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MICROBIAL MODELS OF MAMMALIAN METABOLISM: FUNGAL METABOLISM OF THE DITERPENE SCLAREOL BY *CUNNINGHAMELLA* SPECIES

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ABSTRACT.—Microbial metabolism of the diterpene sclareol [1] was studied. Screening studies have shown a number of microorganisms capable of metabolizing sclareol. Preparative scale fermentation with *Cunninghamella* species NRRL 5695 has resulted in the production of two fungal metabolites that have been characterized as 3β-hydroxysclareol [2] and 18-hydroxy-sclareol [3] with the use of 2D nmr techniques. The yield of the two metabolites was improved by utilizing resting-cell suspensions of *Cunninghamella* species NRRL 5695.

Sclareol [labd-14-ene-8α,13β-diol] [515-03-7] [1] is a labdane diterpene ditertiary alcohol widely distributed in nature. It was first isolated from the essential oil of *Salvia sclarea* L. (Labiatae) in 1931 (1). Sclareol has been reported to be a fungal growth regulator and a plant growth inhibitor (2–4) as well as to have a high antibacterial activity (5). Commercially, sclareol is used as a fixative, modifier, and natural body for the perfume industry, as a flavoring agent in the tobacco industry, and as a synthon for the preparation of a series of Ambra odorants in perfumery (6,7). Sclareol has also been reported to be unusually inert biologically in intact rats (8). The essential oil of *S. sclarea* (Clary Sage Oil) is widely used as a flavoring component in food products, alcoholic beverages, and liqueurs and a fragrance component in soaps, detergents, creams, lotions, and tabac-type fragrances in perfumery, as well as in folk medicine (9,10).

Synthesis and the configuration at C-13 of sclareol were studied by Bigley *et al.* (11). The 13*R* configuration at C-13 has been confirmed by determining the crystal structure of (−)-sclareol-8-acetate (12). The natural product **1** is an epimeric mixture at C-13 in a ratio of approximately 9:1, where the (13*R*)-epimer is the major epimer in the mixture (6,12). Functionalization of the A ring in sclareol is very difficult to achieve by chemical reactions. Oxidation of sclareol by the "Gif system" (13) has afforded 2-keto-(2.5% yield) and 3-ketosclareol (0.7% yield) in extremely low yields.

Microbial transformation studies of sclareol have been reported by Hieda *et al.* (14,15) utilizing two soil bacteria: *Nocardia restricta* and *Rhodococcus erythropolis*. Eight metabolites of sclareol were isolated and identified. The plasmids responsible for the C-18 oxidation were also identified in both bacteria (16,17). In 1982, a Brazilian tobacco company (Companhia Souza Cruz Industria e Comercio) patented the preparation of 3α-, 3β-, and 3-ketosclareol as microbial metabolites of sclareol utilizing several

1

2 R=OH, R¹=Me
3 R=H, R¹=CH₂OH
4 R=OAc, R¹=Me
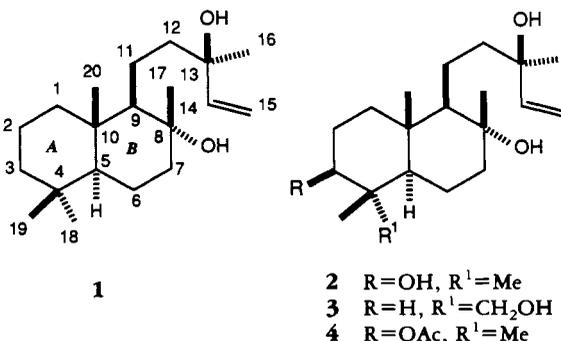
microorganisms (18). These three metabolites were also reported to improve tobacco smoke flavor at very low concentrations.

Because there have been no reports on the mammalian metabolism of sclareol, a prospective approach was undertaken to study the mammalian metabolism of this diterpene utilizing microorganisms, particularly fungi, as in vitro models for the prediction, comparison, and preparation of the mammalian metabolites (19–23) of sclareol. Microbial systems are also used to prepare useful odorants, fragrances, and flavorings compounds from the parent diterpene sclareol and as biocatalysts for conducting highly stereo- and regioselective transformations of sclareol to compounds that are not accessible or accessible with difficulty by chemical reactions and that could be useful as synthons in natural product chemistry.

