Microbial Transformations of Isocupressic Acid

Shwu-Jiuan Lin and John P. N. Rosazza*

Division of Medicinal and Natural Products Chemistry, and Center for Biocatalysis and Bioprocessing, College of Pharmacy, University of Iowa, Iowa City, Iowa 52242

Received February 26, 1998

Microbial transformations of the labdane-diterpene isocupressic acid (**1**) with different microorganisms yielded several oxygenated metabolites that were isolated and characterized by MS and NMR spectroscopic analyses. *Nocardia aurantia* (ATCC 12674) catalyzed the cleavage of the 13,14-double bond to yield a new *nor*-labdane metabolite, **2**. *Cunninghamella elegans* (−) (NRRL 1393) gave 7β-hydroxyisocupressic acid (**3**) and labda-7,13(*E*)-diene-6β,15,-17-triol-19-oic acid (**4**), and *Mucor mucedo* (ATCC 20094) gave 2α-hydroxyisocupressic acid (**5**) and labda-8(17),14-diene-2α,13-diol-19-oic acid (**6**).

Isocupressic acid [15-hydroxylabda-8(17),13(*E*)-dien-19-oic acid] (**1**) is a labdane-diterpene found in species of *Pinus*. Isocupressic acid (**1**) is one compound thought to be responsible for abortion in pregnant cattle following the consumption of *Pinus ponderosa* (Pinaceae) pine needles.[1] Following ingestion, terpenes such as isocupressic acid (**1**) can be subjected to metabolic transformations leading to metabolites that are potentially more or less active, toxic, or inactive compared the terpene starting material. There are no reports concerned with the mammalian metabolism of isocupressic acid (**1**). However, microbial transformations have been reported for labdane-diterpenes, including sclareol [labd-14-ene-8α,13β-diol][2−9] and its analogues,[10,11] and with ruminal metabolism of isocupressic acid (**1**).[12]

This investigation utilized microorganisms as in vitro models to prospectively mimic and predict oxidative mammalian biotransformations of isocupressic acid (**1**) and to produce novel metabolites of the terpene for use as analytical standards for mammalian metabolic studies. The use of selected organisms as "models for xenobiotic metabolism"[13,14] has been successfully exploited in mammalian metabolism studies of many classes of biologically active natural and synthetic compounds.[15−18] This report describes the metabolic products produced by cultures of *Nocardia aurantia*, *Cunninghamella elegans* (−), and *Mucor mucedo*.

Results and Discussion

Of 31 microorganisms screened for their abilities to catalyze the bioconversion of isocupressic acid (**1**), *N. aurantia* ATCC 12674, *C. elegans* (−) NRRL 1393, and *M. mucedo* ATCC 20094 were selected for preparative-scale transformations because they reproducibly formed metabolites. All of the metabolites were isolated from preparative-scale incubations by solvent extraction, purified by chromatography, and subjected to spectral analyses. Signals for protons and carbons of isocupressic acid (**1**) were assigned initially by HMBC and HMQC NMR spectral analyses.[12,20,21]

N. aurantia gave one major metabolite, which was identified as **2**. The preparative-scale incubation with 200 mg of isocupressic acid (**1**) was terminated after 144 h. Solvent extraction and column chromatography afforded 36 mg (18%) of **2** for spectral analysis. Most ^{1}H and ^{13}C NMR spectral data for **2** were similar to those for **1** except for signals attributed to the side chain involving positions 13−16. Signals for the double bond (positions 13 and 14) and for the alcohol functional group at position 15 were absent in the metabolite spectrum. Position 13 in **2**, on the other hand, appeared as a ketone carbonyl singlet signal at δ 209.6 in the ^{13}C NMR spectrum and a three-proton singlet at δ 2.10 in the ^{1}H NMR spectrum. The high-resolution EIMS gave an $[M]^+$ at *m/z* 292.2062 for $C_{18}H_{28}O_3$ (calcd 292.2039) and a $[M - Me_2CO]^+$ peak at *m/z* 234 likely formed by McLafferty rearrangement and cleavage. Oxidative cleavage at the isocupressic acid side chain, possibly involving an initial epoxidation at the side chain olefin between positions 13 and 15 of **1**, would account for the formation of *nor*-labdane metabolite **2** (Figure 1). Thus, metabolite **2** is assigned the structure 14,15-dinorlabd-8(17)-en-13-on-19-oic acid.

