Microbial Transformation of Hypoestenone

Khalid A. El Sayed*,†

Pharmacognosy Department, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt

Received November 17, 2000

Preparative-scale fermentation of hypoestenone (**1**) with *Mucor ramannianus* (ATCC 9628) has resulted in the isolation of 8(9)α-epoxyhypoestenone (**2**) and the new metabolites 8(9)α-epoxy-12,13-anhydrohypoestenone (**3**), 17-hydroxyhypoestenone (**4**), 13α,18-dihydroxyhypoestenone (**5**), and 13*β*,18-dihydroxyhypoestenone (**6**). Structure elucidation of these metabolites was based primarily on 1D and 2D NMR analyses.

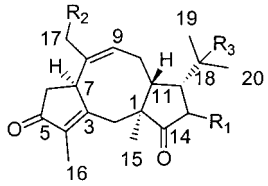
Fusicoccanes are significant biosynthetic plant growth regulators.[1] This group of diterpenoids possessing a dicyclopenta[*a*,*d*]cyclooctane (5 → 8 → 5 ring system) occurs in fungi, lower and higher plants, and insects.[1] Fusicoccanes bear structural resemblance to gibberellins.[1,2] Fusicoccin, the first member of this group reported from the phytopathogenic fungus *Fusicoccum amygdali* Del.,[3] was found to stimulate plant growth through the elongation mechanism, promotion of the opening of leaf stomata, acceleration of seed germination, and induction of root formation.[1,4]

Hypoestenone (**1**) is a fusicoccane diterpene ketone reported from the aerial parts of *Hypoests forskalei* (Acanthaceae).[5] The structure and relative stereochemistry of **1** were based on 1D and 2D NMR data in addition to X-ray crystallographic analysis of its 8(9)α-epoxy derivative.[5] Hypoestenone (**1**) was chosen for a microbial bioconversion study in an attempt to prepare new bioactive analogues. The present study represents the first microbial metabolism study of a member of this significant class of bioactive natural products. Fungi have enzyme systems similar to those of mammals since they are eukaryotic.[6] Hence, many fungi have recently been used as in vitro models for predicting mammalian drug metabolism.[7]

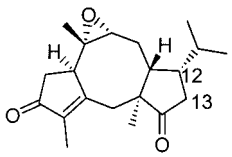
Twenty-three growing microbial cultures were screened for their ability to biotransform **1**. Few cultures were able to transform **1** to more polar metabolites. *Mucor ramannianus* ATCC 9628 was selected for preparative-scale fermentation because it entirely depleted **1** and converted it into five more polar metabolites (**2**−**6**).

The ^{1}H and ^{13}C NMR data of **2** indicated that it was 8(9)α-epoxyhypoestenone (**2**), which was previously reported as a semisynthetic derivative of **1**.[5] The reported structure and relative stereochemistry of **2** was based on X-ray crystallography.[5] This is the first report of **2** as a natural product.

The HRFTMS spectrum of **3** displayed a molecular ion peak at *m*/*z* 315.1988 $[M + H]^+$, suggesting the molecular formula $C_{20}H_{26}O_3$ and eight degrees of unsaturation. The ^{1}H and ^{13}C NMR data of **3** suggested close similarity with **2**, with an additional $\Delta^{12,13}$ system. A broad proton singlet resonating at *δ* 5.94 was assigned as H-13 on the basis of its ^3J HMBC correlation with C-1 and C-11. The downfield quaternary carbon at *δ* 184.0 was assigned as C-12 on the basis of its ^3J HMBC correlation with both methyl doublets H_3-19 and H_3-20 as well as with H_2-10. Hence, metabolite **3** was shown to be 8(9)α-epoxy-12,13-anhydrohypoestenone.



	R_1	R_2	R_3
Hypoestenone (**1**)	H	H	H
17-Hydroxyhypoestenone (**4**)	H	OH	H
13α,18-Dihydroxyhypoestenone (**5**)	α-OH	H	OH
13β,18-Dihydroxyhypoestenone (**6**)	β-OH	H	OH



	Other
8(9)α-Epoxyhypoestenone (**2**)	
8(9)α-Epoxy-12(13)-anhydrohypoestenone (**3**)	$\Delta^{12,13}$

The HRFTMS spectrum of **4** displayed a molecular ion peak at *m*/*z* 317.2131 $[M + H]^+$, suggesting the molecular formula $C_{20}H_{28}O_3$ and indicated that **4** was a monohydroxylated derivative of **1**. The IR absorption band at ν_{max} 3450 cm^{-1} confirmed hydroxylation of **1**. The ^{1}H and ^{13}C NMR data of **4** indicated allylic hydroxylation at C-17. The oxygenated methylene proton doublets resonating at *δ* 4.03 and 3.93 were assigned to a C-17 hydroxymethylene group. This was based on the ^3J HMBC correlation of H_2-17 with C-7 and the downfield olefinic C-9 methine carbons, in addition to their ^2J HMBC correlation with the quaternary olefinic carbon C-8. Therefore, metabolite **4** was established as 17-hydroxyhypoestenone.