In the present study, we report the isolation of two fungal metabolites of sclareol from a preparative scale fermentation of growing cultures of *Cunninghamella* species NRRL 5695. Based on the chemical and spectroscopic data, especially of 2D nmr techniques, these two metabolites have been identified as labd-14-ene-3β,8α,13β-triol [2] and labd-14-ene-8α,13β,18-triol [3]. The yield of these metabolites was improved by using resting-cell suspensions of *Cunninghamella* species NRRL 5695. The isolation and structure elucidation of the two metabolites are described herein.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined either on a Fisher digital melting point analyzer model 355 or in open capillary tubes with a Thomas-Hoover capillary melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. Ir spectra were recorded in KBr using a Perkin-Elmer 281B infrared spectrophotometer. Gc analysis was carried out at 280° isothermal on a Hewlett Packard 5890A Gas Chromatograph equipped with a flame ionization detector using a DB-5 30 m × 0.25 mm capillary column with He (flow rate of 1 ml/min) as the carrier gas. The term in vacuo refers to removal of solvent with a rotary evaporator under water aspirator vacuum (15–30 mm Hg). ^{1}H- and ^{13}C-nmr spectra were obtained in Me_2CO-d_6 on a Varian VXR-300 FT spectrometer operating at 300 MHz and 75 MHz, respectively. The chemical shift values are reported in ppm, and the coupling constants (J values) are in Hz. Abbreviations for nmr signals are as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. Standard pulse sequences were used for COSY (24), HETCOR (25), DEPT GL (26), APT (27), and 2D-INADEQUATE (28,29) experiments. Electron impact mass spectra were obtained using a Finnigan model 3200 (70 eV ionization potential) mass spectrometer coupled to a Teknivent Vector/One data system. High-resolution eims data were obtained at the MS Laboratory, Department of Chemistry, The University of Kansas, Lawrence, Kansas 66045.

CARBON-CARBON CONNECTIVITY PLOT OF SCLAREOL [1].—For the 2D-INADEQUATE (CCC2DQ 2D-INADEQUATE pulse sequence) experiment, a spectral width of 11086.5 Hz in both F2 (the carbon chemical shifts axis) and F1 (the double quantum frequency axis) was employed with an acquisition time of 92.4 msec and 64 increments. The sample was prepared by adding 500 mg of 1 to 0.5 ml of $CDCl_3$ in a 5-mm tube, and the measurements were made at ambient temperature with an internal lock and referencing to $CDCl_3$. The repetition rate of 5.0 sec and 768 repetitions per increment resulted in a total data acquisition time of 69 h and 44.9 min, and the data (FT size 2K × 512) was pseudo-echo processed. The resulting 2D array, in which the signals arising from spin-coupled pairs of ^{13}C-nuclei (in natural abundance) are separated according to their double quantum frequencies, was plotted as a contour plot with the ^{13}C chemical shift as the x-axis and the double quantum frequency the y-axis. This connectivity plot is a contour plot of the nmr data in which the doublets arising from the spin-coupled ^{13}C nuclei appear as two pairs of spots on the same horizontal level at the chemical shift frequencies of the coupled nuclei, and the signals are symmetrically located about a diagonal line through the plot.

CHROMATOGRAPHIC CONDITIONS.—Tlc chromatographic analyses were carried out on precoated Silica G-25 UV$_{254}$ plates (Macherey-Nagel Düren). The adsorbent used for cc was flash Si gel 60/230–400 mesh (E. Merck, Darmstädt). Developing system used for tlc was EtOAc-hexane (9:1) solution, and visualization of the tlc plates was performed using anisaldehyde/H_2SO_4 spray reagent. The spots were visualized by spraying the plate and then heating it at 110° for 3 min in an oven.

MICROORGANISMS.—The cultures were obtained from the University of Mississippi, Department

