Biochemical Pharmacology, Vol. 57, pp. 83–88, 1999.

ISSN 0006-2952/99/$–see front matter
PII S006-2952(98)00273-1

Metabolism of Antitumor Acylfulvene by Rat Liver Cytosol

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ABSTRACT. Illudins are novel compounds from which a potent class of antitumor agents, called acylfulvenes, have been synthesized. The model illudin, illudin S, has marked *in vitro* and *in vivo* toxicity but displays a poor therapeutic index. The toxicity of illudin S is believed to involve a combination of enzymatic reduction and chemical reaction. Enzymatic reduction by a cytosolic NADPH-dependent enzyme produces an aromatic metabolite, as does reaction with thiols. Acylfulvene is formed from illudin S by reverse Prins reaction. Acylfulvene is 100-fold less toxic *in vitro* and *in vivo* than illudin S but possesses marked antitumor efficacy *in vivo*, thus displaying opposite properties from illudin S. For this reason we investigated the *in vitro* metabolism of acylfulvene. Incubation of acylfulvene with NADPH and rat liver cytosol yielded two metabolites. One metabolite, the aromatic product, is similar to that obtained with illudin S in this *in vitro* system and was anticipated. The other metabolite, the hydroxylated product, was not expected and no corresponding metabolite for illudin S could be detected. The production of this hydroxylated metabolite from acylfulvene may explain, in part, the increased antitumor activity of novel acylfulvenes as compared with the illudins. BIOCHEM PHARMACOL **57**;1:83–88, 1999. © 1998 Elsevier Science Inc.

KEY WORDS. acylfulvene; illudin S; antitumor agents; rat liver cytosol; acylfulvene metabolism

Illudin S (Fig. 1; **1**) is a highly toxic sesquiterpene produced in cultures of the basidiomycete *Omphalotus illudens*, formerly *Clitocybe illudens* [1]. This compound is believed to be responsible for the poisoning that occurs when *Omphalotus* is mistaken for an edible mushroom [2]. Illudins have generated scientific interest because of their antitumor activity [3]. However, they possess a low therapeutic index when tested in tumor-bearing animals. We have prepared many analogs of illudins, several of which display increased histiospecific toxicity toward malignant cells versus normal cells [4, 5]. In particular, treatment of illudin S with dilute sulfuric acid yields acylfulvene (**2**, cf. Fig. 1), which has a greatly improved therapeutic index compared with that of illudin S [6]. One acylfulvene derivative is currently in a human phase I clinical trial and is scheduled for many phase II trials by the NCI [7].

Studies of the mechanism of toxicity of illudins indicate that they behave as alkylating agents. Illudin S reacts spontaneously at room temperature with thiols, such as cysteine or glutathione, at an optimum pH of about 6, and toxicity to myeloid leukemia cells (HL60) can be modulated by altering glutathione levels in cells [8]. The reaction of illudin S with GSH is illustrated in Fig. 2. A Michael-type addition to the α,β-unsaturated ketone gives a cyclohexadiene intermediate, an extremely reactive alkylating agent, which is converted rapidly to a stable aromatic product.

Illudin S has been found to undergo bioreductive activation with NADPH in a rat liver cytosol preparation [9]. Addition of hydride to the α,β-unsaturated ketone presumably occurs producing a highly unstable intermediate, as in the reaction with thiols (cf. Fig. 2). This intermediate is a potent alkylating agent. Aromatic products (**3** and **4**) were isolated from the enzyme-catalyzed reaction.

Acylfulvene (**2**) is about two orders of magnitude less toxic than illudin S but displays an increased antitumor effect [5]. It has been found to react very slowly with thiols, compared with the parent compound. It is, therefore, of interest to study the metabolism of acylfulvene (**2**) with rat liver enzymes in order to obtain further information on the mechanism of toxicity. Identification of metabolites may be important in comprehending the pharmacokinetics of acylfulvenes and in explaining why acylfulvenes have antitumor activity (in contrast to illudins).

