Microbial Epoxidation of the Tricyclic Sesquiterpene Presilphiperfolane Angelate Ester

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Microbial Transformation, *Mucor ramannianus,* (2'*R*,3'*R*)-(+)-2β-(2',3'-epoxyangeloyloxy)-5β,8β-dihydroxypresilphiperfolane

Microbial transformation studies on 2β-angeloyloxy-5β,8β-dihydroxypresilphiperfolane have revealed that it was metabolized by a number of microorganisms. Using a standard two-stage fermentation technique, *Mucor ramannianus* (ATCC 9628) produced three metabolites. One of them was characterized as the novel metabolite (2'*R*,3'*R*)-(+)-2β-(2',3'-epoxyangeloyloxy)-5β,8β-dihydroxypresilphiperfolane on the basis of spectral data. The absolute configuration at both oxirane carbons was confirmed by spectral and optical activity data of the hydrolysis product of the novel metabolite which is (2*R*,3*R*)-(+)-2,3-epoxyangelic acid.

2β-angeloyloxy-5β,8β-dihydroxypresilphiperfolane (**1**), a rare tricyclic sesquiterpene angelate ester, was isolated from the aerial parts of *Senecio hadiensis* Forssk. (Astraceae) (Al-Balawi, 1999). This sesquiterpene was also chemically obtained by simple alkaline hydrolysis of its naturally occurring 5β-actetate ester (Bohlmann *et al.*, 1982). Both compounds are derivatives of presilphiperfolane, the common precursor of several tricyclic sesquiterpenes (Bohlmann *et al.*, 1981). *Senecio* species leaves are widely used as a folk medicine in the southern region of Saudi Arabia for their antipyretic properties (Ghazanfar, 1994).

The utilization of microbes as models for mammalian metabolism of xenobiotics has been well established since this concept was first introduced by Smith and Rosazza in the early seventies (Orabi, 2000; Rosazza and Smith, 1979). This concept depends on the fact that fungi, being eukaryotes, possess a metabolizing enzymatic machinery similar to those of mammals. Hence, the outcome of xenobiotic metabolism in both systems is expected to be similar, if not identical, and, thus, fungi can serve as reliable, convenient, predictive models for mammalian metabolism of various xenobiotics.

It is anticipated that the microbial metabolism of **1** would produce significant quantities of metabolites that would be difficult to obtain from either animal systems or chemical synthesis. Moreover, microbial metabolism may provide some novel metabolites that may serve as starting compounds for semi-synthesis of other derivatives, or as analytical standards for mammalian metabolic studies.

This report deals with the microbial bioconversion of **1** and the isolation and characterization of one of its metabolites.

Results and Discussion

Of twenty microbial cultures screened for their ability to catalyze bioconversion of 2β-angeloyloxy-5β,8β-dihydroxypresilphiperfolane (**1**), *Absidia glauca* ATCC 22752, *Cunninghamella species* NRRL 5695, *Mucor ramannianus* ATCC 9628, *Penicillium chrysogenum* ATCC 9480 and *Streptomyces spectabilis* ATCC 27465 showed definite metabolism. These cultures produced several more polar metabolites, some of which have been produced by more than one culture. It was shown (TLC basis) that *M. ramannianus* ATCC 9628 was the most efficient microorganism to metabolize **1** into three metabolites and, therefore, was selected for preparative-scale fermentation. These metabolites were isolated by solvent extraction, and only metabolite **2** was purified by chromatography and subjected to spectral analyses, while the other two were too unstable to be purified and recognized. Complete unambiguous assignments of ^{1}H and ^{13}C resonances of **1** were previously reported (Al-Balawi, 1999; Bohlmann *et al.*, 1982).