of Pharmacognosy Culture collection, and were originally from the American Type Culture Collection (ATCC), Rockville, Maryland, or from Northern Regional Research Laboratories (NRRL), Peoria, Illinois. UI cultures were obtained from Dr. John P. Rosazza, Department of Medicinal Chemistry and Natural Products, College of Pharmacy, University of Iowa, Iowa City, Iowa 52242. Cultures used for preliminary screening of sclareol that showed one or more metabolites by tlc are as follows: *Aspergillus flavipes* ATCC 1030, *A. flavipes* ATCC 11013, *A. flavipes* ATCC 16795, *Aspergillus flavus* ATCC 9170, *A. flavus* NRRL 501, *Aspergillus niger* ATCC 16888, *A. niger* ATCC 10549, *A. niger* ATCC 10581, *A. niger* ATCC 11394, *Aspergillus parasiticus* ATCC 15517, *Beauvaria bassiana* ATCC 7159, *Calonectria decora* ATCC 14767, *Chaetomium cochloides* NRRL 2320, *Cunninghamella blakesleeana* ATCC 8688a, *Cunninghamella bainieri* UI-3605, *Cunninghamella elegans* ATCC 9245, *Cunninghamella echinulata* NRRL 3655, *Cu. echinulata* ATCC 9244, *Cunninghamella* species NRRL 5695, *Fusarium oxysporum* f. *cepae* ATCC 11711, *F. oxysporum* ATCC 7601, *Mucor griseo-cyanus* ATCC 1207a, *Mucor ramannianus* 1839 (sih), *Nocardia corallina* ATCC 19070, *Rhizopus arrhizus* ATCC 11145, *Sporobolomyces pararoseus* ATCC 11386, *Streptomyces lavendulae* L-105, *Streptomyces platensis* NRRL 2364, *Stysanus microsporus* ATCC 2833.

MEDIA.—All the preliminary screening and scale-up experiments were carried out in a medium consisting of: dextrose, 20 g; yeast extract, 5 g; peptone, 5 g; NaCl, 5 g; K_2HPO_4, 5 g; distilled H_2O, 1000 ml. Stock cultures of fungi and bacteria were stored on slants of Mycophil (BBL, Cockeysville, Maryland) and Eugon (Difco, Detroit, Michigan) agar, respectively, at 4°. The 0.1 M phosphate buffer (pH 7.2), used for the resting-cell suspensions of *Cunninghamella* species NRRL 5695, consists of KH_2PO_4, 4.08 g; K_2HPO_4, 10.6 g; distilled H_2O, 1000 ml.

FERMENTATION PROCEDURES.—Microbial metabolism studies were carried out by incubating the cultures with shaking on the model G-10 Gyrotory shaker (New Brunswick Scientific Co., New Jersey), operating at 250 rpm, at 25°. Preliminary screening experiments were carried out in 125-ml stainless-steel-capped DeLong culture flasks containing 25 ml of medium. The media were sterilized at 121° and 18 psi for 15 min. Fermentations were carried out according to a standard two-stage protocol. In general, the substrate was added to the incubation media 24 h after the inoculation of the stage II cultures as a 10% solution in EtOH or DMF at a concentration of 0.2 mg/ml of stage II medium. The fermentations were sampled at 24-h intervals by extraction of 3 ml culture with 3 ml EtOAc. The extracts were concentrated and chromatographed on tlc plates. Substrate controls were composed of sterile medium to which the substrate 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. Substrate-autoclaved culture controls consisted of microbial cultures that were grown under the usual conditions to maturity (usually 5–7 days), autoclaved for 30 min, and then incubated after the substrate was added.

SCLAREOL [1].—The natural product was obtained as an off-white to amber-colored solid resin from R.J. Reynolds Tobacco Company, Flavor Technology Division, produced by the hydrocarbon solvent extraction of the plant *S. sclarea*. Flash Si gel cc, using EtOAc-hexane (1:4) as an eluent, and recrystallization of the homogeneous fractions from hexane yielded pure sclareol [1] (by tlc and gc) as white needles (85% yield from the resin) (R_f 0.62): mp 96–97° [lit. (6) mp 99–100°]; $[\alpha]^{25}D$ −2.82° ($c = 1.45$ g/100 ml, $CHCl_3$) [lit. (6) $[\alpha]^{20}D$ −2.17° ($c = 1.45$ g/100 ml, $CHCl_3$)]; eims m/z $[M − H_2O]^+$ 290; ir (KBr) ν max (cm^{-1}) 3280, 2910, 1635, 1450, 1380, 1360, 985, 910, 890; ^1H nmr 5.93 (1H, dd, J_{cis} = 10.8 Hz and J_{trans} = 17.0 Hz, H-14), 5.20 (1H, dd, J_{trans} = 17.0 Hz and J_{gem} = 1.9 Hz, H$_{trans}$-15), 4.92 (1H, dd, J_{cis} = 10.8 Hz and J_{gem} = 1.9 Hz, H$_{cis}$-15), 3.70 (1H, br s, exchangeable with D_2O, OH), 3.23 (1H, br s, exchangeable with D_2O, OH), 1.79 (1H, dt, J = 3.2, 12.2 Hz, H-7), 1.71 (1H, m, H-1), 1.58–1.60 (2H, m, H-6, H-2), 1.54 (3H, m, H-11, H$_a$-12, H$_b$-12), 1.50 (1H, m, H-7), 1.35–1.46 (3H, m, H-6, H-11, H-3), 1.32 (1H, m, H-1), 1.21 (3H, s, Me-16), 1.12 (3H, s, Me-17), 0.94 (1H, dd, J = 2.2, 12.0 Hz, H-5), 0.87 (3H, s, Me-18), 0.81 (6H, s, Me-19, Me-20); ^{13}C nmr see Table 1.

