Microbial Transformation of Pyrethrosin

Ahmed M. Galal*

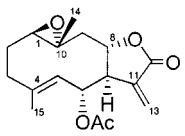
National Center for Natural Products Research, School of Pharmacy, University of Mississippi, University, Mississippi 38677

Received January 12, 2001

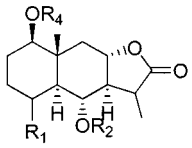
Microbial transformation of the germacranolide pyrethrosin (**1**) using *Rhizopus nigricans* NRRL 1477 has resulted in the isolation of 6α-acetoxy-1*β*,4α-dihydroxy-5,7α*H*,8*β*H-eudesm-11*β*,13-dihydro-8,12-olide (**5**), a new eudesmanolide-type metabolite, in addition to the previously reported eudesmanolides: **2**, **3**, **4**, and **6**. The structure elucidation of these metabolites was based primarily on 1D and 2D NMR analyses. The isolated metabolites exhibited cytotoxic, antifungal, and antiprotozoal activities.

Pyrethrosin (**1**), the first recognized germacrane sesquiterpene lactone,[1,2] was isolated from the flowering heads of *Chrysanthemum cinerariaefolium* Visiani[3] (Asteraceae). Pyrethrosin exhibits diverse biological activities including cytotoxicity,[4] antibacterial,[5] antifungal,[6] molluscicidal,[7] and phytotoxicity.[8]

Microbial metabolism studies have been used as inexpensive in vitro model systems to predict mammalian metabolism or to increase the efficacy of a drug through metabolic activation.[9] Pyrethrosin (**1**), with its diverse bioactivity range, was subjected to microbial metabolism in an attempt to find more active analogues. Twenty-four growing cultures were screened for their ability to biotransform **1**. Of these, *Cunninghamella elegans* NRRL 1392 and *Rhizopus nigricans* NRRL 1477 were able to completely deplete and transform **1** into the same five more polar metabolites. *R. nigricans* (NRRL 1477) was chosen for preparative-scale fermentation due to its higher metabolite yield as compared to *C. elegans* NRRL. Repeated column chromatography of the crude metabolite mixture furnished five compounds, **2**, **3**, **4**, **5**, and **6**. Compound **2** was previously isolated from the flowers of *Chrysanthemum cinerariaefolium* Visiani (Pyrethrum flower), while **6** was isolated from *Cassinia loevis* R.Br. Compounds **3**, **4**, and **6** were previously synthesized from pyrethrosin (**1**) using $BF_3{\cdot}OEt_2$ and *p*-toluenesulfonic acid to convert the germacrane ring into the eudesmane skeleton.[10]



1



	R_1	R_2	R_3	R_4	Other
2	$\Delta^{4,15}$	Ac	α-CH_3	H	-
3	CH_3	H	α-CH_3	H	$\Delta^{3,4}$
4	$\Delta^{4,15}$	H	α-CH_3	H	-
5	α-OH, β-CH_3	Ac	α-CH_3	H	-
6	α-OH, β-CH_3	Ac	$\Delta^{11,13}$	H	-
7	α-OH, β-CH_3	Ac	α-CH_3	Ac	-

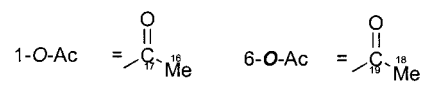


Table 1. ^{13}C NMR Chemical Shift Assignments of Compounds **2**, **3**, **5**, and **7**[a]

position	**2**	**3**	**5**	**7**[b]
1	78.6, d	76.1, d	78.3, d	79.1, d
2	31.8, t	33.5, t	27.9, t	24.2, t
3	34.7, t	123.3, d	40.2, t	40.2, t
4	143.1, s	133.8, s	72.3, s	71.5, s
5	54.0, d	54.3, d	57.2, d	56.9, d
6	69.9, d	71.6, d	72.6, d	71.8, d
7	56.6, d	59.6, d	57.9, d	57.3, d
8	76.5, d	76.9, d	76.4, d	75.3, d
9	40.4, t	39.4, t	43.2, t	42.4, t
10	43.0, s	41.1, s	41.8, s	40.5, s
11	40.9, d	41.9, d	41.1, d	40.3, d
12	178.9, s	179.6, s	178.6, s	177.6, s
13	14.2, q	15.1, q	14.4, q	13.9, q
14	14.2, q	13.3, q	16.2, q	16.8, q
15	109.0, t	24.0, q	24.7, q	24.2, q
16				21.0, q
17				169.6, s
18	21.3, q		21.8, q	21.3, q
19	171.3, s		170.1, s	170.2, s

[a] In $CDCl_3$ at 125 MHz. Carbon multiplicities were determined by DEPT 135° experiments. [b] At 75 MHz.