Metabolites **3** and **4** were obtained in 3% and 2.5% yields, respectively, by preparative-scale incubations of isocupressic acid (**1**) with *C. elegans* (−) after 72 h of incubation. Following solvent extraction and flash column chromatographic purification, samples of metabolites were subjected to spectral analyses. Methylation of **3** with diazomethane gave a GC/MS peak with *m/z* 332 for $[M - H_2O]^+$. HRFABMS of the methyl ester gave a molecular ion at *m/z* 373.2349 corresponding to $C_{21}H_{34}O_4Na$ (calcd 373.2354), indicating a metabolite **3** containing one more oxygen atom than isocupressic acid (**1**). NMR spectral data for **3** were largely similar to that for **1** except for carbon signals for positions 6 and 7, which resonated at δ 36.6 ($\Delta\delta$ +10.5 ppm vs **1**) and δ 75.0 ($\Delta\delta$ +36.2 ppm vs **1**). In addition to other signals similar to those for **1**, the ^{1}H NMR spectrum contained a single carbinol-methine signal at δ 3.83 (dd, J = 5.0, 11.5 Hz), the coupling constants of which indicated a 7-equatorial hydroxyl group.[22] The lack of a shielding effect on C-20 and significant γ-effects at positions 5 and 9 in the ^{13}C NMR spectrum rule out an axial-hydroxyl group at position 7.[23] The dramatic shift of 17-Hb from δ 4.85 to δ 5.24 also supports a 7-equatorial hydroxyl

* To whom correspondence should be addressed. Tel.: (319) 335-8842. Fax: (319) 335-4901. E-mail: john-rosazza@uiowa.edu.

S0163-3864(98)00064-0 CCC: $15.00
Published on Web 06/17/1998

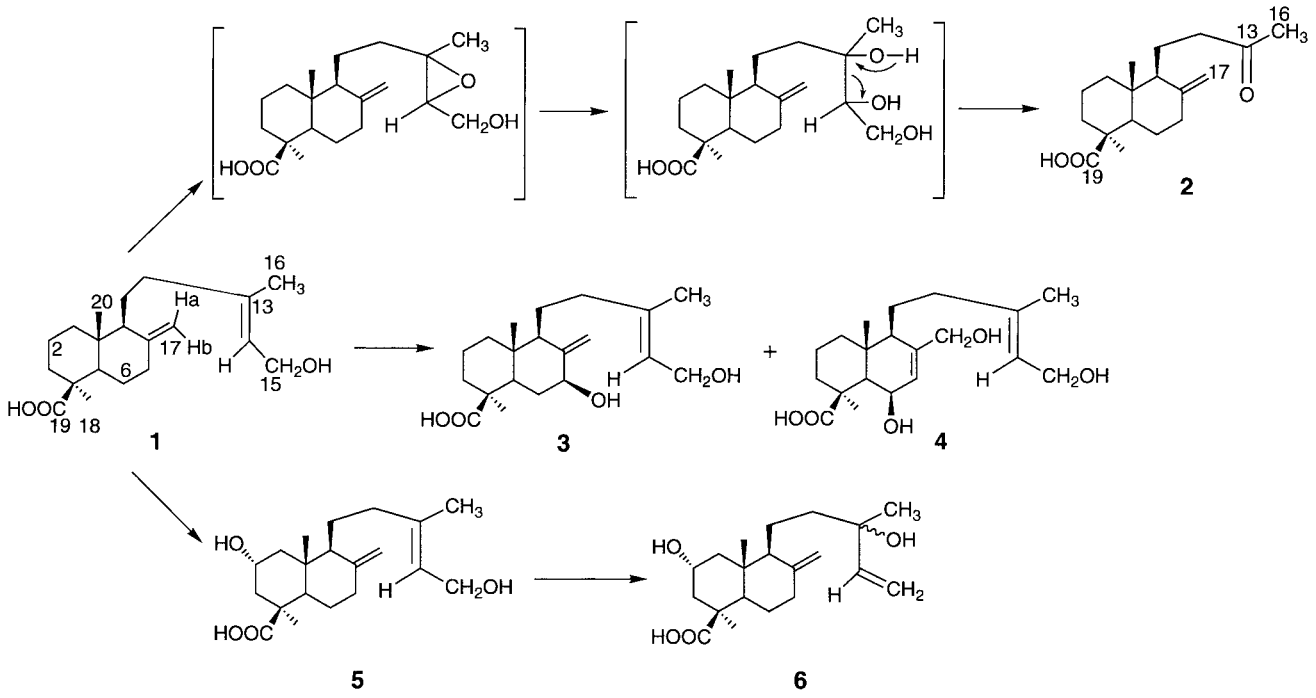

Figure 1. Structures of isocupressic acid (**1**) and metabolites **2**−**6**.

group in the structure of **3**. Thus, by these spectral results, HMBC and HMQC correlation spectra, and comparison with spectra for related labdanes,[24] the metabolite structure is 7β-hydroxyisocupressic acid (**3**).