REAGENTS.—4-Dimethylaminopyridine was obtained from Aldrich Chemical Company, and Ac_2O was purchased from Mallinckrodt. Pyridine and DMF were distilled from calcium hydride and stored over 4 Å molecular sieves.

FUNGAL METABOLISM OF SCLAREOL [1] TO METABOLITES 2 AND 3.—A total of 600 mg of sclareol [1] was dissolved in 2 ml of absolute EtOH and distributed equally among fifteen 1-liter culture flasks each containing 200 ml of 24-h-old *Cunninghamella* species NRRL 5695 stage II culture. After 8 days, the entire incubation mixtures were combined and filtered, and the cells were washed with distilled H_2O. The combined aqueous filtrates (3.2 liters) were extracted with 4 × 700 ml of EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and filtered, and the solvent was evaporated in vacuo to afford 1.27 g of brownish-yellow residue. The residue was chromatographed on a Si gel (127 g) column (65 × 4 cm) using EtOAc-hexane as an eluent, and 10-ml fractions were collected. After elution with EtOAc-hexane (3:7),

TABLE 1. ^{13}C-nmr Chemical Shift Assignments for Compounds **1–4**.[a]

Carbon	Compound			
	1	**2**	**3**	**4**
C-1	40.4(2)[b]	38.8(2)	40.1(2)	38.0(2)
C-2	19.0(2)	27.9(2)	18.4(2)	23.9(2)
C-3	42.7(2)	78.5(1)	36.0(2)	80.8(1)
C-4	33.7(0)	39.4(0)[c]	38.2(0)	38.1(0)
C-5	56.9(1)	56.1(1)	49.5(1)	55.7(1)
C-6	21.1(2)	20.9(2)	20.7(2)	20.5(2)
C-7	45.1(2)	45.2(2)	44.9(2)	44.6(2)
C-8	73.9(0)	73.7(0)	73.8(0)	73.6(0)
C-9	62.3(1)	62.2(1)	62.3(1)	61.7(1)
C-10	39.8(0)	39.7(0)[c]	39.7(0)	39.3(0)
C-11	20.0(2)	20.1(2)	20.0(2)	19.8(2)
C-12	46.2(2)	46.2(2)	46.2(2)	45.8(2)
C-13	73.3(0)	73.3(0)	73.3(0)	73.2(0)
C-14	147.5(1)	147.4(1)	147.4(1)	147.1(1)
C-15	110.7(2)	110.7(2)	110.6(2)	110.7(2)
C-16	27.8(3)	28.1(3)	28.0(3)	27.8(3)
C-17	24.5(3)	24.3(3)	24.3(3)	24.2(3)
C-18	33.7(3)	28.6(3)	71.5(2)	28.2(3)
C-19	21.8(3)	15.9(3)[d]	17.8(3)	16.6(3)
C-20	15.8(3)	15.8(3)[d]	16.0(3)	15.8(3)
C-21				170.5(0)
C-22				20.9(3)

[a] Assignments in ppm are based on ^{1}H-^{1}H and ^{1}H-^{13}C chemical shift correlated 2D nmr spectroscopy and comparisons to the assignments of compound **1**. The assignments for compound **1** are also based on ^{13}C-^{13}C correlated 2D-INADEQUATE experiment.
[b] The number in parentheses indicates the number of hydrogens attached to the corresponding carbon and was determined from the DEPT-GL experiments.
[c,d] Assignments may be reversed.

