding to cellular macromolecules including DNA and proteins, which is thought to be an important mode of toxicity for acylfulvenes.[9] Acylfulvene−DNA lesions inhibit DNA synthesis,[15] and the influences of protein adducts are unknown. Both cellular transport and adduct formation are likely to be sensitive to substrate stereochemistry and may be important contributing factors.

Conclusions

We have synthesized both enantiomers of acylfulvene and HMAF by two preparative routes: chiral resolution and asymmetric synthesis. Both pathways are effective for obtaining enantiomerically pure acylfulvenes. The relative toxicities of this series of compounds were compared in 293 cells transfected to overexpress the metabolic enzyme AOR. Additionally, kinetic parameters for AOR-catalyzed metabolism were determined independently with isolated enzyme. (−)-HMAF is the best AOR substrate, and (+)-isomers are metabolized with lower overall efficiency compared to the (−)-isomers; these data are consistent with cytotoxicity data. However, the natural stereochemistry of the tertiary alcohol only contributed to an approximate 2-fold enhancement of catalytic efficiency, whereas toxicities were over an order of magnitude higher. Furthermore, AOR-mediated metabolism is influenced more strongly by the presence or absence of the hydroxylmethyl substituent than by absolute stereochemistry. This assertion is illustrated by an approximate 2-fold *increase* in V_{max}/K_m for (+)-HMAF over (−)-AF, but a 10-fold *decrease* in cytotoxicity. Overall, these data support that metabolic activation contributes to the relative toxicities of acylfulvene analogues but indicate that other biochemical interactions sensitive to stereochemistry are important. These may include differences in cellular transport as well as subsequent binding to DNA and protein targets. Current studies are focused on the use of synthetic analogues of acylfulvenes to probe further mechanisms of selective toxicity.

Experimental Section

General. All chemicals were purchased from Sigma-Aldrich and used without further purification. Solvents were dried on an M. Braun (Stratham, NH) solvent purification system prior to use. ^{1}H NMR spectra were acquired on a 300 MHz Varian Mercury NMR spectrometer, and chemical shifts were reported relative to the residual nondeuterated solvent signals. HPLC analysis was carried out on an Agilent 1100 series instrument with diode array detector. HRMS were recorded on a Bruker BioTOF II mass spectrometer with an electrospray ionization source. Polypropylene glycol (PPG) was used as matrix in all HRMS experiments. Flash chromatography (silica gel, 200−400 mesh) was used for product purification. Normal phase TLC was used for product checking and the spots were detected by exposure to UV-lamp or I_2.

IBX was prepared by a published procedure.[24;25] Racemic **10** was prepared as previously reported[23] and was used as precursor for chiral resolution. (4*S*,5*S*)-**23** and (4*R*,5*R*)-**23** were prepared using a published method.[21] (−)- and (+)-**3**, and (−)- and (+)-**4** were prepared by chiral resolution of diastereomeric intermediates using the procedures described here, or using (4*S*,5*S*)-**23** and (4*R*,5*R*)-**23** as precursors, by published methods.[21;22] The enantiomeric excesses of compounds obtained by asymmetric synthesis were determined by derivatization of the intermediates (1*R*,2*S*)-*trans*-**10** and (1*S*,2*R*)-*trans*-**10** with camphanic acid, as described below, and analysis by ^{1}H NMR and HPLC.

Chemical Synthesis. (1*R,2*S**)-1,2-Dihydroxy-2,4-dimethyl-3-spiro-cyclopropyl-bicyclo[4.3.0]nona-5,9-dien-7-one (*rac cis*-14).** TBAF (3 mL, 1.0 M solution in THF) was added to an ice-cold solution of **10** (50 mg, 0.15 mmol) in 2 mL of THF. The

solution was stirred at 0 °C for 10 min and was then warmed slowly to 25 °C and stirred for additional 30 min. The solvent was evaporated under reduced pressure, and the residue was purified by flash chromatography eluting with 50% EtOAc/CH_2Cl_2 to yield the title compound as a white solid. 97% yield. mp 75−77 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.96 (m, 4H), 1.09 (s, 3H), 1.27 (s, 3H), 2.19 (s, 1H), 2.29 (d, J = 8.4 Hz, 1H), 3.02 (s, 2H), 4.43 (d, J = 7.5 Hz, 1H), 6.26 (s, 1H); HRMS calcd for $C_{13}H_{16}O_3$ [M + Na]$^+$: 243.0997. Found: 243.1025.

