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Microbiological conversion of a β- and γ-eudesmol mixture by *Rhizopus*

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A mixture of β-and γ-eudesmols was microbiologically biotransformed by *Rhizopus stolonifer* ATCC 6227. Positions-2 and 3, in both substrates, proved to be accessible by hydroxylase enzyme. Four different metabolites were isolated and their structures were elucidated by different spectroscopic methods. The structures of these metabolites were established as eudesma-3-en-2β,11-diol; eudesma-4-en-3β,11-diol; eudesma-4(15)-en-2β,11-diol and eudesma-4(15)-en-3β,11-diol.

1. Introduction

Beta- and gamma-eudesmols (**1**, **2**) are sesquiterpene alcohols that could serve as precursors for pharmaceuticals, agrochemicals or even synthons in organic synthesis. Biologically active eudesmane-derivatives have been reported previously e.g. argentone, santonin and panellon [1–3]. Both β- and γ-eudesmols were isolated and characterized from the resin of the hybrid *Parthenium argentatum* × *P. tomentosa* and *P. argentatum* cultivars (guayule) [4]. Relatively, large quantities of these compounds were obtained in a semi-crude mixture while searching for antifungal agents [1]. This paper describes the biotransformation of a mixture of these eudesmols by microorganisms.

2. Investigations, results and discussion

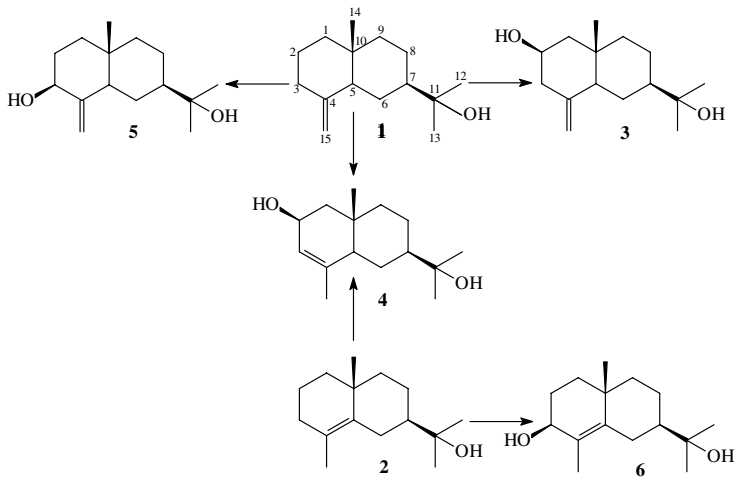
For the biotransformation of a mixture of β- and γ-eudesmols (**1**, **2**) the following microbes were subjected to screening tests: *Aspergillus niger* UA 172–1, *Aspergillus fumigatus* ATCC 13073, *Rhizopus arrhizus* ATCC 2433, *Rhizopus stolonifer* ATCC 6227, *Gibberella suabinetti* ATCC 20193, *Calonectria decora* ATCC 14767, *Septomyxa affinis* ATCC 6737, *Coniothyrium hellebori* ATCC 12527, *Rhodotorula glutinis* 15125, *Nocardia corallina* var. *taoka* ATCC 31338, *Mucor circinelloides* ATCC 15242, *Streptomyces antibioticus* ATCC 11891, *Streptomyces coeruleorubidus* ATCC 31276, *Staphyllococcus aureus* ATCC 27664 and *Bacillus subtilis* ATCC 21394.
Rhizopus stonlonifer ATCC 6227 demonstrated good results and was able to convert the eudesmol mixture into several metabolites. Scale up of this biotransformation reaction afforded the isolation of four metabolites (**3**–**6**).
Metabolite **3** possesses NMR and mass spectral data similar to those reported for eudesma-4(15)-en-2α,11-diol (pterocarpol) with few differences [6, 7]. The hydroxy methine proton signal is moved downfield to δ 4.21 ppm, in addition to slight deviations in ^{13}C-data, especially at ring-A (Table). This indicated that metabolite **3** is most likely an isomer of pterocarpol and its structure should be 2-epi-pterocarpol, or eudesma-4(15)-en-2β,11-diol.