the homogeneous fractions which showed a single spot with R_f 0.45 were combined and evaporated in vacuo to give 200 mg of metabolite **2**. Recrystallization from EtOAc/hexane afforded white needles of **2** (181 mg, 28% yield): mp 162–163°; [α]^{25}D −7.33° (c = 0.15 g/100 ml, CHCl$_3$); hrms m/z calcd for C$_{20}$H$_{34}$O$_2$ [M − H$_2$O]$^+$ 306.2557, found 306.2546; ir (KBr) ν max (cm^{-1}) 3400, 2980, 2960, 2930, 2860, 1455, 1385, 1050, 995, 920; ^{1}H nmr 5.93 (1H, dd, J_{cis} = 10.7 Hz, J_{trans} = 17.3 Hz, H-14), 5.20 (1H, dd, J_{trans} = 17.3 Hz, J_{gem} = 1.9 Hz, H$_{trans}$-15), 4.93 (1H, dd, J_{cis} = 10.7 Hz, J_{gem} = 1.9 Hz, H$_{cis}$-15), 3.55 (1H, s, exchangeable with D$_2$O, OH), 3.36 (1H, d, J = 5.4 Hz, exchangeable with D$_2$O, OH-3), 3.16 (1H, ddd, J = 5.4, 5.4, 10.5 Hz, H-3; on D$_2$O treatment, the ddd collapses to a dd, J = 6.5, 8.9 Hz), 3.07 (1H, s, exchangeable with D$_2$O, OH), 1.74 (1H, m, H-7), 1.64 (1H, m, H-1), 1.57 (3H, m, H-6, H$_a$-2, H$_b$-2), 1.54 (2H, m, H-11), 1.40 (2H, m, H-6, H-7), 1.21 (3H, s, Me-16), 1.11 (3H, s, Me-17), 1.05 (2H, m, H-1, H-9), 0.98 (3H, s, Me-18), 0.91 (1H, dd, J = 2.4, 11.6 Hz, H-5), 0.82 (3H, s, Me-20), 0.75 (3H, s, Me-19); ^{13}C nmr see Table 1.
     After increasing the polarity of the eluent to EtOAc-hexane (2:3), the homogeneous fractions which showed a single spot with R_f 0.28 were combined and evaporated in vacuo to give 260 mg of metabolite **3**. Recrystallization from EtOAc/hexane yielded colorless crystals of **3** (233 mg, 37% yield): mp 108–109°; [α]^{25}D +1.91° (c = 0.157 g/100 ml, CHCl$_3$); hrms m/z calcd for C$_{20}$H$_{34}$O$_2$ [M − H$_2$O]$^+$ 306.2557, found 306.2551; ir (KBr) ν max (cm^{-1}) 3370, 2990, 2960, 2930, 2860, 1460, 1385, 1045, 990, 910; ^{1}H nmr 5.91 (1H, dd, J_{cis} = 10.8 Hz, J_{trans} = 17.4 Hz, H-14), 5.18 (1H, dd, J_{trans} = 17.4 Hz, J_{gem} = 1.9 Hz, H$_{trans}$-15), 4.91 (1H, dd, J_{cis} = 10.8 Hz and J_{gem} = 1.9 Hz, H$_{cis}$-15), 3.55 (1H, t, J = 5.5 Hz, exchangeable with D$_2$O, OH-18), 3.35 [1H, dd, J = 5.5, 10.6, Hz, H$_A$-18; on D$_2$O treatment, the dd collapses to a d at 3.32 ppm (1H, J = 10.6 Hz, H$_A$-18)], 3.08 (1H, br s, exchangeable with D$_2$O, OH), 2.97 [1H, dd, J = 5.5, 10.6 Hz, H$_B$-18; on D$_2$O treatment, the dd collapses to a d at 2.95 ppm (1H, J = 10.6 Hz, H$_B$-18)], 2.86 (1H, br s, exchangeable with D$_2$O, OH), 1.62–1.65 (3H, m, H$_a$-7, H$_b$-7, and H-1), 1.47–1.57 (4H, m, H-3, H$_a$-6, H$_b$-6, H-11), 1.43 (1H, dd, J = 3.0, 12.5 Hz, H-5), 1.21

(3H, s, Me-16), 1.14 (1H, t, J = 3.7 Hz, H-9), 1.11 (3H, s, Me-17), 0.92 (1H, m, H-1), 0.83 (3H, s, Me-20), 0.70 (3H, s, Me-19); ^{13}C nmr see Table 1.