Metabolites **5** and **6** both analyzed for the molecular formula $C_{20}H_{28}O_4$, which suggested they were dihydroxyhypoestenone derivatives. The IR and ^{1}H and ^{13}C NMR data of **5** and **6** indicated hydroxylation at C-13 and C-18. The quaternary carbons resonating at *δ* 72.4 and 73.6 were assigned to C-18 in **5** and **6**, respectively. These assignments were based on the ^2J HMBC correlation of C-18 with the methyl singlets H_3-19, H_3-20 and the proton H-12 in both compounds. Carbon C-18 of **5** and **6** also displayed a ^3J HMBC correlation with H-11. The downfield oxygenated protons resonating at *δ* 3.91 and 4.44 were assigned to H-13 in **5** and **6**, respectively, on the basis of the ^3J HMBC correlation of H-13 in each of **5** and **6** with C-1, C-11, and C-18 (Figure 1). H-13 also showed ^2J HMBC correlation with C-12 and the ketone C-14. The difference in the proton

* To whom correspondence should be addressed. Tel: (662) 915-7184. Fax: (662) 915-7026. E-mail: elsayed99@yahoo.com.
† Current address: Department of Pharmacognosy, School of Pharmacy, University of Mississippi, University, MS 38677.

10.1021/np0005255 CCC: $20.00
Published on Web 02/22/2001

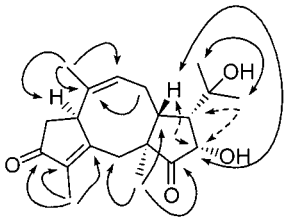


Figure 1. Important HMBC (solid arrows) and NOESY (dotted arrow) correlation of **5**.

chemical shift value (0.53 ppm) and the splitting pattern of H-13, in addition to the significant difference in carbon chemical shift value (10.8 ppm) between C-13 in both **5** and **6**, suggested the epimeric nature of the compounds at this position. Assignment of relative stereochemistry at C-13 was based on NOESY data. In **5**, H-13 displayed strong NOESY correlation with the β-oriented protons H-11 and H-12 (Figure 1), suggesting a similar stereo orientation. On the other hand, H-13 in **6** was proved to be α-oriented because it displayed a NOESY correlation with the α-oriented methyl singlet H-15. Metabolites **5** and **6** were therefore identified as 13α,18-dihydroxyhypoestenone and 13β,18-dihydroxyhypoestenone, respectively.

Compounds **1**−**6** were tested for antimicrobial activity against a wide range of microorganisms using microtiter-plate assay.[10] Hypoestenone (**1**) and 8(9)α-epoxy-hypoestenone (**2**) show moderate antimicrobial activity against *Cryptococcus neoformans* with MIC 20 and 50 μg/mL, respectively. Hypoestenone also shows moderate activity against *Pseudomonas aeruginosa* with MIC 45 μg/mL and *Plasmodium falciparium* (W2 Clone) with IC_{50} 2800 ng/mL and selectivity index >1.7.

Experiment Section

General Experimental Procedures. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. UV spectra were run on a Perkin-Elmer Lambda 3B UV/vis spectrophotometer. The IR spectra were recorded on a ATI Mattson Genesis Series FTIR spectrophotometer. The ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$, on a Bruker DRX NMR spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C NMR. The LRMS data were obtained using an E.I. Finnigan model 4600 (70 eV ionization potential) quadruple system. HRMS spectra were measured on a Bioapex FTMS with electrospray ionization. TLC analyses were carried out on precoated silica gel G_{254} 500 μm (E-Merk) plates, with the following developing system: EtOAc−cyclohexane (4:5). For column chromatography, silica gel 60, 40 μm was used.