(1*S,2*S**)-1,2-Dihydroxy-2,4-dimethyl-3-spiro-cyclopropyl-bicyclo[4.3.0]nona-5,9-dien-7-one (*rac trans*-14).** The title compound was prepared by the same procedure as *rac cis*-**14**, with TBAF (3 mL, 1.0 M solution in THF) and **10** (50 mg, 0.15 mmol) in 2 mL of THF. In this case, the solution was stirred at 0 °C for 10 min and was then warmed slowly to 25 °C and stirred for additional 2 h. The solvent was evaporated under reduced pressure, and the residue was purified by flash chromatography eluting with 50% EtOAc/CH_2Cl_2 to yield the title compound as a white solid. 95% yield. mp 72−74 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.53 (M, 1H), 0.80 (M, 1H), 1.05 (M, 1H), 1.16 (s, 3H), 1.34 (M, 1H), 1.56 (s, 3H), 2.42 (d, J = 8.4 Hz, 1H), 1.94 (s, 2H), 4.64 (d, J = 7.5 Hz, 1H), 6.20 (s, 1H). HRMS calcd for $C_{13}H_{16}O_3$ [M + Na]$^+$: 243.0997. Found: 243.1005.

(1*R,2*S**)-2-Dihydroxy-2,4-dimethyl-3-spiro-cyclopropyl-1-[(2S)-2-methoxy-phenylacetyloxy]bicyclo[4.3.0]nona-5,9-dien-7-one (15).** To an oven-dried 10 mL round-bottomed flask was added *rac cis*-**14** (50 mg, 0.23 mmol), (+)-methoxyphenylacetic acid **11** (115 mg, 0.69 mmol), *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyl-uronium hexafluorophosphate (HBTU) (259 mg, 0.69 mmol), DMAP (27.7 mg, 0.23 mmol), Et_3N (0.143 mL, 1.15 mmol), and 2 mL of THF. The mixture was stirred at 25 °C for 1 day. After this time, the reaction mixture was poured into 20 mL of H_2O and extracted with EtOAc. The organic solution was washed with a 5% aqueous K_2CO_3 solution and then dried over anhydrous Na_2SO_4. Solvent was evaporated under reduced pressure, and the resulting residue was purified by flash chromatography eluting first with 20% then 35% EtOAc/CH_2Cl_2 to yield the corresponding ester as a white solid. 70% yield. ^{1}H NMR ($CDCl_3$) δ 0.85 (m, 4H); 1.20 (m, 10H), 1.51 (s, 3H), 1.53 (s, 3H), 2.22 (s, 6H), 2.85 (m, 2H), 2.97 (s, 1H), 2.99 (s, 1H), 5.68 (s, 1H), 5.81 (s, 1H), 5.88 (s, 1H), 5.98 (s, 1H), 6.19 (s, 1H), 7.32−7.58 (m, 10H); HRMS calcd for $C_{23}H_{24}O_6$ [M + Na]$^+$ = 419.1471. Found 419.1482.

(1*R,2*S**)-2-Dihydroxy-2,4-dimethyl-3-spiro-cyclopropyl-1-[(2S)-2-acetyloxy-phenylacetyloxy]bicyclo[4.3.0]nona-5,9-dien-7-one (16).** The title compound was prepared as a white solid by the same procedure as ester-**15**, with *rac cis*-**14** (50 mg, 0.23 mmol), (−)-*O*-acetylmandelic acid **12** (134 mg, 0.69 mmol), HBTU (259 mg, 0.69 mmol), DMAP (27.7 mg, 0.23 mmol), and Et_3N (0.143 mL, 1.15 mmol) in 2 mL of THF. 80% yield. ^{1}H NMR ($CDCl_3$) δ 0.63−0.95 (m, 8H), 1.07 (s, 3H), 1.52 (s, 3H), 1.54 (s, 3H), 1.58 (s, 3H), 2.86 (s, 2H), 2.93 (s, 2H), 3.42 (s, 3H), 3.44 (s, 3H), 4.80 (s, 1H), 4.90 (s, 1H), 5.65 (s, 1H), 5.74 (s, 1H), 5.78 (s, 1H), 6.10 (s, 1H), 7.38−7.45 (m, 10H); HRMS calcd for $C_{22}H_{24}O_5$ [M + Na]$^+$ = 391.1521, found 391.1517.