The positive ion HRESIFTMS spectrum of **5** displayed a molecular ion peak $[M + H]^+$ at *m/z* 327.1822, suggesting the molecular formula $C_{17}H_{24}O_6$. The IR spectrum of **5** showed a broad absorption band at 3427 cm^{-1}, suggesting the presence of a hydroxyl group. It also showed a strong absorption band at 1767 cm^{-1}, consistent with the existence of a *γ*-lactone carbonyl functionality. The ^{13}C and ^1H NMR data of **5** (Table 1 and experimental) were in agreement with a eudesmanolide skeleton.[12] Inspection of the NMR spectra of **5** revealed close similarity to those of the previously reported compound 6α-acetoxy-1*β*,4α-dihydroxy-5,7α*H*,8*β*H-eudes-11-en-8,12-olide (**6**),[12] except for the presence of a methyl group at C-11, resonating at *δ* 14.4 (d, Me-13) in **5**, instead of an exocyclic methylene functionality in **6**. The assignment of the relative stereochemistry of the stereocenter C-11 was deduced from NOESY and ROESY data. The *β*-oriented Me-15 displayed NOESY correlation with the downfield H-11, suggesting a similar stereoorientation. Moreover, the α-oriented acetate methyl at C-6 showed ROESY correlation with Me-13, suggesting α-orientation for Me-13 and supporting the relative stereochemistry assignment of C-13. Therefore, metabolite **5** was

* To whom correspondence should be addressed. Tel: (662) 915-1019. Fax: (662) 915-7989. E-mail: amgalalv@yahoo.com.

10.1021/np0100082 CCC: $20.00
Published on Web 07/21/2001

established as 6α-acetoxy-1β,4α-dihydroxy-5,7α*H*,8β*H*-eudesm-11β,13-dihydro-8,12-olide.

Metabolites **2**, **3**, **4**, and **6** were identified by comparing their spectral data with literature.[3,10] All isolated new and known compounds were tested for cytotoxicity, antimicrobial, and antimalarial activities using standard methods described elsewhere.[11−13]

Metabolite **5** and its 1-*O*-acetate derivative (**7**) exhibited antiprotozoal activity against *Plasmodium falciparum* (D6 clone) with IC_{50} 0.88 and 0.32 μg/mL, respectively, without significant toxicity. Compound **7** also displayed a pronounced activity against the chloroquine-resistant strain of *P. falciparum* (W2 clone) with IC_{50} 0.38 μg/mL. Compound **2** showed considerable in vitro cytotoxic activity against human epidermoid carcinoma (KB) and against human ovary carcinoma (SK-OV-3) with IC_{50} <1.1 and 8.0 μg/mL, respectively. Pyrethrosin (**1**) and compound **3** exhibited cytotoxic activity against human malignant melanoma (SK-MEL) with IC_{50} 4.20 and 7.5 μg/mL, respectively. Compounds **4** and **7** were active against *Cryptococcus neoformans* with IC_{50} 35.0 and 25 μg/mL, respectively, while **1** and **5** displayed anticandidal activity (*Candida albicans* B311) with IC_{50} 30 and 10 μg/mL, respectively. Metabolites **2**, **3**, **4**, **5**, and **7** are more cytotoxic and antifungal than the parent compound (**1**).[4]

Experimental Section

General Experimental Procedures. IR spectra were recorded with an ATI Mattson Genesis Series Fourier transform (FT-IR) spectrophotometer. Optical rotations were recorded at ambient temperature using a JASCO DIP-370 digital polarimeter. 1D and 2D NMR spectra were obtained on Bruker Avance DRX 400 and 500 spectrometers. HRESIFTMS was obtained using a Bruker Bioapex FT-MS in ESI mode. For TLC, glass-supported silica gel 60 plates (0.25 mm layer, F_{250}, E. Merck) were used. Silica gel 60 (230−400 mesh, E. Merck) was used for column chromatography. Visualization was accomplished by spraying with *p*-anisaldehyde spray reagent followed by heating at 110 °C.