Comparison among MS and ^{1}H and ^{13}C NMR spectra of **1** and **4** clearly revealed the presence of hydroxyl groups at positions 6 and 17 and a double bond that had shifted from $\Delta^{8,17}$ in **1** to $\Delta^{7,8}$ in metabolite **4**. The pair of singlets for H-17 in the exocyclic double bond of **1** was absent, and a new two-proton doublet at δ 4.23 (J = 5.8 Hz) was present in the spectrum of **4**. The ^{1}H NMR spectrum of **4** also displayed a new one-proton olefinic singlet signal at δ 6.14. The shift of one olefinic carbon signal to δ 119.5 from δ 106.5 in the ^{13}C NMR spectrum suggested the presence of an allylic alcohol functional group in the structure of **4**. HMQC and HMBC spectral analyses showed that the olefinic carbon was three-bond correlated with carbons 9, 17, and 5, thus confirming the 7,8-position of the double bond. HMBC correlations of the position-6 methine proton at δ 4.89 showed three-bond connectivities to carbons 4, 10, and 8, thus confirming the location of the hydroxyl group at C-6. The position 20-methyl group was shifted significantly downfield in both carbon and proton NMR spectra to δ 18.1 ($\Delta\delta$ +5.3 ppm vs **1**) and δ 0.82 ($\Delta\delta$ +0.23 ppm vs **1**), consistent with an axial C-6 hydroxyl group.[25] Furthermore, metabolite **4** showed HRFABMS at m/z 341.2301 [M + Li − H_2O]$^+$ [calcd for ($C_{20}H_{32}O_5$-Li - H_2O), 341.2304]. Thus, the structure of metabolite **4** is labda-7,13(*E*)-diene-6β,15,17-triol-19-oic acid.

Incubation of isocupressic acid (**1**) with *M. mucedo* ATCC 20094 afforded metabolites **5** and **6**. The ^{13}C NMR spectrum of **5** showed a secondary hydroxyl group at δ 65.6. HRFABMS of the methyl ester of **5** gave m/z 373.2358 for $C_{21}H_{34}O_4Na$, indicating a metabolite structure containing one additional oxygen atom vs **1**. The ^{1}H NMR spectrum of **5** contained a one-proton signal at δ 4.09 (m, br) that was partially overlapped with signals for the hydroxymethylene group at C-15. As with **6**, HMBC and HMQC analyses suggested the presence of a C-2 hydroxyl group in the metabolite structure. The shifting downfield of protons for H-1 and H-3 as with **6** were consistent with a 2α-hydroxyl group. A NOESY experiment also showed significant NOE correlations among H_{ax}-2 and Me-20; H_{eq}-1 and Me-20; H_{ax}-6 and Me-20. Thus, the C-2 hydroxyl group was equatorial. On the basis of spectral analyses, **5** was assigned the structure of 2α-hydroxyisocupressic acid.

HRFABMS of methyl ester of **6** gave m/z 373.2328 for $C_{21}H_{34}O_4Na$, suggesting a structure containing one additional oxygen atom vs **1**. ^{1}H NMR spectral data of **6** were comparable to **1** except for the presence of a new one-proton methine signal and signals for the side chain involving positions 13−16. HMBC and HMQC analyses suggested that C-2 was the most likely position of hydroxylation within the ring system. For **6**, signals for H-1 and H-3 were coupled to the new C-2 methine signal. In the spectrum of **6**, signals for H-1 and H-3 were shifted from δ 1.77−1.96 to δ 2.16−2.20, and δ 2.13−2.17 to δ 2.40−2.43, respectively, vs **1**. The signal for the C-2 methine proton occurred as a characteristic triplet of triplets ($J_{2\beta,1\alpha} = J_{2\beta,3\alpha} = 11.4$ Hz, $J_{2\beta,1\beta} = J_{2\beta,3\beta} = 4.2$ Hz) defining the new hydroxyl group as secondary and equatorial.[6,8] Resonances observed for the side chain of **6** included signals for two equivalent methylene protons at δ 5.18 (d, J = 17.4 Hz, H-15 trans to H-14) and δ 5.04 (d, J = 10.8 Hz, H-15 cis to H-14). Furthermore, the 16-methyl group signal was shifted from δ 1.65 to δ 1.30. A new 1 H-methine signal at δ 5.86 (dd, $J_{cis} = 10.8$ Hz, $J_{trans} = 17.4$ Hz, H-14) indicated that for the side chain the double bond and alcohol functional groups had been rearranged. Thus, the structure of **6** is labda-8(17),14-diene-2α,13-diol-19-oic acid.