Chemicals. Hypoestenone (**1**) was isolated from a Saudi Arabian collection of *Hypoestes verticillaris* (Acanthaceae) using the previously reported procedure.[5] The compound was authenticated by comparing its R_f and mp values with those of a standard hypoestenone sample, kindly provided by Dr. Farouk El-Feraly of King Saud University, and comparison of its ^{1}H and ^{13}C NMR data with the literature.[5]

Organisms. Microbial metabolism studies were conducted as previously reported.[8,9] Twenty-three microbial cultures, obtained from the University of Mississippi, Department of Pharmacognosy culture collection, were used for screening. These microbes were reported earlier,[9,11] in addition to *Bullera alba ATCC 18568, Cylinderocephalum aureum* ATCC 12720, *Mucor griseo-cynus* ATCC 1207a, *Penicillium frequentans* ATCC 10444, *Penicillium frequentans* UM-ATCC 10444, *Piptocephalis corymbifera* ATCC 12665, *Ramichloridium anceps* ATCC 15672, *Rhizopogen* species ATCC 36060, *Rhodotorula glutinus* ATCC 15125, *Schizosaccharomyces pombe* ATCC 20130, *Septomyxa affinis* ATCC 6737, *Talaromyces ucrainicus* ATCC 18352, *Tricophyton mentagrophytes* ATCC 9972, *Volutella buxi* ATCC 13545, and *Zopfiella pleuropora* ATCC 18994.

Microbial Metabolism of Hypoestenone (1). *M. ramannianus* ATCC 9628 was grown in 11 1-L culture flasks, each containing 250 mL of compound medium α.[9] A total of 275 mg of **1** was mixed with 1.3 mL of DMF and evenly distributed among the stage II (24 h) cultures at a concentration of 25 mg/1 L flask. After 7 days, the incubation mixtures were pooled and filtered. The filtrate (2.5 L) was exhaustively extracted with $CHCl_3$ (4 × 2 L), which were then dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue (480 mg) was flash chromatographed over 50 g of silica gel 60 using cyclohexane, gradient elution with increasing proportions of ethyl acetate, and finally MeOH. The polar fractions were subjected to repeated prep TLC on silica gel G (EtOAc−cyclohexane, 4:5) to afford **2** (4.1 mg, R_f 0.45), **3** (1.6 mg, R_f 0.43), **4** (4.6 mg, R_f 0.21), **5** (2.6 mg, R_f 0.20), and **6** (1.4 mg, R_f 0.15).

8(9)α-Epoxyhypoestenone (2): colorless needles, mp 176−178 °C, $[\alpha]^{25}_D$ +68 (*c* 0.14, $CHCl_3$); UV λ_{max} (log ϵ) (MeOH) 237 (3.39) nm; IR ν_{max} ($CHCl_3$) 1740 (C═O), 1690 (C═O), 1630, 1070, 890 cm^{-1}; ^{1}H and ^{13}C NMR, identical to the literature.[5]

8(9)α-Epoxy-12,13-anhydrohypoestenone (3): colorless needles, mp 160−162 °C, $[\alpha]^{25}_D$ +11 (*c* 0.18, $CHCl_3$); UV λ_{max} (log ϵ) (MeOH) 236 (3.25) nm; IR ν_{max} ($CHCl_3$) 1700 (C═O), 1690 (C═O), 1630, 1450, 1365, 1070, 890 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.94 (1H, brs, H-13), 3.65 (1H, d, J = 16.9 Hz, H-2a), 3.03 (1H, dd, J = 9.6, 5.5 Hz, H-9), 2.85 (1H, brd, J = 7.1 Hz, H-7), 2.70 (1H, ddd, J = 13.6, 9.3, 5.7 Hz, H-10a), 2.67 (1H, m, H-11), 2.64 (1H, brq, J = 6.9 Hz, H-18), 2.58 (2H, m, H_2-6), 1.82 (3H, brs, H_3-16), 1.60 (1H, m, H-10b), 1.23 (3H, d, J = 6.9 Hz, H_3-19),[a] 1.20 (3H, d, J = 7.0 Hz, H_3-20),[a] 1.10 (3H, s, H_3-17), 0.96 (3H, s, H_3-15); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 209.3 (s, C-14), 207.6 (s, C-5), 184.0 (s, C-12), 167.3 (s, C-3), 141.6 (s, C-4), 123.3 (d, C-13), 64.8 (d, C-9), 59.7 (s, C-8), 52.2 (s, C-1), 52.0 (d, C-11), 45.0 (d, C-7), 36.4 (t, C-6), 34.0 (t, C-2), 29.2 (d, C-18), 23.1 (t, C-10), 21.1 (q, C-15), 20.4 (q, C-20),[b] 20.3 (q, C-19),[b] 16.7 (q, C-17), 8.6 (q, C-16), [a,b]Interchangeable assignments; LREIMS *m*/*z* 314 $(M)^+$; HRFTMS *m*/*z* calcd for $C_{20}H_{27}O_3$ $(M + H)^+$ 315.1960, found 315.1988.