Metabolite **2** was isolated as colorless oil in a 7.0% yield. High resolution electrospray ionization mass spectroscopy (HRESIMS) of **2** gave the

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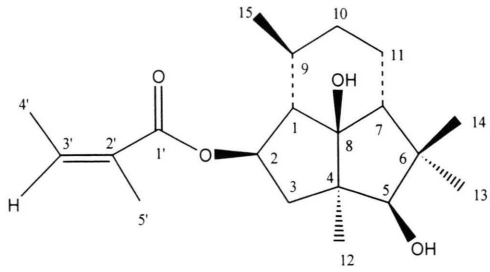

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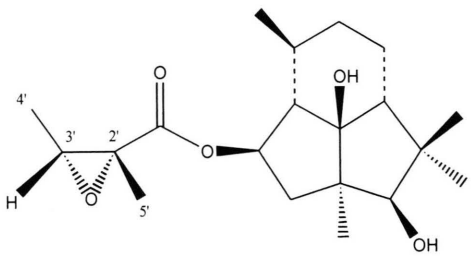

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characteristic ion peak at *m/z* 353.2342 $[M+H]^+$, suggesting the molecular formula $C_{20}H_{32}O_5$. Most ^1H and ^{13}C NMR (Table I) spectral data of **2** were similar to those of **1** except for resonances attributed to the angelate ester moiety. The ^{13}C NMR spectra revealed the conversion of the olefinic carbons, C-2' at δ 128.5 and C-3' at δ 137.7, into an oxygenated quaternary carbon at δ 60.1 (C-2') and an oxygenated methine carbon at δ 60.2 (C-3'). These changes suggested the conversion of the olefinic system into an oxirane ring. This was further supported by the presence of three-bond correlations in the "heteronuclear multiple bonds connectivity" spectra (^3J-HMBC) between C-1' (δ 169.9, s) and H-3' (δ 3.02, q, J = 5.4), C-1' and H_3-5' (δ 1.53, s), C-2' (δ 60.1, s) and H_3-4' (δ 1.31, d, J = 5.4), C-3' (δ 60.2, d) and H_3-5', and C-5' (δ 19.5, q) and H-3'. The exchangeable proton OH-5 was assigned to resonate at δ 3.84 (br s), due to the presence of a cross peak in the COSY spectrum with H-5 (δ 3.34, d, J = 4.4).

The nuclear Overhauser effect spectroscopy (NOESY) of **2** suggested a *cis* orientation for the

Table I. ^1H and ^{13}C NMR assignments of compounds **1** and **2**[a].

	1		**2**	
Position	δ_C[b]	δ_H (m, J Hz)	δ_C	δ_H (m, J Hz)
1	57.4, d	1.44 (d, 11.1)	57.4, d	1.39 (dd, 10.3, 2.2)
2	84.6, d	5.46 (ddd, 7.1, 7.1, 2.5)	85.5, d	5.49 (ddd, 7.0, 7.0, 2.2)
3 α	38.9, t	2.0 (m)	38.9, t	1.96 (dd, 11.5, 7.0)
3 β		2.24 (m)		2.17 (dd, 11.5, 7.0)
4	58.3, s		58.3, s	
5	87.1, d	3.34 (m)	87.0, d	3.34 (d, 4.4)
6	51.9, s		52.0, s	
7	53.3, d	1.74 (dd, 11.9, 7.8)	53.4, d	1.73 (dd, 12.4, 7.7)
8	95.6, s		95.4, s	
9	36.0, d	1.53 (m)	35.8, d	1.51 (m)
10 α	34.3, t	1.05 (m)	34.2, t	1.05 (m)
10 β		1.68 (m)		1.67 (m)
11 α	26.6, t	1.32 (m)	26.5, t	1.32 (m)
11 β		1.66 (m)		1.65 (m)
12	27.9, q	1.21 (s)	27.9, q	1.20 (s)
13	27.6, q	1.20 (s)	27.6, q	1.19 (s)
14	30.3, q	1.23 (s)	30.3, q	1.22 (s)
15	21.8, q	0.96 (d, 6.0)	21.7, q	0.93 (d, 6.2)
1'	168.3, s		169.9, s	
2'	128.5, s		60.1, s	
3'	137.7, d	6.02 (m)	60.2, d	3.02 (q, 5.4)
4'	16.1 q	1.96 (d, 7.1)	14.1 q	1.31 (d, 5.4)
5'	20.9, q	1.87 (d, 1.5)	19.5, q	1.53 (s)
OH-5		3.55 (m)		3.84 (br s)
OH-8		3.89 (m)		3.40 (br s)

[a] Spectra recorded in $CDCl_3$. [b] ^{13}C multiplicities were determined by DEPT 135°.

oxirane ring, due to the presence of a cross peak between H-3' and H-5'. Moreover, the configuration at both oxirane carbons was concluded to be *R*, based on the chemical shift values of the two epoxyangelate methyl groups, as previously reported for several natural products (Valencia *et al.*, 1998a; Bohlmann *et al.*, 1979). Thus, CH_3-4' and CH_3-5' were found to resonate at δ 1.31 and 1.53, respectively, giving a $\Delta\ \delta_{5'\text{-}4'} = 0.22$, identical to other previously reported (2*R*,3*R*)-(+)-2,3-epoxyangelates, which is in a full agreement with that of the previously reported method (Valencia *et al.*, 1998a).