Chemicals. Pyrethrosin was kindly supplied by Dr. F. S. El-Feraly, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. The compound was authenticated by comparing its physical and spectral data with literature values.[2]

Organisms. Microbial metabolism studies were conducted as previously reported.[14] Twenty-four microbial cultures were obtained from either American Type Culture Collection (ATCC) or National Center for Agricultural Utilization Research (NCAUR) (formerly Northern Regional Research Laboratories, NRRL); organisms were maintained on Sabourad dextrose agar (Oxoid) slants at 4 °C and were used for the preliminary screening. All fermentation experiments were carried out in a medium of the following composition: 10 g of dextrose, 10 mL of glycerol, 5 g of peptone, 5 g of K_2HPO_4, 5 g of NaCl, and 1000 mL of distilled H_2O. The pH was adjusted to 6.0 before autoclaving at 121 °C for 15 min.[14]

Fermentation Procedures.[14] Cells of microorganisms were transferred from 2 week old slants into 200 mL of sterile culture media per 1 L flask and kept on a gyratory shaker at room temperature, 80 rpm, for 72 h to give stage I culture. Stage I cultures (5 mL) were used as inocula for stage II cultures (50 mL/250 mL flask). After 24 h incubation of stage II cultures, **1** was added as a solution in Me_2CO (5 mg/0.25 mL), per flask. Both substrate and culture controls were run. Each fermentation was sampled by extracting 5 mL of the culture medium with $CHCl_3$ (5 mL), every 3 and 6 days.

Fermentation of Pyrethrosin (1). Pyrethrosin (**1**, 410 mg), dissolved in Me_2CO, was added at 10 mL per flask in 41 flasks (250 mL) containing Stage I cultures of *Rhizopus nigricans* NRRL 1477. Fermentation was stopped after 6 days. The mixture was filtered, and the fermentation broth was extracted three times with equal volumes of $CHCl_3$. The combined extracts were dried over anhydrous Na_2SO_4, and the solvent was evaporated to give 425 mg of translucent residue. The crude residue was loaded on a silica gel column (45 g). Elution of the column utilizing a gradient of EtOAc in hexane (50 → 100%) yielded **3** as a yellowish powder (32.0 mg) and **5** (75 mg). The impure **2** (49 mg) was rechromatographed on a silica gel column (7.5 g) and eluted with 40% EtOAc in hexane. This column afforded **2** (17 mg, colorless amorphous solid). Impure **4** was also rechromatographed over a silica gel column (10.0 g). Elution with 50% EtOAc in hexane afforded **4** (18 mg, colorless amorphous solid).

6α-Acetoxy-1β,4α-dihydroxy-5,7α*H*,8β*H*-eudesm-11β,13-dihydro-8,12-olide 5: pale yellow gum, $[\alpha]_D$ +28.3 (*c* 0.06, $CHCl_3$); IR ν_{max} (film) 3427 (OH), 2939, 2876, 1767 (C═O), 1745 (C═O), 1456, 1375, 1251, 1149, 1092, 1030 cm^{-1}; ^1H NMR (500 MHz, CDCl3) δ 1.05 (3H, s, Me-14), 1.17 (3H, d, *J* = 6.8 Hz, Me-13), 1.21 (3H, s, Me-15), 1.36 (1H, m, H-9′), 1.63 (2H, m, H-2′, H-3′), 1.77 (2H, m, H-2, H-3), 1.84 (2H, m, H-5, H-7) 2.15 (3H, s, Me-18), 2.50 (1H, dd, *J* = 3.8, 3.1 Hz, H-9), 2.62 (1H, septet, *J* = 6.7 Hz, H-11), 3.10 (1H, s, OH-4), 3.74 (1H, dd, *J* = 10.3, 4.0 Hz, H-1), 4.07 (1H, ddd, *J* = 11.7,3.9,3.7 Hz, H-8), 5.47 (1H, t, *J* = 10.6 Hz, H-6); ^{13}C NMR, see Table 1; HRESIFTMS *m*/*z* 327.1822 $[M + 1]^+$ (calcd for $C_{17}H_{26}O_6$, 327.1808).