Metabolism of isocupressic acid (**1**) by the bacterium *N. aurantia* ATCC 12674 produced metabolite **2** in which only the side chain was altered by apparent double-bond oxidation and cleavage. We suggest the possibility that initial epoxidation of the 13,14 double

bond (Figure 1) gives a diol that undergoes periodate-like cleavage to give the ketone **2**. A similar oxidation pathway has been used to explain oxidative-ring fission of the naphthoquinone, lapachol.[26] Enzymes known to mediate diol-cleavage reactions include peroxidases and copper oxidases in the oxidative ring fission of vinblastine[27,28] and cytochrome P-450 in cholesterol side chain degradation.[29] Under typical aerobic incubation conditions, *Nocardia restricta* and *N. aurantia* catalyzed the degradation of the side chains of sclareol and isocupressic acid, respectively. Interestingly, the side chain of isocupressic acid was also the target of metabolic transformation by a consortium of bacterial species in cow ruminal fluid incubated under anaerobic conditions.[12]

Both of the fungi examined here yielded hydroxylated metabolites of isocupressic acid (**1**). Metabolites **3** and **5** are simple hydroxylated metabolites likely formed by activation and insertion of molecular oxygen into the respective 7β- and 2α-positions of **1**. Pathways to metabolites **4** and **6** are more complex. Metabolite **4** can be obtained by 8,17-epoxidation of 6β-hydroxyisocupressic acid and subsequent base-catalyzed proton abstraction and 7,8-double-bond formation. Reactions involved in the formation of **6** from **1** likely involve two separate steps: 2α-hydroxylation and side-chain alteration. Side-chain alteration involves exchange of the hydroxyl group and olefinic functional groups, which can occur by concerted acid-catalyzed dehydration and double-bond migration followed by addition of a hydroxyl group from water to a tertiary carbonium ion at position 13. Microbial transformation studies with sclareol and its analogues show that hydroxylations commonly occur at positions 2α, 3β, and 18, different from those observed for isocupressic acid. The presence of a carboxylic acid functional group at position 19 in **1** likely has a directing influence on the different patterns of microbial hydroxylations obtained with **1**.

Experimental Section

General Experimental Procedures. NMR spectra were recorded with Bruker WM-360 and AMX-600 (HMQC, HMBC) spectrometers using TMS as the internal standard. ^{1}H and ^{13}C NMR chemical shifts were assigned on the basis of HMQC and HMBC experiments.[20,21] Gas chromatography/mass spectrometry (GC/MS) was conducted with a Trio-1 mass spectrometer operating at 70 eV, linked with a Hewlett-Packard 5890 A gas chromatograph using a DB-1 methylsilicone column (15 m × 0.32 mm i.d.) (J&W Scientific, Folsom, CA). FAB mass spectra were measured with a VG ZAB-HF mass spectrometer. Optical rotations were obtained with a Perkin-Elmer 141 polarimeter. Isocupressic acid (**1**) was obtained as previously reported.[12]

Microorganisms, Media, and Incubation Conditions. Microbial studies were conducted by selecting 31 microorganisms from the culture collection of the College of Pharmacy, University of Iowa. All cultures were stored on Sabouraud-maltose agar slants at 4 °C. Preliminary screening experiments were carried out in 125 mL stainless steel-capped DeLong culture flasks containing 25 mL of medium. The incubation medium consisted of yeast extract (5 g), NaCl (5 g), K_2HPO_4 (5 g), dextrose (20 g), soybean meal (5 g), and 1 L of distilled water, adjusted to pH 7.0 and then autoclaved at 121 °C for 20 min. Cultures were incubated with shaking at 250 rpm at 28 °C on New Brunswick Scientific G25 Gyrotory shakers. Incubations were initiated by suspending the surface growth from slants in 5 mL of sterile medium and using the suspensions to inoculate stage-I cultures. Thick, 72 h stage-I cultures were used to inoculate stage-II fermentations. The inoculum was 10% of the volume of medium held in stage-II culture flasks. Isocupressic acid (**1**) [10 mg in 0.1 mL of dimethylformamide (DMF)] was added to each 24-h-old stage-II culture. Culture controls were composed of sterile medium and microorganisms without substrate and were grown under identical conditions. Substrate controls consisted of sterile medium and substrate, incubated without microorganisms. Samples (4 mL) were withdrawn at 24, 72, 144 h after substrate addition, acidified with 6 N HCl, and extracted with 1 mL of EtOAc−butanol (9:1). The organic and aqueous layers were separated by centrifugation in a desk-top centrifuge. The organic layers were spotted onto TLC plates (Merck Kieselgel 60 F_{254}) for analyses, and developed plates were visualized by spraying with anisaldehyde−H_2SO_4, followed by heating. On the basis of screening experiments, five metabolites were reproducibly produced by *N. aurantia*, *C. elegans* (−), and *M. mucedo* and displayed TLC R_f values at 0.67 (**2**), 0.11 (**3**), 0.16 (**4**), 0.05 (**5**), and 0.09 (**6**) vs 0.43 (**1**) (solvent system: CH_2Cl_2−CH_3OH, 20:1).