Furthermore, the alkaline hydrolysis of **2**, using NaOH in MeOH, yielded (2*R*,3*R*)-(+)-2,3-epoxyangelate sodium salt, which upon neutralization with HCl in aqueous NH_4Cl solution gave the corresponding (2*R*,3*R*)-(+)-2,3-epoxyangelic acid. The specific optical activity of this acid was found to be + 25.9°. Valencia *et al.* (1998b) reported the optical activities for both (2*R*,3*R*)-(+)- and (2*S*,3*S*)-(−)-2,3-epoxyangelic acids to be +30.0° and −29.0°, respectively. Other spectral data were indistinguishable from those previously reported for (2*R*,3*R*)-(+)-2,3-epoxyangelic acid (Valencia *et al.*, 1998b).

These aforementioned data constituted a solid body of evidence to confirm the absolute stereochemistry of the epoxyangelate moiety to be 2*R*, 3*R*.

Since no other stereoisomers of **2** was detected in the culture media, microbial epoxidation must have proceeded stereospecifically, and **2** can be formulated as (2'*R*,3'*R*)-(+)-2β-(2',3'-epoxyangeloyloxy)-5β,8β-dihydroxypresilphiperfolane.

The oxidation of aliphatic C=C double bonds with the formation of optically active epoxides is the domain of bacteria. However, some fungi can form epoxides in cyclic systems like steroids with accessible double bonds as well as exocyclic double bonds of non-steroidal compounds (Kieslich, 1997, 1976), as shown in this work.

It is worth mentioning that diverse compounds containing epoxyangelate moieties possess important biological activities as antibiotics (Kato *et al.*, 1996), tumor inhibitors (Kimura *et al.*, 1987), or anti-inflammatory agents (Alam *et al.*, 1992), and that in one case, the biological activity has been attributed to the presence of the epoxyangelate moiety (Alam *et al.*, 1992).

Experimental section

General experimental procedures

The IR spectra were recorded in KBr disk using an ATI Mattson Genesis Series FTIR spectrophotometer. Optical rotations were taken with a Perkin-Elmer 241 MC polarimeter. The ^1H and ^{13}C NMR were obtained by a Bruker DRX-500 spectrometer operating at 500 and 125 MHz, respectively. Both ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$, and the chemical shift values were expressed in δ (ppm) relative to the internal standard TMS. For the ^{13}C NMR spectra, the number of attached protons was determined by "distortionless enhancement by polarization transfer" (DEPT) 135° experiments. 2D NMR data were obtained using the standard pulse sequence of the Bruker DRX-500 for COSY, heteronuclear multiple quantum coherence (HMQC), HMBC and NOESY. HRMS was carried out using a Bruker Bioapex FTMS with electrospray ionization spectrometer.

Cultures and fermentation screening procedure

The microbial cultures were originally obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, or from the USDA Northern Regional Research Laboratories (NRRL), Peoria, Illinois, and are maintained in King Saud University, Department of Pharmacognosy Culture Collection. Stock Cultures were maintained on agar slants of media recommended by the ATCC and were stored at 4 °C.

Preliminary screening and preparative-scale experiments were carried out as reported (Orabi *et al.*, 1999), according to a standard two-stage protocol (Orabi, 2000). Substrate **1** was prepared as a 15% (w/v) solution in *N,N*-dimethylformamide (DMF) and added to the 24-h-old stage II culture medium of the microorganism at a concentration 0.3 mg/ml medium. Substrate controls were composed of sterile medium to which the substrate (4 mg/100μl DMF) was added and incubated without microorganisms. Culture controls consisted of fermentation blanks in which the microorganisms were grown under identical conditions but without the substrate addition. After two weeks of incubation, each control was harvested and analyzed by TLC.