MATERIALS AND METHODS

Materials

Illudin S was isolated by extraction of the culture medium of *O. illudens* [10]. Acylfulvene was obtained by treatment of illudin S with 1 M H_2SO_4 at room temperature [4]. Stock

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Received 23 March 1998; accepted 15 July 1998.

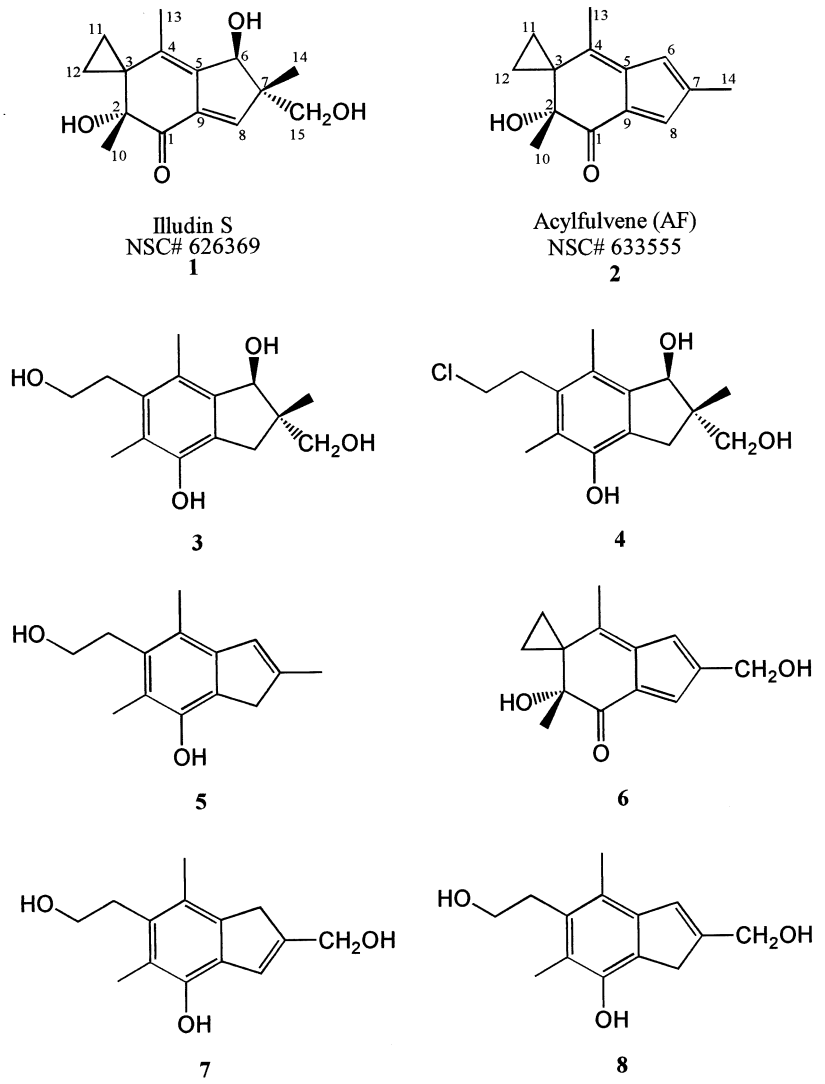

FIG. 1. Illudin S, acylfulvene, and metabolites.

solutions of illudin S and $MgCl_2$ were made in water. All water used was filtered through a "Mega Pure System" (Corning Medical and Scientific Glassworks) to a final resistance greater than 18 Mohms/cm to eliminate trace metals. Catalase (from bovine liver, EC 1.11.1.6), glutathione peroxidase (from bovine erythrocytes, EC 1.11.1.9), and superoxide dismutase (from bovine erythrocytes, EC 1.15.1.1) were all purchased from the Sigma Chemical Co.