Preparative Conversion of Isocupressic Acid (1) by *N. aurantia*. *N. aurantia* (ATCC 12674) was grown in 20 125 mL stainless steel-capped DeLong culture flasks, each containing 25 mL of medium. Isocupressic acid (**1**) (200 mg) was dissolved in DMF (2 mL) and evenly distributed among the 24-h-old stage-II cultures. After 144 h, the incubation mixtures were pooled and acidified with 6 N HCl and then filtered to remove cells. The filtrate (480 mL) was extracted with EtOAc−butanol (9:1) (3 × 240 mL). The combined extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure to afford 160 mg of oil. The oil was purified by repeated column chromatography over SiO_2 (1.5 × 9 cm) using a CH_2Cl_2 as an eluting solvent to yield 2 mg of **2** (1%). The cell fraction was extracted by stirring with 300 mL of acetone and filtered. The filtrate was evaporated to remove acetone, water (30 mL) was added, and the mixture was extracted with EtOAc (3 × 30 mL). The combined extracts were dried and evaporated under reduced pressure to afford 270 mg of brown oil. The brown oil was purified by flash column chromatography over a silica gel column (2.8 × 25 cm) using hexanes−EtOAc (3:1) or CH_2Cl_2−CH_3OH (10:1) as eluting systems to yield 34 mg of **2** (17%) and 20 mg of **1** (10%).

Compound **2**: colorless oil; $[\alpha]^{25}_D$ +32.3 ° (*c* 0.43, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 360 MHz) δ 4.85 (1H, s, H-17b), 4.45 (1H, s, H-17a), 2.56 (1H, m, H-12), 2.38 (1H, m, H-7), 2.13 (1H, m, H-3), 2.29 (1H, m, H-12), 2.10 (3H, s, Me-16), 1.94 (1H, m, H-6), 1.81−1.89 (5H, m, H-1, H-2, H-6, H-7, H-11), 1.50−1.57 (3H, m, H-2, H-9α, H-11), 1.30 (1H, m, H-5), 1.23 (3H, s, Me-18), 1.09 (1H, m, H-1), 1.01 (1H, m, H-3), 0.59 (3H, s, Me-20); ^{13}C NMR ($CDCl_3$, 90 MHz) δ 209.6 (C-13), 184.2 (C-19), 148.0 (C-8), 106.5

(C-17), 56.2 (C-5), 55.5 (C-9), 44.2 (C-4), 42.8 (C-12), 40.6 (C-10), 39.0 (C-1), 38.6 (C-7), 37.9 (C-3), 30.0 (C-16), 29.0 (C-18), 26.0 (C-6), 19.9 (C-2), 17.7 (C-11), 12.6 (C-20); EIMS (70 eV) *m/z* 292 $[M]^+$ (1), 274 $[M - H_2O]^+$ (13), 259 $[M - Me]^+$ (5), 246 $[M - HCOOH]^+$ (6), 234 $[M - Me_2CO$, McLafferty$]^+$ (13), 221 (9), 213 (16), 188 (27), 173 (20), 121 $[C_9H_{13}]^+$ (100); HREIMS *m/z* $[M]^+$ 292.2062 (calcd for $C_{18}H_{28}O_3$, 292.2038).

Preparative Conversion of Isocupressic Acid (1) by *C. elegans*. *C. elegans* (−) (NRRL 1393) bioconversions of isocupressic acid were conducted as described for *N. aurantia* except that the incubation reaction was terminated 72 h after substrate addition. The culture was filtered and extracted as before to afford 240 mg of a brown oil that was purified by flash column chromatography over silica gel (3 × 25 cm, 8 psi) using $CH_2Cl_2-CH_3OH$ (15:1) to give 7 mg of **3** (3.5%) and 2 mg of **4** (1%) with R_f values of 0.11 and 0.16, respectively, with $CH_2Cl_2-CH_3OH$, 20:1. The cell fraction contained no metabolites.