Fermentation sampling and chromatographic conditions

The fermentations were sampled by withdrawing 5 ml of culture and extracting it with 5 ml of $CHCl_3$. The concentrated organic phase was analyzed by TLC for the presence of metabolites. TLC analyses were performed on precoated Si gel 60 F_{254} (Merck) plates using 4% MeOH in $CHCl_3$ as the solvent system. Visualization was accomplished by spraying with *p*-anisaldehyde spray reagent. The adsorbent used for column chromatography was Si gel 60/230–400 mesh (EM Science). 2β-angeloyloxy-5β,8β-dihydroxypresilphiperfolane (**1**), the substrate used in this project, was generously supplied by Dr. Farid Al-Muhtadi, and Mr. Sulaiman Al-Balawi of the College of Pharmacy, King Saud University, and was originally isolated from the aerial parts of *Senecio hadiensis* as reported (Al-Balawi, 1999). All solvents used for chromatographic purposes were reagent grade.

Preparative scale fermentation of **1** *by Mucor ramannianus*

M. ramannianus ATCC 9628 was grown in six 1-l culture flasks each containing 200 ml of medium α (Orabi *et al.*, 1999). A total of 480 mg of **1** (in 3.2 ml DMF) was evenly distributed among the 24-h-old stage II culture. After two weeks, the incubation mixtures were checked by TLC. TLC revealed that most of **1** was transformed and three metabolites were produced.

The incubation mixtures were combined and filtered to remove the mycelia, and the filtrate (1.2 l) was extracted with $CHCl_3$ (1 l × 4). The combined extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo* at 40 °C to afford a brownish residue (0.64 g), which was purified by column chromatography over Si gel (60 g, 3 × 26 cm) using MeOH- $CHCl_3$ (1:20 v/v) as the eluting solvent system. Fractions of 20 ml each were collected and pooled on the basis of TLC analyses. Fractions 12–18 yielded semi-pure (2'*R*,3'*R*)-(+)-2β-(2',3'-epoxyangeloyloxy)-5β,8β-dihydroxypresilphiperfolane (**2**) (70 mg), which upon further purification afforded 35 mg of pure **2** (7.0% yield) with R_f = 0.56. Moreover, fractions 24–33 and 37–41 yielded 7.1 and 18.6 mg of other two more polar metabolites with R_f = 0.42 and 0.26, respectively. Those two metabolites were too unstable to be purified and recognized.

(2'R,3'R)-(+)-2β-(2',3'-epoxyangeloyloxy)-5β,8β-dihydroxypresilphiperfolane (**2**):

Colorless oil; $[\alpha]_D^{25}$ + 7.9° (*c* 0.20, $CHCl_3$). IR (KBr) cm^{-1}: 3360, 2940, 1740, 1250. ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) see Table I. HRESIMS *m/z*: 353.2342 (Calcd. for $C_{20}H_{33}O_5$ $[M+H]^+$: 353.2327).

Alkaline hydrolysis of **2**

A solution of **2** (30 mg) in MeOH (1.5 ml) was stirred at room temperature with NaOH (5 mg) for 18 h. The solvent was removed with an N_2 stream, and acetone (3 ml) was added to precipitate the sodium salt. The suspension was filtered off and washed with acetone (3 ml X 2) to yield a colorless residue [(2*R*,3*R*)-(+)-2,3-epoxyangelate sodium salt, 10 mg, 83% yield]. This residue was dissolved in a saturated solution of NH_4Cl (3 ml), cooled on an ice bath, neutralized with 0.5 N HCl and then extracted with EtOAc (3 ml X 3). The EtOAc layer was dried over anhydrous Na_2SO_4 and evaporated to dryness at room temperature to yield (2*R*,3*R*)-(+)-2,3-epoxyangelic acid (5.4 mg, 64% yield).

(2R,3R)-(+)-2,3-epoxyangelic acid

colorless oil; $[\alpha]_D^{25}$ + 25.9° (*c* 0.11, $CHCl_3$). IR (neat) cm^{-1}: 3660, 3460, 1720, 1250. ^1H and ^{13}C NMR ($CDCl_3$) were indistinguishable from those previously reported (Valencia *et al.*, 1998 b). HRESIMS *m/z*: 117.0569 (Calcd. for $C_5H_9O_3$ $[M+H]^+$: 117.0552).

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