Metabolite **4** gave m/z 238 analyzed for $C_{15}H_{26}O_2$. This indicated that **4** has one more oxygen atom than the substrates. The ^{1}H NMR and ^{13}C NMR spectra displayed olefinic signals different from those for either β- or γ-eudesmol. The methine carbon signals at δ 126.8 and the quaternary carbon signal at δ 136.7 ppm, together with the proton doublet at δ 5.49 ppm pointed to a double bond at position-3 rather than position-4 (γ-eudesmol) or position-4(15) (β-eudesmol). The downfield shifted methyl protons singlet at δ 1.66 ppm was assigned to the 15-methyl group. The new carbon signal at δ 74.5 ppm which is correlated to the new proton multiplet at δ 4.73 ppm was assigned to a carbinol methine group at position-2 [6]. The relative stereochemistry of the hydroxyl group at position-2 was assigned to be in a beta configuration, based on ^{13}C-chemical shift value of this position, compared to some eudesmanoid analogs [6]. Therefore, the structure of **4** is concluded to be eudesma-3-en-2β,11-diol.
The ^{1}H NMR spectrum of **5** showed three methyl groups singlets at δ 0.67, 1.19 and 1.19 ppm assigned to positions-14, 12 and 13, respectively. A pair of protons doublets at δ 4.62 and 4.94 ppm (J = 1 Hz each), were assigned to an exomethylene group at position-15. In the ^{13}C NMR spectrum, the methylene carbon signal at δ 109.6 ppm and the quaternary carbon signal at δ 149.8 ppm were assigned to positions-4 and 15, respectively. These results pointed to a β-eudesmol metabolite. The new proton triplet at δ 4.32 ppm which was correlated to the carbon signal at δ 74.2 ppm was assigned to a proton of a β-hydroxymethine group at position-3 in eudesmane skelton [6]. Metabolite **5** gave m/z 220 assigned to $[M-H_2O]^+$ which concluded a molecular weight of 238 calculated for $C_{15}H_{26}O_2$. This established the structure of **5** as eudesma-4(15)-en-3β,11-diol.
Metabolite **6** gave m/z 238 analyzed for $C_{15}H_{26}O_2$, indicating the possible presence of a hydroxylation product. The two quaternary carbon signals at δ 126.8 and 139.4 ppm indicated the presence of a double bond at position-4. This was verified by the presence of a methyl

Table: ^{13}C NMR data of β- and γ-eudesmol and their metabolites*

	1	2	3	4	5	6
1	41.1	40.3	47.7	32.5	41.6	41.6
2	23.5	19.2	69.2	74.5	24.4	36.1
3	36.9	33.2	44.5	126.8	74.2	72.7
4	151.1	124.5	146.7	136.7	149.8	126.8
5	49.4	134.9	50.2	36.9	44.2	139.4
6	25.0	26.4	41.0	27.3	30.3	28.8
7	49.8	50.6	49.3	46.9	49.6	50.5
8	22.4	23.3	24.6	22.5	22.4	23.1
9	41.9	42.3	21.6	31.7	36.1	26.5
10	35.9	34.5	35.8	31.1	35.8	35.1
11	72.9	72.8	72.9	72.9	72.7	71.4
12++	27.2	27.2	27.1	27.5	27.6	27.1
13++	27.2	26.9	27.3	27.6	27.7	27.2
14	16.3	24.7	18.6	19.2	16.3	15.0
15	105.3	19.2	108.8	22.6	109.6	24.6

* At 62.5 MHz, using $CDCl_3$ as a solvent,TMS is the internal standard and the chemical shifts are expressed in ppm
++ Assignment may be interchangeable

Scheme



proton singlet at δ 1.71 ppm, which was assigned to position-15. The broad proton triplet at δ 3.99 ppm, which was correlated to the carbon signal at δ 72.7 ppm was assigned to an α-hydroxymethine proton at position-3 [6]. These observations pointed to the γ-eudesmol metabolite **6**, eudesma-4-en-3β,11-diol.