Compound **3**: colorless oil; $[\alpha]^{25}_D$ +34.8 ° (*c* 0.34, CH_3OH); ^{1}H NMR (CD_3OD, 360 MHz) δ 5.30 (1H, t, J = 6.8 Hz, H-14), 5.24 (1H, s, H-17b), 4.69 (1H, s, H-17a), 4.06 (2H, d, J = 6.8 Hz, H-15), 3.83 (1H, dd, J = 5.0, 11.5 Hz, H-7α), 2.24 (1H, ddd, J = 2.8, 5.0, 12.6 Hz, H-6), 2.13−2.17 (2H, m, H-3, H-12), 1.91 (1H, m, H-6), 1.81−1.91 (3H, m, H-1, H-2, H-12), 1.68 (1H, m, H-11), 1.66 (3H, s, Me-16), 1.49−1.57 (3H, m, H-2, H-9α, H-11), 1.39 (1H, dd, J = 2.8, 13.3 Hz, H-5), 1.22 (3H, s, Me-18), 1.04−1.14 (2H, m, H-1, H-3), 0.62 (3H, s, Me-20); ^{13}C NMR (CD_3OD, 90 MHz) δ 181.1 (C-19), 151.6 (C-8), 139.9 (C-13), 125.0 (C-14), 103.7 (C-17), 75.0 (C-7), 59.4 (C-15), 54.9 (C-5), 54.7 (C-9), 45.0 (C-4), 40.9 (C-10), 40.3 (C-1), 39.3 (C-3), 39.2 (C-12), 36.6 (C-6), 29.5 (C-18), 22.7 (C-11), 21.2 (C-2), 16.2 (C-16), 13.4 (C-20); EIMS (methyl ester) (70 eV) *m/z* 332 $[M - H_2O]^+$ (7), 314 (10), 299 (6), 272 (15), 255 (19), 239 (27), 221 (22), 199 (13), 185 (20), 173 (42), 161 (39), 147 (33), 131 (39), 123 (100), 121 (71); HRFABMS (methyl ester) *m/z* $[M + Na]^+$ 373.2349 (calcd for $C_{21}H_{34}O_4Na$, 373.2354).

Compound **4**: colorless oil; $[\alpha]^{25}_D$ −27.5 ° (*c* 0.07, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 360 MHz) δ 6.14 (1H, br s, H-7), 5.41 (1H, t, J = 6.8 Hz, H-14), 4.89 (1H, br s, H-6α), 4.23 (2H, d, J = 5.8 Hz, H-17), 4.16 (2H, d, J = 6.8 Hz, H-15), 2.24 (1H, m, H-12), 2.09 (1H, m, H-3), 2.02 (1H, m, H-12), 1.86 (1H, m, H-9α), 1.75 (1H, m, H-5), 1.69 (3H, s, Me-16), 1.46−1.67 (6H, m, H-1, H-2, H-3, H-11), 1.31 (3H, s, Me-18), 1.21 (1H, m, H-1), 0.82 (3H, s, Me-20); ^{13}C NMR ($CDCl_3$, 90 MHz) δ 182.2 (C-19), 147.1 (C-8), 139.1 (C-13), 124.3 (C-14), 119.5 (C-7), 73.0 (C-6), 64.3 (C-17), 59.4 (C-15), 51.1 (C-5), 49.1 (C-9), 42.8 (C-4), 40.9 (C-12), 34.5 (C-10), 33.4 (C-1), 28.1 (C-3), 24.3 (C-11), 24.0 (C-18), 18.1 (C-20), 18.0 (C-2), 16.4 (C-16); HRFABMS *m/z* $[M + Li - H_2O]^+$ 341.2301 [calcd for $(C_{20}H_{32}O_5Li - H_2O)$, 341.2304].

Preparative Conversion of Isocupressic Acid (1) by *M. mucedo*. *M. mucedo* (ATCC 20094) bioconversions were conducted as with other preparative-scale reactions and were terminated 24 h following substrate addition. Extraction as before afforded 350 mg of brown oil that was purified by flash column chromatography over silica gel (2.8 × 24 cm, 5 psi) eluted with $CH_2Cl_2-CH_3OH$ (10:1). We obtained 5 mg of **5** (2.5%) and 6 mg of **6** (3%) with R_f values of 0.56 and 0.62, respectively, with $CH_2Cl_2-CH_3OH$, 10:1. The cell fraction was treated as before to afford 20 mg (10%) of **1** and a mixture of **5** and **6** (10 mg).