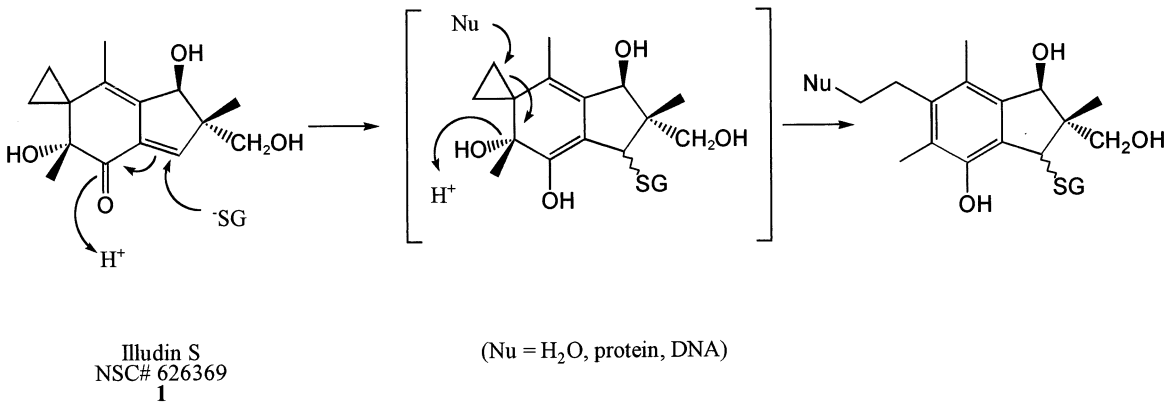

FIG. 2. Reaction of illudin S with glutathione.

TABLE 1. ^{1}H NMR data for acylfulvene (2) and derivatives

Proton	Acylfulvene (2)	5	6	7	8
6-H	6.43 s	6.56 s	6.67 s	3.25 m	6.79 s
8-H	7.16 s	3.21 s	7.30 s	6.87 s	3.31 s
10-H	1.38 s	2.27 s	1.38 s	2.23 s	4.48 s
11-H	0.72 m	3.01 t (7.5)	0.77 m	2.93 t (7.5)	2.94 t (7.5)
11-H	1.30 m		1.3 m		
12-H	1.09 m	3.75 t (7.5)	1.14 m	3.56 t (7.5)	3.57 t (7.5)
12-H	1.30 m		1.4 m		
13-H	2.15 s	2.33 s	2.05 s	2.23 s	2.24 s
14-H	2.00 s	2.16 s	4.61 s	4.46 s	2.33 s
OH	3.95 br s	4.45 br s	3.92 br s	2.31 s	not observed

Spectra were measured at 300 MHz in $CDCl_3$ solutions. Data are ppm from tetramethylsilane. Values in parentheses are coupling constants (Hz).

All other chemicals and reagents were commercially available.

Rat Liver Preparations

Cytosolic and microsomal fractions were obtained from ABS Bioreagents. Rats were killed, and their livers were removed quickly and placed into ice-cold 50 mM K_3PO_4 buffer containing 4 mM $MgCl_2$, 1 mM EDTA, 0.1 M dithiothreitol. Livers were minced with a scalpel and homogenized (1 g/2 mL of buffer). The homogenate was centrifuged at 5000 *g* for 10 min. The supernatant was filtered through two layers of cheesecloth and centrifuged at 100,000 *g* for 60 min. The resulting supernatant, or cytosolic fraction, was stored in 2-mL aliquots. The pellet was resuspended in the original volume of buffer and centrifuged at 100,000 *g* for 60 min. The pellet, or microsomal fraction, was resuspended in 1.5 vol. of buffer (original weight), and then was stored in 2-mL aliquots at −80°.