17-Hydroxyhypoestenone (4): colorless needles, mp 118−120 °C, $[\alpha]^{25}_D$ +80 (*c* 0.10, $CHCl_3$); UV λ_{max} (log ϵ) (MeOH) 204 (3.85), 239 (3.95) nm; IR ν_{max} ($CHCl_3$) 3450 (OH), 1740 (C═O), 1695 (C═O), 1635, 1465, 1395, 1065 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.99 (1H, dd, J = 8.2, 8.1 Hz, H-9), 4.03 (1H, brd, J = 12.5 Hz, H-17a), 3.93 (1H, d, J = 12.5 Hz, H-17b), 3.93 (1H, brs, H-7), 3.31 (1H, d, J = 15.3 Hz, H-2a), 2.67 (1H, m, H-10a), 2.58 (1H, m, H-6a), 2.51 (1H, m, H-6b), 2.50 (1H, m, H-13a), 2.48 (1H, m, H-10b), 2.41 (1H, m, H-13b), 2.15 (1H, m, H-11), 2.05 (1H, m, H-12), 1.95 (1H, m, H-18), 1.90 (1H, d, J = 15.3 Hz, H-2b), 1.77 (3H, brs, H_3-16), 1.07 (3H, d, J = 7.0 Hz, H_3-20),[a] 0.95 (3H, d, J = 7.0 Hz, H_3-19),[a] 0.92 (3H, s, H_3-15); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 220.0 (s, C-14), 209.5 (s, C-5), 171.5 (s, C-3), 138.5 (s, C-4), 137.0 (s, C-8), 131.6 (d, C-9), 64.5 (t, C-17), 54.9 (d, C-11), 50.0 (s, C-1), 44.8 (d, C-12), 42.8 (d, C-7), 42.5 (t, C-13), 36.8 (t, C-6), 36.7 (t, C-2), 31.5 (d, C-18), 26.5 (t, C-10), 24.0 (q, C-20),[b] 23.7 (q, C-19),[b] 17.1 (q, C-15), 8.7 (q, C-16), [a,b]Interchangeable assignments; LREIMS *m*/*z* 316 $(M)^+$; HRFTMS *m*/*z* calcd for $C_{20}H_{29}O_3$ $(M + H)^+$ 317.2117, found 317.2131.

13α,18-Dihydroxyhypoestenone (5): colorless needles, mp 141−142°, $[\alpha]^{25}_D$ +33 (*c* 0.12, $CHCl_3$); UV λ_{max} (log ϵ) (MeOH) 204 (3.85), 239 (3.61) nm; IR ν_{max} ($CHCl_3$) 3250−3480 (OH), 1735 (C═O), 1695 (C═O), 1635, 1460, 1395, 1065 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.76 (1H, dd, J = 8.3, 7.9 Hz, H-9), 4.01 (1H, brs, H-7), 3.91 (1H, s, H-13), 3.24 (1H, d, J = 15.7 Hz, H-2a), 2.56 (1H, m, H-6a), 2.53 (1H, m, H-6b), 2.51 (1H, m, H-10a), 2.28 (1H, m, H-10b), 2.23 (1H, d, J = 16.0 Hz, H-2b), 1.98 (1H, m, H-11), 1.95 (1H, m, H-12), 1.75 (3H, s, H_3-16), 1.56 (3H, s, H_3-17), 1.39 (3H, s, H_3-19),[a] 1.34 (3H, s, H_3-20),[a] 0.72 (3H, s, H_3-15); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 216.0 (s, C-14), 209.0 (s, C-5), 173.2 (s, C-3), 139.1 (s, C-4), 135.5 (s, C-8), 127.8 (d, C-9), 84.8 (d, C-13), 72.4 (s, C-18), 57.7 (d, C-11), 47.6 (d, C-12), 45.0 (s, C-1), 42.8 (d, C-7), 37.4 (t, C-6), 36.7 (t, C-2), 28.9 (q, C-19),[b] 28.8 (q, C-20),[b] 27.1 (t, C-10), 19.1 (q,

C-17), 12.3 (q, C-15), 9.0 (q, C-16), [a,b]Interchangeable assignments; LREIMS *m/z* 332 $(M)^+$; HRFTMS *m/z* calcd for $C_{20}H_{29}O_4$ $(M + H)^+$ 333.2060, found 333.2057.