PREPARATION OF METABOLITES **2** AND **3** USING RESTING-CELL SUSPENSIONS OF *CUNNING-HAMELLA* SPECIES NRRL 5695.—The yield of both metabolites was improved by using whole-cell suspensions of *Cunninghamella* species NRRL 5695. Following the standard two-stage fermentation protocol, eight 2-liter culture flasks each containing 400 ml of *Cunninghamella* species NRRL 5695 stage II culture were incubated on the shaker for 5 days to get as much cell-mass as possible. The mycelium (ca. 40 g, wet wt) was harvested from each flask by suction filtration through a Büchner funnel, washed several times with sterile distilled H_2O, and suspended in a 1-liter flask containing 200 ml of sterile 0.1 M phosphate buffer (pH 7.2). A total of 400 mg of sclareol was then dissolved in 1.6 ml of DMF and distributed equally among the eight resting-cell suspension flasks. After 3 days of incubation on the shaker, the suspensions were harvested by filtration and the cells were washed with distilled H_2O. The aqueous filtrate (1.7 liters) was extracted with 4 × 400 ml of EtOAc. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated in vacuo to afford 1.14 g of a yellowish residue. The residue was chromatographed on a flash Si gel (114 g) column (65 × 4 cm) using EtOAc/hexane as an eluent, and 10-ml fractions were collected. Elution with EtOAc-hexane (3:7) and recrystallization of the homogeneous fractions from EtOAc/hexane afforded metabolite **2** (155 mg, 36% yield). Increasing the polarity of the eluent to EtOAc-hexane (2:3) and recrystallization of the homogeneous fractions from EtOAc/hexane yielded metabolite **3** (211 mg, 50% yield).

PREPARATION OF 3β-ACETOXY-LABD-14-ENE-8α,13β-DIOL [**4**].—A total of 35 mg (0.10 mmol) of metabolite **2** was dissolved in 2 ml of dry pyridine. A catalytic amount of 4-dimethylaminopyridine was added, and the mixture was stirred in a flask stoppered with a drying tube containing Drierite®, at 0° in an ice-bath. Ac_2O (3 ml) was then added slowly to the reaction mixture that was stirred at 0° for 1 h. Iced distilled H_2O was then added to the mixture, which was stirred for 10 min and extracted with EtOAc. The organic layer was washed successively with 10% HCl until the aqueous layer remained acidic, 10% NH_4OH until the aqueous layer remained basic, and distilled H_2O, and then dried over anhydrous Na_2SO_4. Evaporation of the organic layer in vacuo yielded 40 mg of a yellowish oily residue. Purification of the residue over Si gel (5 g) by cc (52 × 2 cm), using EtOAc-hexane (1:3) as an eluent, yielded compound **4** (R_f 0.54) as an oil (37 mg, 93% yield): $[\alpha]^{25}$D +2.0° (c = 0.05 g/100 ml, CHCl$_3$); hrms m/z calcd for $C_{22}H_{36}O_3$ [M − H$_2$O]$^+$ 348.2664, found 348.2646; ir (CHCl$_3$) ν max (cm^{-1}) 3580, 3380, 2940, 1720, 1450, 1370, 1250, 1120, 1020, 990, 920; ^{1}H nmr 5.92 (1H, dd, J_{cis} = 10.7 Hz, J_{trans} = 17.3 Hz, H-14), 5.20 (1H, dd, J_{trans} = 17.3 Hz, J_{gem} = 1.9 Hz, H$_{trans}$-15), 4.92 (1H, dd, J_{cis} = 10.7 Hz, J_{gem} = 1.9 Hz, H$_{cis}$-15), 4.44 (1H, dd, J = 5.1, 9.0 Hz, H-3), 3.72 (1H, br s, exchangeable with D$_2$O, OH), 3.31 (1H, br s, exchangeable with D$_2$O, OH), 1.99 (3H, s, Me-22), 1.80 (1H, dt, J = 3.0, 12.2 Hz, H-7), 1.73 (1H, m, H-1), 1.54–1.67 (5H, m, H$_a$-2, H$_b$-2, H-6, H-11, H-12), 1.27–1.51 (3H, m, H-7, H-6, H-11), 1.21 (3H, s, Me-16), 1.12 (3H, s, Me-17), 1.02 (1H, dd, J = 2.2, 10.6 Hz, H-5), 0.86 (3H, s, Me-18), 0.84 (6H, s, Me-19, Me-20); ^{13}C nmr see Table 1.

RESULTS AND DISCUSSION

A total of 45 microorganisms were screened for their ability to biotransform sclareol [**1**]. *Cunninghamella* species was selected for preparative scale transformation of **1** because no sclareol was detected by tlc.