Incubation Conditions

One milliliter of cytosol was added to illudin S and $MgCl_2$ solutions and 60 mM phosphate buffer, pH 7.4. Then NADPH, in phosphate buffer, was added for a total volume of 5 mL. Final concentrations were: 5.4 mM illudin S, 20 mM $MgCl_2$, and 4 mM NADPH. The phosphate buffer concentration was greater than 10 mM. The solution was incubated at 37° for 30 min with shaking.

Acylfulvene was dissolved initially in DMSO and then diluted with water until the final concentration of DMSO was less than 1%. The cytosol (2.5 to 3 mL) was incubated at 37° for 2 hr, with shaking, in 2.8 mM acylfulvene, 20 mM $MgCl_2$, 4 mM NADPH, and phosphate buffer, pH 7.4, to give a total volume of 5 mL.

Analytical Procedures

The reaction was quenched by cooling to 0° and the metabolites were extracted with ethyl acetate. Extracts were pooled and dried over $MgSO_4$, and the solvent was removed *in vacuo*. The residue was analyzed by TLC, which was performed on Whatman 4410 222 silica gel plates using ethyl acetate:benzene (3:2). Compounds were detected under UV light (254 nm) and by a 1% vanillin/H_2SO_4 spray, followed by heating at 120°. Column chromatography was carried out with silica gel (Davisil 230–425 mesh, Fisher Scientific). ^{1}H NMR spectra were obtained at 300 MHz and taken of solutions in $CDCl_3$ with the internal standard Me_4Si. High-resolution mass spectra were determined at the University of Minnesota Mass Spectrometry Service Laboratory. X-ray crystallographic analysis was performed in the Department of Chemistry and Biochemistry at the University of California, San Diego.

Isolation of Metabolites 3 and 4

Incubation of illudin S was carried out as described above. The metabolites **3** and **4** obtained upon purification by chromatography had properties identical to those previously reported [9].

Isolation of Metabolites 5 and 6

Incubation of acylfulvene was carried out with 2–3 times the amount of rat liver than that with illudin S and for 2 hr at 37°. Chromatography of the crude product gave unchanged acylfulvene, metabolite **5** and metabolite **6**. Properties for **5** include: m.p. 200–202°; UV (MeOH) λ_{max} 265 nm, 300 nm, and 310 nm, ϵ 9321, 2810, and 2658, respectively. ^{1}H NMR data are given in Table 1; HRMS $C_{14}H_{18}O_2$ M^+ *m/z* 218.1304, M^+ 187.1119 (M^+-CH_2OH, base peak, $C_{13}H_{15}O$). For **6**: UV (MeOH) λ_{max} 235 nm and 327 nm, ϵ 16,800 and 7631, respectively. ^{1}H NMR data are given in Table 1; HRMS $C_{14}H_{16}O_3$ M^+ *m/z* 232.1097 (base peak), 217.0854 (M^+-CH_3, $C_{13}H_{13}O_3$).

Isolation of Metabolite 8

A sample of **6** (3.5 mg, 0.015 mmol) was incubated with 2.7 mL rat liver cytosol at 37° for 2 hr similarly to the incubation of acylfulvene. Isolation of metabolite **8** was carried out in the usual way. The ^{1}H NMR spectrum (Table

1) shows slight differences from that of compound **7** obtained by non-enzymatic reduction of **6** (*vide infra*). However, **7** and **8** had the same R_f on chromatography. Mass spectra of **7** and **8** showed the same molecular ion $C_{14}H_{18}O_3$ M^+ *m/z* 234.1263 (base peak), 203.1055 (M^+ -CH_2OH, $C_{13}H_{15}O_2$), with slightly different fragmentation patterns.