(1*R,2*S**)-2-Dihydroxy-2,4-dimethyl-3-spiro-cyclopropyl-1-{4,7,7-trimethyl-3-oxo-2-oxabicyclo[2,2,1]heptane-1-carboxyl}-bicyclo[4.3.0]nona-5,9-dien-7-one (17).** To an oven-dried 10 mL round-bottomed flask were added *rac cis*-**14** (50 mg, 0.23 mmol), (−)-camphanic acid chloride **13** (149 mg, 0.69 mmol), DMAP (27.7 mg, 0.23 mmol), Et_3N (0.143 mL, 1.15 mmol), and 2 mL of THF. The mixture was stirred at 25 °C for 1 day. After this time, the reaction mixture was poured into 20 mL of H_2O and extracted with EtOAc. The organic solution was washed with a 5% aqueous K_2CO_3 solution and then dried over anhydrous Na_2SO_4. Solvent was evaporated under reduced pressure, and the resulting residue was purified by flash chromatography eluting first with 20% then 35% EtOAc/CH_2Cl_2 to yield the corresponding ester as a white solid. 85% yield; ^{1}H NMR (C_6D_6) δ 0.29 (s, 4H) 0.60 (s, 3H), 0.63 (s, 3H), 0.71 (s, 4H), 0.78 (s, 3H), 0.80 (s, 3H), 0.88 (m, 12H), 1.35 (s, 6H), 1.56−1.68 (m, 4H), 1.90−2.14 (m, 4H), 2.51 (m, 2H),2.54 (s, 1H), 2.55 (s, 1H), 5.67 (s, 1H), 5.69 (s, 1H), 6.25 (s, 2H); HRMS calcd for $C_{23}H_{28}O_6$ [M + Na]$^+$ = 423.1784, found 423.1843.

(1*S,2*S**)-2-Dihydroxy-2,4-dimethyl-3-spiro-cyclopropyl-1-[(2S)-2-methoxyphenylacetyloxy]bicyclo[4.3.0]nona-5,9-dien-7-one (18).** The title compound was prepared as a white solid by the same procedure as ester-**15**, with *rac trans*-**14** (50 mg, 0.23 mmol), (+)-methoxyphenylacetic acid **11** (115 mg, 0.69 mmol), HBTU (259 mg, 0.69 mmol), DMAP (27.7 mg, 0.23 mmol), and Et_3N (0.143 mL, 1.15 mmol) in 2 mL of THF. 74% yield. ^{1}H NMR (C_6D_6) δ 0.05−0.16 (m, 2H), 0.53−0.66 (m, 2H), 0.68−0.75 (m, 1H), 0.84 (s, 3H), 0.88−0.93 (m, 1H), 0.98 (s, 3H), 1.16−1.22 (m, 4H), 1.24−1.40 (m, 4H), 1.65 (s, 3H), 1.70 (s, 3H), 2.44 (m, 2H), 2.47 (s, 1H), 2.49 (s, 1H), 2.71 (m, 1H), 5.57 (d, J = 1.7 Hz, 1H), 5.91 (s, 1H), 5.94 (s, 1H), 5.99 (s, 1H), 6.04 (s, 1H), 6.33 (d, J = 1.7 Hz, 1H), 7.01−7.09 (m, 6H), 7.42−7.47 (m, 4H); HRMS calcd for $C_{23}H_{24}O_6$ [M + Na]$^+$ = 419.1482. Found 419.1528.

(1*S,2*S**)-2-Dihydroxy-2,4-dimethyl-3-spiro-cyclopropyl-1-[(2*S*)-2-acetyloxy-phenylacetyloxy]bicyclo[4.3.0]nona-5,9-dien-7-one (19).** The title compound was prepared as a white solid by the same procedure as ester-**15**, with *rac trans*-**14** (50 mg, 0.23 mmol), (−)-*O*-acetylmandelic acid **12** (134 mg, 0.69 mmol), HBTU (259 mg, 0.69 mmol), DMAP (27.7 mg, 0.23 mmol), Et_3N (0.143 mL, 1.15 mmol) in 2 mL THF. 75% yield. ^{1}H NMR (C_6D_6) δ 0.52−0.60 (m, 1H), 0.81−0.97 (m, 2H), 0.98−1.08 (m, 2H), 1.14 (s, 3H), 1.21 (s, 3H), 1.29−1.42 (m, 3H), 1.57 (s, 3H), 1.58 (s, 3H), 2.93 (s, 2H), 3.39 (m, 3H), 3.41 (s, 3H), 3.50 (s, 2H), 3.70 (m, 1H), 4.28 (m, 1H), 4.83 (s, 1H), 4.92 (s, 1H), 5.03 (s, 1H), 5.07 (s, 1H), 5.60 (s, 1H), 5.84 (s, 1H), 7.35−7.46 (m, 7H), 7.51−7.55 (m, 3H); HRMS calcd for $C_{22}H_{24}O_5$ [M + Na]$^+$ = 391.1521. Found 391.1372.