The ^{13}C-nmr chemical shift assignments of **1** were first reported by two research groups (30,31) with ambiguous assignments for C-2, C-6, C-11, C-7, and C-12. This ambiguity was clarified by Barton *et al.* (13) who reported complete and unambiguous ^{13}C-nmr chemical shift assignments of **1** by parallel use of homo- and heteronuclear chemical shift correlation spectroscopy. This was also confirmed in our laboratory by the ^{13}C-^{13}C correlated 2D-INADEQUATE experiment. This carbon-carbon connectivity experiment unambiguously confirmed 18 of the 21 C-C bonds present in **1**. Of the remaining three bonds, two bonds (C-1 to C-10 and C-4 to C-18) would not show cross peaks because the chemical shifts are nearly coincidental; therefore only one bond (C-14 to C-15) did not show any cross peaks. On the other hand, C-14 and C-15 can be easily assigned based on chemical shifts and multiplicities. C-4 and C-10 could also be assigned by comparing the data for **1** and **2** and by noting the down-field shift of C-4 when C-3 is hydroxylated in **2**. ^{1}H-nmr chemical shift assignments of **1** were based on ^{1}H-^{1}H and ^{1}H-^{13}C chemical shift correlated 2D nmr spectroscopy.

A preparative scale fermentation with *Cunninghamella* species was performed, and two metabolites **2** and **3** were isolated and purified by chromatography. The mass spectra of metabolite **2** ($[M - H_2O]^+$ at *m/z* 306) suggested that a single oxygen atom had been added to the substrate molecule. The ^{1}H-nmr spectrum of metabolite **2** showed a new ddd ($J = 5.4$, 5.4, 10.5 Hz) at 3.16 ppm, which collapses to a dd ($J = 6.5$ and 8.9 Hz) after D_2O exchange, and a new d ($J = 5.4$ Hz) at 3.36 ppm which is exchangeable on treatment with D_2O. The DEPT GL experiment showed the disappearance of a CH_2 group and the appearance of a new CH group at 78.5 ppm. All of these data indicated that metabolite **2** is a monohydroxylated (secondary) metabolite of sclareol. Comparison of the ^{13}C-nmr spectral data of **1** and metabolite **2** supported C-3 as the position of hydroxylation. The stereochemistry of the new hydroxyl group at C-3 was established as beta from the ^{1}H-nmr data. H-3 is coupled to H-2 protons and to the proton of the new OH-group at C-3. On treatment with D_2O, the proton of the OH-group at C-3 exchanges with deuterium, leading to the disappearance of the coupling with H-3. H-3 couples with the H-2 protons with $J = 6.5$ and 8.9 Hz, and therefore must be axial. In $CDCl_3$, the coupling between H-3 and the proton of the new OH group at C-3 was not observed, and H-3 resonated at 3.21 ppm as a dd ($J = 4.5$, 11.0 Hz). This was further confirmed by the acetylation of metabolite **2** to produce the monoacetate **4**. Based on all of the evidence, metabolite **2** was characterized as 3β-hydroxysclareol. ^{13}C- and ^{1}H-nmr chemical shift assignments of metabolite **2** and its monoacetate **4** were based on ^{1}H-^{1}H and ^{1}H-^{13}C chemical shift correlated 2D nmr spectroscopy. ^{1}H- and ^{13}C-nmr chemical shift assignments of the five methyl groups in **2**, Me-16, -17, -18, -19, and -20, were also based on long-range HETCOR (LR HETCOR) experiment where the *J* value was optimized to 10 Hz to detect ^{13}C-^{1}H 3-bonds coupling. The LR HETCOR spectrum of compound **2** showed coupling between Me-19 and C-18, Me-20 and C-1, Me-20 and C-5, Me-20 and C-9, Me-18 and C-5, Me-18 and C-19, Me-17 and C-9, Me-16 and C-12, and Me-16 and C-14.