Synthesis of 5

To a mixture of illudin S (105 mg, 0.396 mmol), zinc dust (1 g, 15.7 mmol) and 10 mL of water was added 0.5 mL of 2 M H_2SO_4, and the mixture was stirred at room temperature. H_2SO_4 (0.5 mL) was added to the reaction mixture after 5 and 17 hr. After 24 hr, the reaction mixture was extracted with ethyl acetate and the organic extract was washed with $NaHCO_3$ solution and saline. After drying over $MgSO_4$, the extract was concentrated and chromatographed to give 45 mg reduced product. The reduced product was dissolved in 3 mL of 1 M H_2SO_4 and the solution was stirred at room temperature for 2 days. It was extracted with ethyl acetate, and then the organic extract was washed with $NaHCO_3$ solution and saline. The extract was dried over $MgSO_4$. After concentration, the crude product was chromatographed to give 15 mg of compound **5** with spectral properties identical to those of the metabolite from acylfulvene.

Synthesis of 6

To a solution of acylfulvene (**2**), (6.9 g, 31.9 mmol) in 70 mL ethyl acetate was added selenium dioxide (1.75 g, 15.9 mmol) and 6 mL of 90% *t*-butyl-hydroperoxide (54.1 mmol, d 0.901). The mixture was stirred at room temperature for 24 hr, and then was washed with Na_2SO_3 solution and saline. The organic layer was dried over $MgSO_4$. After filtration, the crude product was chromatographed to give 923 mg of **6**, with 5.81 g of recovered **2**. Spectral properties of the synthetic product were identical to those of the hydroxylated metabolite of acylfulvene.

Synthesis of 7

To a mixture of **6** (100 mg, 0.431 mmol), zinc dust (1.12 g,17.1 mmol), 5 mL water and 5 mL acetone was added 0.5 mL of 2 M H_2SO_4. The mixture was stirred for 90 min at room temperature. Work-up as above afforded 28.0 mg of crystalline **7**: m.p. 195–197°. ^{1}H NMR data are included in Table 1.

X-ray Crystal Structure Analysis of 7, $C_{14}H_{18}O_3$, *at 298 K*

P1, a = 8.494 (4) Å, b = 8.735 (4) Å, c = 9.417 (4) Å, α = 102.19 (4)°, β = 116.37 (4)°, γ = 90.61 (4)°, *z* = 2, calculated density 1.264 g/cm^3, 5°/min 0.6° Ω scan range, 1347 observed [$I > 2\sigma(I)$] from 2.40 to 25° θ, R = 0.1458, residual electron density 0.541 and −0.666 e/A^3.

RESULTS AND DISCUSSION

Illudin S was reduced enzymatically with either NADPH or NADP as cofactors, rat liver cytosol and $MgCl_2$, to form metabolites **3** and **4**, as reported previously by Tanaka *et al.* [11]. However, chlorinated metabolite **4** was obtained only when a 10-fold increase in concentration of $MgCl_2$ was used. All of the illudin S was consumed after incubation for 30 min. Metabolism of illudin S by rat liver microsomal fraction was much slower than that with the cytosolic fraction. Only about 5% of the amount of **3** and **4** was isolated from the microsomal experiments. It is possible that metabolism attributed to the microsomes was actually caused by enzymes localized in the cytosol that were not removed completely in the separation of cytosol from microsomes.

Metabolism of the yellow acylfulvene (**2**) by rat liver showed significant differences from that of illudin S. The metabolism was slower, but by increasing the proportion of cytosol and allowing the incubation to proceed for 2 hr, metabolites **5** and **6** could be isolated. Metabolite **5**, from acylfulvene (**2**), is analogous to metabolite **3** from illudin S. However, there was no metabolite corresponding to **6** in the illudin S system. TLC analysis showed **5** and **6** as more polar compounds compared with acylfulvene (**2**) (**2**, R_f 0.9; **5**, R_f 0.7; **6**, R_f 0.54); **5** turned blue upon development with vanillin reagent. These polar compounds, **5** and **6**, were clearly metabolites of acylfulvene (**2**), since they were not produced in the incubation mixture in the absence of substrate or NADPH. The metabolites were also not observed when the cytosolic fraction was boiled for 15 min prior to the addition of substrate and cofactor. About 2% of acylfulvene (**2**) was metabolized to **6**, with a trace amount of **5** detected by TLC, and 24% of acylfulvene (**2**) was recovered unchanged. When NAD was used as a cofactor, the yield of **5** was increased to 10%, a trace amount of **6** was detected by TLC, and 30% of acylfulvene (**2**) was recovered. The remainder of acylfulvene presumably is bound to macromolecules, in agreement with work previously reported for illudin S [12].