In conclusion, four new eudesmanoidal metabolites (**3**–**6**) were isolated from the biotransformation reactions of a mixture of β- and γ-eudesmols (Scheme) by *Rhizopus stolonifer* ATCC 6227. Metabolite **4** could be produced either from β- or γ-eudesmol. The hydroxylase enzymatic system of the bug behaves in a similar way with both substrates, where positions-2 and 3 are proved to be accessible and ideal for β-oxidation.

3. Experimental

3.1. Instrumentation

Melting points are uncorrected. ^{1}H NMR and ^{13}C NMR were measured on a Bruker WM 250 NMR spectrometer, at 250 MHz and 62.5 MHz, respectively, with $CDCl_3$ or acetone-d_6 as a solvent and TMS as the internal standard. The chemical shifts are expressed in ppm. DEPT and HETCOR were measured on a Bruker WM 300 NMR Spectrometer. EI-MS and GC-EI-MS were conducted on Hawlett Packard 5988 A, at 70 eV equipped with a Hawlett Packard RTE-6/VM data system and a Hawlett Packard 5890 GC, using a 25 meter HP-5 capillary column, 0.2 mm ID, film thickness 0.33 μm, cross-linked 5% phenyl-methyl silicone, helium with head pressure of 18 psi (124.2 kPa), 1 μl injection, split ratio 1 : 50; injector 200 °C, detector 300 °C, temperature program was 70 °C, hold for 1 min, 20 °C/min^{-1} to 300 °C, hold for 6 min. IR was conducted on Beckman Acculab I, IR spectrometer. The optical rotations were measured on Autopole III, automatic polarimeter (Rudolph Scientific).

3.2. Substrate material

Beta and gamma-eudesmols were isolated from the resin of *Parthenium argentatum* × *tomentosa* and were characterized by ^{1}H-, ^{13}C NMR and mass spectrometry [6, 8–11]. A semi-crude mixture of both materials was used in these biotransformation reactions.

3.3. Fermentation methods

Several microbial cultures were grown according to the standard two-stage fermentation protocol [12]. Screening experiments were done in 125 ml DeLong culture flasks. The culture flasks held one fifth of their volume of the following medium; 2% glucose, 0.5% soybean meal, 0.5% yeast extract, 0.5% NaCl and 0.5% K_2HPO_4. The pH of the medium adjusted to 7.0 using 6 N HCl before autoclaving for 20 min at 121 °C and 15 psi. After inoculation with the slants, stage I cultures were incubated at 27 °C and 250 rpm for 72 h before being used to inoculate stage II culture flasks. Usually, 10% inoculum volumes are recommended. For screening scale experiments, 10 mg of the eudesmol mixture in 0.1 ml of DMSO was added to 24-h- old stage II cultures, which were incubated again and sampled periodically for analysis.

3.4. Sampling

Samples of 1 ml each were taken after 12, 24, 36 and 48 h. and then every other day for 2 weeks following substrate addition. Each sample was extracted by shaking with 0.5 ml EtOAc and spun at 3.000 × g for 1 min in a desk-top centrifuge. All the EtOAc extracts were spotted on Si gel GF_{254} TLC plates, and developed with varying percentages of EtOAc/C_6H_{14} or $(CH_3)_2CO$/DCM, and visualized after spraying with 0.001% vanillin/H_2SO_4, followed by heating for 5–10 s with a heat gun.