Compound **5**: colorless oil; $[\alpha]^{25}_D$ +17.2 ° (*c* 0.29, CH_3OH); ^{1}H NMR (CD_3OD, 360 MHz) δ 5.30 (1H, t, J = 6.5 Hz, H-14), 4.89 (1H, s, H-17b), 4.57 (1H, s, H-17a), 4.09 (1H, m, H-2β), 4.06 (2H, d, J = 6.5 Hz, H-15), 2.40 (1H, m, H-7), 2.36 (1H, ddd, J = 1.8, 4.2, 12.6 Hz, H-3), 2.14 (1H, m, H-12), 2.10 (1H, ddd, J = 1.8, 4.8, 12 Hz, H-1), 1.76−2.01 (4H, m, H-6, H-7, H-12), 1.68−1.72 (2H, m, H-9α, H-11), 1.67 (3H, s, Me-16), 1.45 (1H, m, H-11), 1.26 (1H, m, H-5), 1.25 (3H, s, Me-18), 0.96−1.02 (2H, m, H-1, H-3), 0.64 (3H, s, Me-20); ^{13}C NMR (CD_3OD, 90 MHz) δ 180.7 (C-19), 149.1 (C-8), 139.9 (C-13), 125.0 (C-14), 107.5 (C-17), 65.6 (C-2), 59.4 (C-15), 56.8 (C-5), 56.6 (C-9), 49.1 (C-1), 47.7 (C-3), 46.1 (C-4), 42.4 (C-10), 39.7 (C-7), 39.5 (C-12), 29.5 (C-18), 27.1 (C-6), 23.2 (C-11), 16.3 (C-16), 14.3 (C-20); EIMS (methyl ester) (70 eV) *m/z* 315 (5), 299 (4), 273 (8), 255 (24), 239 (25), 213 (5), 199 (12), 187 (19), 173 (33), 159 (38), 137 (56), 121 (42), 119 (100); HRFABMS (methyl ester) *m/z* $[M + Na]^+$ 373.2358 (calcd for $C_{21}H_{34}O_4Na$, 373.2354).

Compound **6**: colorless oil; $[\alpha]^{25}_D$ + 26.1 ° (*c* 0.23, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 360 MHz) δ 5.86 (1H, dd, J_{cis} = 10.8 Hz, J_{trans} = 17.4 Hz, H-14), 5.18 (1H, d, J = 17.4 Hz, H-15 trans to H-14), 5.04 (1H, d, J = 10.8 Hz, H-15 cis to H-14), 4.88 (1H, s, H-17b), 4.57 (1H, s, H-17a), 4.15 (1H, tt, J = 4.2, 11.4 Hz, H-2β), 2.40−2.43 (2H, m, H-3, H-7), 2.16 (1H, m, H-1), 2.05 (1H, m, H-6), 1.67−1.96 (3H, m, H-6, H-7, H-12), 1.58−1.63 (2H, m, H-9α, H-11), 1.32−1.41 (3H, m, H-5, H-11, H-12), 1.30 (3H, s, Me-16), 1.28 (3H, s, Me-18), 0.95−1.08 (2H, m, H-1, H-3), 0.61 (3H, s, Me-20); ^{13}C NMR ($CDCl_3$, 90 MHz) δ 181.5 (C-19), 147.1 (C-8), 145.1 (C-14), 111.8 (C-15), 107.6 (C-17), 73.6 (C-13), 64.9 (C-2), 56.4 (C-9), 55.6 (C-5), 47.7 (C-1), 46.6 (C-3), 45.0 (C-4), 41.6 (C-10), 41.3 (C-12), 38.4 (C-7), 28.8 (C-18), 27.8 (C-16), 25.5 (C-6), 18.1 (C-11), 13.7 (C-20); EIMS (methyl ester) (70 eV) *m/z* 332 $[M - H_2O]^+$ (3), 314 (8), 299 (7), 273 (10), 255 (7), 239 (32), 213 (5), 199 (17), 187 (26), 173 (46), 159 (59), 137 (89), 121 (54), 119 (100); HRFABMS (methyl ester) *m/z* $[M + Na]^+$ 373.2328 (calcd for $C_{21}H_{34}O_4Na$, 373.2354).

Acknowledgment. We acknowledge financial support through a USDA grant and financial support through a fellowship afforded by the National Science Council, Taipei, Taiwan.