(1*S,2*S**)-2-Dihydroxy-2,4-dimethyl-3-spiro-cyclopropyl-1-{4,7,7-trimethyl-3-oxo-2-oxabicyclo[2,2,1]heptane-1-carboxyl}-bicyclo[4.3.0]nona-5,9-dien-7-one (20).** The title compound was prepared as a white solid by the same procedure as ester-**17**, with *rac trans*-**14** (50 mg, 0.23 mmol), (−)-camphanic acid chloride **13** (149 mg, 0.69 mmol), DMAP (27.7 mg, 0.23 mmol), and Et_3N (0.143 mL, 1.15 mmol) in 2 mL of THF. 85% yield; ^{1}H NMR (C_6D_6) δ 0.12−0.21 (m, 2H); 0.58−0.68 (m, 2H), 0.70 (s, 3H), 0.72 (s, 3H), 0.89 (s, 3H), 0.91 (s, 3H), 1.08 (s, 3H), 1.10 (s, 3H), 1.12−1.37 (m, 7H), 1.65−1.77 (m, 2H), 1.88−1.92 (m, 1H), 1.97−2.11 (m, 2H), 2.53−2.58 (m, 4H), 5.97 (s, 1H), 5.99 (s, 1H), 6.07 (d, J = 1.7 Hz, 1H,), 6.13 (d,, J = 1.7 Hz, 1H); HRMS calcd for $C_{23}H_{28}O_6$ [M + Na]$^+$ = 423.1784, found 423.1812.

(1*S*,2*R*)-8,9-Dihydroxy-3,6,8-trimethyl-7-*spiro*-cyclopropyl-bicyclo[4.3.0]nona-1,3,5-triene (21). To a 25 mL round-bottomed flask was added $CeCl_3 \cdot 7H_2O$ (232 mg, 0.62 mmol). It was dried at 140 °C under vacuum with stirring for 4 h and was then cooled on ice and suspended in THF. The suspension was stirred at 25 °C for 2 h and then cooled to −78 °C. MeLi (0.4 mL of a 1.6 M solution in ether, 0.62 mmol) was added dropwise, and the resulting brown suspension was stirred at −78 °C for an additional 30 min. A solution of resolved ester (1*S*,2*R*)-**15** (25 mg, 0.063 mmol) in 0.5 mL of THF was added dropwise and the mixture was stirred for 1 h. The mixture was poured into an ice-cold solution of dilute HCl and warmed to 25 °C, resulting in a yellow solution. The mixture was extracted twice with EtOAc, and the combined organic extracts were washed with 5% K_2CO_3 solution, dried over anhydrous Na_2SO_4, and filtered. The solvent was evaporated under reduced pressure, and the residue was purified by flash chromatography eluting with 20% EtOAc/hexanes to yield **21** (11 mg, 0.05 mmol, 90% yield) as a yellow gum. ^{1}H NMR ($CDCl_3$) δ 0.80 (m, 1H), 0.90 (m, 1H), 0.97 (m, 1H), 1.15 (s, 1H), 1.20 (m, 1H), 1.58 (d, J = 7.8 Hz, 1H), 1.84 (s, 1H), 2.05 (s, 3H), 2.83 (s, 1H), 4.30 (d, J = 7.8 Hz, 1H), 6.05 (s, H), 6.32 (s, 1H). $[\alpha]^{25}_D$ = −15.7 (c 2.1 mg/mL, $CHCl_3$).

(−)-Acylfulvene ((−)-3) (by IBX-mediated oxidation). The title compound was prepared according to the published procedure[22] with diol (8*R*,9*S*)-**21** (30 mg, 0.14 mmol) and IBX (110 mg, 0.28 mmol) in 4 mL of DMSO. 80% yield. ^{1}H NMR ($CDCl_3$) δ 0.70 (m, 1H), 1.10 (m, 1H), 1.30 (m, 1H), 1.38 (s, 3H), 1.50 (m, 1H), 2.00 (s, 3H), 2.15 (s, 3H), 3.92 (s, 1H), 6.43 (s, 1H), 7.18 (s, 1H).

$[\alpha]^{25}_D = -487.2$ (c 3.5 mg/mL, EtOH). (+)-Acylfulvene ((+)-**3**) was prepared in the same manner: $[\alpha]^{25}_D = +493.2$ (*c* 4.1 mg/mL, EtOH).