13β,18-Dihydroxyhypoestenone (6): colorless needles, mp 145−147°, $[\alpha]^{25}_D$ +13 (*c* 0.10, $CHCl_3$); UV λ_{max} (log ϵ) (MeOH) 205 (3.85), 239 (3.60) nm; IR ν_{max} ($CHCl_3$) 3250−3490 (OH), 1730 (C═O), 1690 (C═O), 1630, 1460, 1390, 1055 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.76 (1H, dd, J = 8.3, 7.9 Hz, H-9), 4.44 (1H, d, J = 6.6, H-13), 3.92 (1H, brs, H-7), 3.46 (1H, d, J = 15.8 Hz, H-2a), 3.07 (1H, dd, J = 14.1, 7.8, H-10a), 2.54 (1H, m, H-10b), 2.51 (1H, m, H-6a), 2.49 (1H, m, H-6b), 2.49 (1H, d, J = 15.8 Hz, H-2b), 2.33 (1H, dd, J = 11.6, 11.1, H-11), 2.23 (1H, dd, J = 11.6, 6.6, H-12), 1.77 (3H, s, H_3-16), 1.49 (3H, s, H_3-17), 1.46 (3H, s, H_3-19),[a] 1.43 (3H, s, H_3-20),[a] 1.11 (3H, s, H_3-15); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 216.1 (s, C-14), 209.5 (s, C-5), 172.5 (s, C-3), 139.5 (s, C-4), 135.0 (s, C-8), 128.7 (d, C-9), 74.0 (d, C-13), 73.6 (s, C-18), 54.2 (d, C-11), 50.8 (d, C-12), 49.2 (s, C-1), 43.0 (d, C-7), 37.3 (t, C-6), 34.9 (t, C-2), 32.5 (q, C-19),[b] 30.1 (q, C-20),[b] 23.8 (t, C-10), 18.9 (q, C-17), 17.8 (q, C-15), 9.1 (q, C-16), [a,b]Interchangeable assignments; LREIMS *m/z* 332 $(M)^+$; HRFTMS *m/z* calcd for $C_{20}H_{29}O_4$ $(M + H)^+$ 333.2060, found 333.2045.

Acknowledgment. The author is grateful to Mr. Abdalla Ibrahim, King Saud University, Saudi Arabia, for assistance during isolation of hypoestenone, and Dr. D. Chuck Dunbar, University of Mississippi, for acquiring HRFTMS data. The National Center for Natural Products Research, University of Mississippi, is acknowledged for antimicrobial assays.

References and Notes

(1) Muromtsev, G. S.; Voblikova, V. D.; Kobrina, N. S.; Koreneva, V. M.; Krasnopolskaya, L. M.; Sadovskaya, V. L. *J. Plant Growth Regul.* **1994**, *13*, 39−49.
(2) Muromtsev, G. S.; Voblikova, V. D.; Kobrina, N. S.; Koreneva, V. M.; Sadovskaya, V. L.; Stolpakova, V. V. *Fiziologiya Rastenii* **1987**, *34*, 980−987.
(3) Ballio, A.; Chain, E. B.; De Leo, P.; Erlanger, B. F.; Mauri, M.; Tonolo, A. *Nature* **1964**, *203*, 297.
(4) Asakawa, Y.; Lin, X.; Tori, M.; Kondo, K. *Phytochemistry* **1990**, *29*, 2597−2603.
(5) Muhammed, I.; Mossa, J. S.; Al-Yahya, M.; El-Feraly, F. S.; Mcphail, A. T. *Phytochemistry*. **1997**, *44*, 125−129.
(6) Rosazza, J. P. N.; Duffel, M. W. In *Alkaloids: Chemistry and Pharmacology*; Brossi, A., Ed.; Academic Press: New York, 1986; Vol. 27, pp 391−392.
(7) Clark, A. M.; McChesney, J. D.; Hufford, C. D. *Med. Res. Rev*. **1985**, *5*, 231−253.
(8) Lee, I.-S.; ElSohly, H. N.; Hufford, C. D. *Pharm. Res*. **1990**, *7*, 199−203.
(9) El Sayed, K. A. *Phytochemistry* **2000**, *53*, 675−678.
(10) National Committee for Clinical Laboratory Standards. *Methods for Dilution. Antimicrobial Susceptibility Test for Bacteria That Grow Aerobically. Approved Standard M7-A*, 4th ed.; National Committee for Clinical Laboratory Standards: Wayne, PA, 1997.
(11) El Sayed, K. A.; Halim, A. F.; Zaghloul, A. M.; Dunbar, D. C.; McChesney, J. D. *Phytochemistry* **2000**, *55*, 19−22.

NP0005255