References and Notes

(1) Gardner, D. R.; Molyneux, R. J.; James, L. F., Panter, K. E.; Stegelmeier, B. L. *J. Agric. Food Chem*. **1994**, *42*, 756−761.
(2) Hieda, T.; Mikami, Y.; Obi, Y.; Kisaki, T. *Agric. Biol. Chem*. **1982**, *46*, 2477−2484.
(3) Hieda, T.; Mikami, Y.; Obi, Y.; Kisaki, T. *Agric. Biol. Chem*. **1983**, *47*, 243−250.
(4) Kouzi, S. A.; McChesney, J. D. *Xenobiotica* **1991**, *21*, 1311−1323.
(5) Aranda, G.; Lallemand, J. Y. *Tetrahedron Lett*. **1991**, *32*, 1783−1786.
(6) Aranda, G.; Kortbi EI, M. S.; Lallemand, J. Y.; Neuman, A.; Hammoumi, A.; Facon, I.; Azerad, R. *Tetrahedron* **1991**, *47*, 8339−8350.
(7) Kouzi, S. A.; McChesney, J. D. *J. Nat. Prod*. **1991**, *54*, 483−490.
(8) Kouzi, S. A.; McChesney, J. D. *Helv. Chim. Acta* **1990**, *73*, 2157−2164.
(9) Hanson, J. R.; Hitchcock, P. B.; Nasir, H.; Truneh, A. *Phytochemistry* **1994**, *36*, 903−906.
(10) Hieda, T.; Mikami, Y.; Obi, Y.; Kisaki, T. *Agric. Biol. Chem*. **1982**, *46*, 2249−2255.

(11) Hieda, T.; Mikami, Y.; Obi, Y. *Agric. Biol. Chem*. **1983**, *47*, 787−794.
(12) Lin, S. J.; Short, R. E.; Ford, S. P.; Grings, E. E.; Rosazza, J. P. N. *J. Nat. Prod*. **1998**, *61*, 51−56.
(13) Rosazza, J. P.; Smith, R. V. *Adv. Appl. Microbiol*. **1979**, *25*, 169−208.
(14) Smith, R. V.; Rosazza, J. P. *J. Pharm. Sci*. **1975**, *64*, 1737−1753.
(15) Beukers, R.; Marx, A. F.; Zuidweg, M. H. In *Drug Design*; Ariens, E. J., Ed.; Academic Press: New York, 1972; Vol. 3.
(16) Davis, P. J. *Dev. Ind. Microbiol*. **1987**, *29*, 197−219.
(17) Gibson, D. T. *Microbial Degradation of Organic Compounds*; Marcel Dekker: New York, 1984.
(18) Rosazza, J. P.; Duffel, M. W. In *The Alkaloids*; Manske, R. H. F., Rodrigo, R. G. A., Eds.; Academic Press: New York, 1986; pp 323−405.
(19) Abourashed, E. A.; Hufford, C. D. *J. Nat. Prod*. **1996**, *59*, 251−253.
(20) Bax, A.; Subramanian, S. *J. Magn. Reson*. **1986**, *67*, 565−569.
(21) Bax, A.; Summers, M. F. *J. Am. Chem. Soc*. **1986**, *108*, 2093−2094.
(22) Oshima, Y.; Saito, J. I.; Hikino, H. *Tetrahedron* **1986**, *42*, 6443−6446.
(23) Bastard, J.; Duc, D. K.; Fetizon, M. *J. Nat. Prod*. **1984**, *47*, 592−599.
(24) Noma, M.; Suzuki, F.; Gamou, K.; Kawashima, N. *Phytochemistry* **1982**, *21*, 395−397.
(25) Grover, S. H.; Guthrie, J. P.; Stothers, J. B.; Tan, C. T. *J. Magn. Reson*. **1973**, *10*, 227−230.
(26) Otten, S. L.; Rosazza, J. P. *J. Biol. Chem*. **1983**, *258*, 1610−1613.
(27) Elmarakby, S. A.; Duffel, M. W.; Goswami, A.; Sariaslani, F. S.; Rosazza, J. P. *J. Med. Chem*. **1989**, *32*, 674−679.
(28) Elmarakby, S. A.; Duffel, M. W.; Rosazza, J. P. *J. Med. Chem*. **1989**, *32*, 2158−2162.
(29) Ortiz de Montellano, P. R. In *Cytochrome P-450 Structure, Mechanism, and Biochemistry*; Ortiz de Montellano, P. R., Ed.; Plenum Press: New York, 1986; Chapter 7, pp 249−251.

NP980064B