(−)-HMAF ((−)-4). The title compound was prepared according to the published procedure[6] with (−)-**3** (20 mg, 0.09 mmol) and formaldehyde (55 mg, 1.85 mmol) in 2 mL of acetone and 2 mL of 5% H_2SO_4 solution. 60% yield. ^{1}H NMR ($CDCl_3$) δ 0.71 (m, 1H), 1.09 (m, 1H), 1.36 (m, 1H), 1.38 (s, 3H), 1.49 (m, 1H), 2.15 (s, 3H), 2.19 (s, 3H), 3.90 (s, 1H), 4.68 (s, 1H), 7.10 (s, 1H). $[\alpha]^{25}_D = -609.8$ (c 2.5 mg/mL, EtOH). (+)-**4** was prepared in the same manner: $[\alpha]^{25}_D = +618.4$ (c 1.4 mg/mL, EtOH).

Expression of AOR in *Escherichia coli* and Purification. Recombinant rAOR was purified mainly following what was previously described.[20] Briefly, plasmid pTrcHisA containing rAOR cDNA with a 6-His tag at N-terminus was transformed into chemically competent *E. coli* strain BL21. AOR was expressed in Luria-Bertani medium containing 100 μg/mL carbenicillin as described previously.[30] Recombinant AOR was purified using nickel-nitrilotriacetic acid superflow resin (Qiagen Inc.) according to manufacturer's recommendations. Elutions were analyzed by SDS-polyacrylamide gel electrphoresis using Coomassie Blue staining. Fractions with >95% purity were pooled and dialyzed overnight against 1 L of dialysis buffer (10 mM potassium phosphate, pH 7.3, 1 mM dithiothreitol, 5 mg/mL complete protease inhibitor cocktail (Roche Inc.), 0.1% Tween-20) at 4 °C overnight. Protein concentration was determined by Bio-Rad protein assay. Aliquots were stored at −80 °C for up to 12 months without significant loss of enzymatic activity.

Cell Culture and Transfection. 293 cells were obtained from ATCC (American Type Culture Collection) and maintained in complete medium (Dulbecco's modified Eagle's medium, high glucose, with 10% heat-deactivated fetal bovine serum). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Plasmid pCEP4-rAOR was cloned as described previously [20] and transfected into 293 cells with Lipofectamine 2000 reagents (Invitrogen) according to the manufacturer's recommendations.

Determination of AOR Enzymatic Activities in Cultured Cells. Cultured cells were collected and lysed in reaction buffer (0.5X PBS (phosphate buffer saline, pH = 7.2), 0.01% Triton X-100) by freezing and thawing in liquid nitrogen and 37 °C water bath three times. Cell lysate was then centrifuged at 12 000*g* for 10 min, and the supernatant was saved. AOR activities were determined spectrophotometrically by monitoring the rate of NADPH oxidation at 340 nm as previously described[20] on a Beckman DU800 spectrophotometer.

Toxicity Studies and Cell Viability Assay. pCEP4 and pCEP4-rAOR transfected 293 cells were maintained in complete medium supplemented with 100 μg/mL hygromycin B (Invitrogen). Cells were plated in 96-well culture plates 18 h prior to treatment at a density of 4000 cells/well. Toxicity studies were conducted by replacing medium with the complete medium containing acylfulvene isomers at desired concentrations. Cell viability was measured 24 h later via MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay). IC_{50} calculation and statistical analyses were performed using Sigmaplot Version 9.0.

Enzyme Kinetic Studies. Enzymatic kinetic studies were performed on a Beckman DU 7400 spectrophotometer. Initial velocities were measured by monitoring the decay of the absorbance at 420 nm ($\epsilon = 2194$ cm^{-1}·M^{-1} for AF, 2210 cm^{-1}·M^{-1} for HMAF) at 37 °C. Stock solutions of analytes were prepared in DMSO (50 mM) and were diluted to a final incubation concentration between 0.05 and 0.4 mM. The percentage of DMSO in each experiment was below 1% of the total volume. NADPH was generated in situ from $NADP^+$, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase.[19] In each experiment, a 1.4 mL UV cuvette containing analyte, 5 mM $NADP^+$, 10 mM glucose-6-phosphate, and 10 units of glucose-6-phosphate dehydrogenase in 1.0 mL of 100 mM phosphate buffer (pH = 7.2) was heated at 37 °C for 5 min. AOR (5.0 μg) was added, and the UV−vis absorbance spectrum was recorded. A 10 and 20 min incubation was performed for HMAF and acylfulvene, respectively.

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