The structure of metabolite **5** was established by spectral analysis. The high-resolution mass spectrum indicated a molecular formula of $C_{14}H_{18}O_2$ (M^+ *m/z* 218.1304, calculated 218.1333). The ^{1}HNMR spectrum (Table 1) exhibited signals for a vinyl methyl and two aromatic methyls (δ 2.16, 2.27, 2.33), the hydroxyethyl side chain (δ 3.01 t, 3.75 t), a methylene singlet (δ 3.21), and a vinyl proton (δ 6.56).

The structure for metabolite **5** was confirmed by reacting illudin S with zinc dust and dilute sulfuric acid at room temperature to give a crude product containing compound **3**. Upon further exposure to dilute sulfuric acid, the product underwent reverse Prins reaction, yielding the indenol **5**.

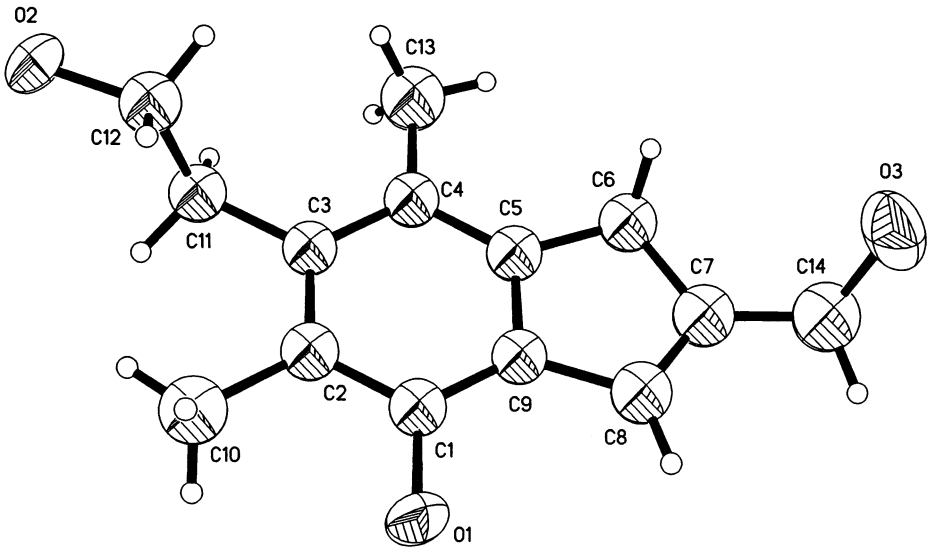

FIG. 3. ORTEP view of x-ray molecular structure of 7.

(This sequence of reactions was used previously to prepare similar derivatives of illudin S [1].)

Metabolite **5** evidently is formed from acylfulvene in a manner similar to that of **3** from illudin S. Thus, bioreductive activation of acylfulvene gives a very reactive intermediate that is converted rapidly to an aromatic product (cf. Fig. 2). Water or macromolecules (protein, DNA) are alkylated in the process.

Metabolite **6** was also characterized by spectral analysis. The yellow color suggested that the acylfulvene chromophore was unchanged: UV λ_{max} 235 and 327 nm, ϵ 16,800 and 7631. The high-resolution mass spectrum indicated a molecular formula of $C_{14}H_{16}O_3$ (M^+ *m/z* 232.1097, calculated 232.1100). The ^1H NMR spectrum (Table 1) gave signals for four cyclopropane protons (δ 0.77, 1.3, 1.14, 1.4), a tertiary methyl (δ 1.38), a vinyl methyl (δ 2.05), hydroxyl (hydrogen bonded δ 3.92), a methylene (δ 4.61) and two vinyl protons (δ 6.67, 7.30).