Acetylation of 5. To a solution of **5** (6.5 mg) in pyridine (0.5 mL) was added acetic anhydride (0.5 mL), and the reaction mixture was kept at room temperature for 7 h. The reaction mixture was diluted with CH_2Cl_2 (10 mL) and shaken with 2% aqueous $NaHCO_3$, followed by shaking with 2% HCl. The CH_2Cl_2 extract was then dried over anhydrous Na_2SO_4 to yield **7** (7.0 mg, 95.4%, colorless amorphous solid, R_f 0.21, 1% MeOH in CH_2Cl_2): [α] +50.0 (*c* 0.04, MeOH); IR ν_{max} (film) 3580−3478 (OH), 2955, 1780 (C═O), 1736 (C═O), 1373, 1241, 1154, 1083, 1030, 980 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 1.12 (3H, s, Me-14), 1.17 (3H, d, *J* = 6.8 Hz, Me-13), 1.22 (3H, S, Me-15), 1.87 (2H, m, H-5, H-7), 2.06 (3H, s, Me-16), 2.15 (3H, s, Me-18), 2.60 (1H, septet, *J* = 6.0 Hz, H-11), 3.06 (1H, s, OH-4), 4.02 (1H, ddd, *J* = 11.6, 3.7, 3.1 Hz, H-8), 4.68 (1H, brd, *J* = 9.4 Hz), 5.45 (1H, t, *J* = 10.5 Hz); ^{13}C NMR, see Table 1; HRESIFTMS *m*/*z* 403.1536 $[M + Cl]^-$ (calcd for $C_{19}H_{28}O_7$, 403.1529).

Acknowledgment. The author is grateful to Mr. Frank T. Wiggers for the 500 MHz NMR and Dr. Chuck Dunbar for the HRESIFTMS analyses.

References and Notes

(1) Barton, D. H. R.; De Mayo, P. *J. Chem. Soc.* **1957**, Part V111, 150−158.
(2) Iriuchijima, S.; Tamura, S. *Agr. Biol. Chem.* **1970**, 204−209.
(3) Doskotch, R. W.; El-Feraly, F. S.; Hufford, C. D. *Can. J. Chem.* **1971**, *49*, 2103-2110.
(4) Abeysekera, B. F.; Abramowski, Z.; Towers, G. H. N. *Biochem. Syst. Ecol.* **1985**, *13*, 365−369.
(5) Picman, A. K.; Towers, G. H. N. *Biochem. Syst. Ecol.* **1983**, *11*, 321−327.
(6) Picman, A. K. *Biochem. Syst Ecol.* **1984**, *12*, 13−18.
(7) Yoke, M. Y.; Balza, F.; Abeysekera, B. F.; Towers, G. H. N. *Biochem. Syst. Ecol.* **1984**, *12*, 285−286.
(8) Iino, Y.; Tanaka, A.; Yamashita, K. *Agr. Biol. Chem.* **1972,** 2505−2509.
(9) Abourashed, E. A.; Clark, A. M.; Hufford, C. D. *Curr. Med. Chem.* **1999**, *6*, 359−374.
(10) Cardona, M. L.; Fernandez, I.; Garcia, B.; Pedro, J. R. *J. Nat. Prod.* **1990**, *53*, 1042−1045.
(11) Borenfreund, E.; Abich, H. B.; Martin-Alguacil, N. *Toxic. In Vitro.* **1988**, *2*, 1−6.
(12) Li, X.; ElSohly, H. N.; Nimrod, A. C.; Clark, A. M. *J. Nat. Prod.* **1999**, *62*, 674−677.
(13) El Sayed, K. A.; Dunbar, D. C.; Goins, D. K.; Cordoya, C. R.; Perry, T. L.; Weeson, K. J.; Sanders, S. C.; Janus, S. A.; Humman, M. *J. Nat. Toxins* **1996**, *5*, 261−285.
(14) Galal, A. M.; Ibrahim, A. S.; Mossa, J. S.; El-Feraly, F. S. *Phytochemistry* **1999**, *51*, 761−765.

NP0100082