The mass spectral data of metabolite **3** ($[M - H_2O]^+$ at *m/z* 306) suggested that a single oxygen atom had been added to the substrate molecule. The ^{1}H-nmr spectrum of **3** showed two new dd at 3.35 and 2.97 ppm ($J = 5.5$, 10.6 Hz), and a new t ($J = 5.5$ Hz) at 3.55 ppm which is exchangeable on treatment with D_2O. The DEPT GL experiment showed the disappearance of a Me group and the appearance of a new CH_2 group at 71.5 ppm. All of these data indicated that metabolite **3** is also a monohydroxylated (primary) metabolite of sclareol. Comparison of the ^{13}C-nmr spectral data of **1** and **3** supported C-18 as the position of hydroxylation. On treatment with D_2O, the two new dd collapsed to an AB-q at 3.14 ppm ($J = 10.6$ Hz). The two H-18 protons are coupled to each other and to the proton of the new OH-group at C-18. After D_2O exchange, the coupling between H-18 protons and the OH group at C-18 disappears, leaving behind an AB-q which represents the two H-18 protons coupled to each other. In $CDCl_3$, the coupling between H-18 protons and the proton of the new OH group at C-18 was not observed, and the H-18 protons resonated as an AB-q at 3.41 ppm (1H, d, $J = 10.8$ Hz, H_A-18) and 3.11 ppm (1H, d, $J = 10.8$ Hz, H_B-18). Based on all of the evidence, metabolite **3** was characterized as 18-hydroxysclareol. ^{13}C- and ^{1}H-nmr chemical shift assignments of metabolite **3** were based on ^{1}H-^{1}H and ^{1}H-^{13}C chemical shift correlated 2D nmr spectroscopy. ^{1}H- and ^{13}C-nmr chemical shift assignments of the four methyl groups in **3**: Me-16, -17, -19, and -20 were also based on long-range HETCOR experiment where the *J* value was optimized to 10 Hz to detect ^{13}C-^{1}H 3-bonds coupling. The LR HETCOR spectrum of compound **3** showed coupling between Me-19 and C-18, Me-19 and C-3, Me-20 and C-9, Me-20 and C-1, Me-17 and C-7, Me-17 and C-9, Me-16 and C-12, and Me-16 and C-14.

Twelve genera (29 species) of microorganisms were found to be able to biotransform

sclareol [1] to one or more metabolites based on tlc analysis (see Experimental). *Cunninghamella* (6 species), *Aspergillus* (2 species), *Chaetomium*, *Mucor* (1 species), and *Sporobolomyces* produced the same pattern of hydroxylation reactions of 1 to give 2 and 3 as the major metabolites, whereas *Aspergillus* (6 species), *Calonectria*, *Mucor* (1 species), and *Streptomyces* (2 species) produced metabolite 2 as the major metabolite. On the other hand, *Aspergillus* (2 species), *Beauvaria*, *Fusarium* (2 species), *Nocardia*, *Rhizopus*, and *Stysanus* showed only a trace of metabolites 2 and/or 3 by tlc. *Cunninghamella* species NRRL 5695 was selected for preparative scale fermentation because of its high yielding conversion of 1 to both metabolites 2 and 3 as shown by tlc. Sampling of the culture at 24-h intervals showed the appearance of both metabolites 2 and 3 48 h after the addition of the substrate (in EtOH), while the disappearance of the substrate was observed by tlc 8 days after the substrate addition. A preparative scale fermentation of *Cunninghamella* species afforded metabolites 2 and 3 in 28% and 37% yield, respectively. Addition of 1 to the growing cultures of *Cunninghamella* species in DMF did not have any effect on the yield or the kinetics of the formation of 2 and 3. Preparative scale fermentation of 1 using growing cultures of *Cunninghamella bainieri* UI-3605 resulted in the production of metabolites 2 and 3 in 32% and 29% yield, respectively.

In an attempt to improve the yield of metabolites 2 and 3 from cultures of *Cunninghamella* species, the use of whole-cell (also called resting-cell) suspensions was investigated, since it has been shown that an increase in yield of microbial transformation products was obtained with cell suspensions as compared to growing cultures of some fungi (22, 32). In resting-cell suspensions, the aqueous medium (0.1 M phosphate buffer) is a much cleaner incubation medium when compared to the complex culture medium used for growing cultures, making the substrate more available to be metabolized by the fungal cells without any interference from the culture medium. Resting-cell biocatalysts, which are not in an actively growing state, afford cleaner reaction mixtures and render analyses and metabolite isolations much simpler (33). Sampling the resting-cell suspensions at 24-h intervals showed the appearance of 2 and 3 only 24 h after the addition of the substrate (in DMF), while the disappearance of the substrate was observed 3 days after the substrate addition. When added in EtOH, the substrate 1 has a poor solubility in the aqueous medium of the resting-cell suspensions of *Cunninghamella* species, and tlc showed that, in addition to metabolites 2 and 3, the substrate can still be detected 3 days after its addition. In growing cultures with their more complex media, 1 was more easily dispersed in the aqueous medium when added in EtOH. Clearly, the components of the complex culture medium used for growing cultures are playing a major role in solubilizing the substrate and facilitating its dispersion in the aqueous culture medium. On the other hand, DMF facilitated the dispersion of the substrate 1 in the whole-cell suspensions making it more accessible to the fungal cells in the incubation medium. By using whole-cell suspensions of *Cunninghamella* species, we were able to obtain metabolites 2 and 3 in 36% and 50% yield, respectively. Further microbial transformation studies and the mammalian metabolism studies on sclareol [1] are in progress.

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