The structure for metabolite **6** was confirmed by synthesis. Acylfulvene (**2**) was oxidized with selenium dioxide and *t*-butyl hydroperoxide in ethyl acetate, forming **6**. Allylic hydroxylation could occur on C14 or the more activated methyl (C13). To distinguish between the two possibilities, a sample of **6** was reduced with zinc dust and sulfuric acid to give an aromatic product, **7**. X-ray crystallographic analysis of **7** established its structure as shown in Fig. 3. Thus, location of the hydroxymethyl group in **6** is assigned unambiguously.

Formation of metabolites **5** and **6**, one by reduction and the other by oxidation of substrate **2**, is unusual. In the case of illudin S, only reduction products were detected. Other investigators who studied this reaction found that the enzyme systems involved may differ from DT-diaphorase, aldehyde oxidase, xanthine oxidase, ketone reductase, aldehyde reductase, and alcohol dehydrogenase, known cytosolic enzymes responsible for xenobiotic metabolism [11]. Enzymes metabolizing illudin S utilize NADPH in preference to NADH, are not affected by oxygen, and have low pH optima.

Metabolism of acylfulvene (**2**) gave compound **5**, which, like **3** and **4**, was formed in the presence of NADPH. However, NADH could also serve as a cofactor. The most intriguing finding was the unexpected formation of hydroxylated derivative **6** from acylfulvene (**2**). Use of the cofactor NADH generated an increased yield of metabolite **5** relative to **6**, whereas using NADPH caused an increase in the amount of **6** relative to **5**.

To test the possibility that cytochrome P450 enzymes were involved, the cytosolic fraction was flushed for 30 min with carbon monoxide, which is known to bind to the active site Fe of P450 and inhibit any reactions it might catalyze [13]. However, this had no effect on the formation of **5** and **6**. Pretreatment of cytosol with carbon monoxide was also found to have no effect on the metabolism of illudin S (**1**) to **3** and **4**, confirming that cytochrome P450 is not involved (in agreement with work previously reported) [11].

Experiments were also carried out to examine the possibility of a non-specific oxidation of acylfulvene (**2**) with H_2O_2 formed during the incubation to produce metabolite **5**. Separate incubations were done with catalase (2000 U/5 mL metabolism experiment), glutathione peroxidase (100 U/5 mL metabolism experiment) and superoxide dismutase (100 U/5 mL metabolism experiment) to eliminate any free peroxides or superoxide. There was no effect on the formation of metabolites **5** and **6**.

Our studies indicate that several enzyme systems are involved in the metabolism of illudin S and acylfulvene. The reduction leading to an aromatic product appears to be unprecedented. Either a novel enzyme or mechanism may be operating. Acylfulvene metabolism differs from illudin S in that while metabolite **5** is analogous to metabolite **3** from illudin S, metabolite **6** has no corresponding metabolite in the illudin S system. The oxidative reaction leading

to **6** is not uncommon, but it is surprising that a P450 enzyme does not seem to be involved. Compound **6**, which is more water-soluble than acylfulvene (**2**), can be metabolized further by rat liver cytosol, producing metabolite **8**. (The structure of **8** was confirmed by spectral analysis.) We conclude that the difference in acylfulvene metabolism compared with illudin S may explain the increased antitumor effect of acylfulvene.

X-ray crystallographic analysis was performed by Dr. Peter K. Gantzel, Department of Chemistry and Biochemistry, University of California, San Diego. This investigation was supported by funds provided by the Cigarette and Tobacco Tax Fund of the State of California through the Tobacco-related Disease Research Program of the University of California (Award 4RT-0226 and 4IT-0194), and by funds provided by MGI PHARMA, Inc., Minneapolis, MN.

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