3.5. Preparative scale conversion

Six 2-liter and three 1-liter stage II culture flasks received 3.0 g of the eudesmol mixture in 15 ml of DMSO (1 mg substrate per ml of culture medium). After incubation for 15 days under the same conditions, the cultures were combined and exhaustively extracted with 3 × 3 liter of 10% MeOH/EtOAc. The extract was dried over anh. Na_2SO_4 and concentrated under reduced pressure to yield a crude dark orange oily residue of 4.6 g.

3.6. Isolation and purification of the metabolites

The crude extract of the reaction (4.6 g) was loaded onto 350 g silica gel (63–200 μ) flash column, 3.5 × 90 cm. The eluting solvent was $(CH_3)_2CO/C_6H_{14}$, one liter each, 5%, 10%, 15%, . . . , 50% and 100%. Fractions of 200–250 ml each were collected and analyzed by TLC. The chromatograms were visualized after spraying with vanillin/H_2SO_4 followed by heating with a heat gun for 5–10 s. Similar fractions were pooled together. This afforded five groups of fractions. Group 1–3 (3.5 g) were eluted with 5–20% $(CH_3)_2CO/C_6H_{14}$, contained the recovered reaction substrates. Group 4 (325 mg) eluted with 20–25% $(CH_3)_2CO/C_6H_{14}$ and group 5 (950 mg) eluted with 30% $(CH_3)_2CO/C_6H_{14}$ and up.

Group 4 (235 mg) was subjected to MPLC, 140 g SiO_2, 15–25 μ, 26 × 460 mm. The eluting solvent was 1 l each of 30%, 40%, 50% and 60% EtOAc/C_6H_{14} with 250 ml fractions collected. Fractions 7, 8, 9 and 10 eluted with 40%–50% EtOAc/C_6H_{14}, gave 85 mg, 40 mg, 49 mg and 70 mg of semi-pure residues, respectively. All fractions were purified separately on prep. TLC plates using 50% EtOAc/C_6H_{14} as a solvent system. This gave 52 mg of **3** as white needles, 24 mg of **4** as oil, 20 mg of **5** as oil and 48 mg of **6** as oil, respectively. Metabolites **3–6** possess $R_f = 0.6$, 0.5, 0.46 and 0.32, respectively, on SiO_2 plates with 40% EtOAc/C_6H_{14} as a solvent system. All gave grayish colors, which changed to reddish-brown after spraying with vanillin/H_2SO_4 spray reagent and heating for 5–10 s with a heat gun.

3.6.1. Metabolite **3***, (-)eudesma-4(15)-en-2β,11-diol*

Needles, m.p. 81–82 °C, α[D]^{25}, −22.0 ($CHCl_3$; c. 0.05), IR $\nu_{max}^{cm^{-1}}$; 3400, 3080, 2940, 2850, 1640, 1450, 1370, 1260, 1040, 880 and 730. EI-MS, 70 eV, m/z (relative intensity); 238 $[M]^+$ (2), 220 $[M-H_2O]^+$ (5), 202 $[M-2H_2O]^+$ (3), 1187 $[M-2H_2O-CH_3]^+$ (5), 180 (12), 162 (32), 147 (42), 132 (20), 121 (27), 105 (32), 79 (28), 59 (100) and 43 (52). ^{1}H NMR (250 MHz, $CDCl_3$, δ ppm, J = Hz); 4.21 (1 H, m, H-2), 1.22 (6 H, s, H-12 and H-13), 0.94 (3 H, s, H-14), 4.64 (1 H, d, 1, H-15) and 4.85 (1 H, d, 1, H-15′).

3.6.2. Metabolite **4***, (+)eudesma-3-en-2β,11-diol*

Yellow oil, $\alpha[D]^{25}$, +32.88 $CHCl_3$; c. 4.50), IR $\nu_{max}^{cm^{-1}}$; 3400, 2970, 2930, 1645, 1440, 1370, 1270, 1020, 960, 920, 800 and 720. EI-MS, 70 eV, m/z (relative intensity); 238 $[M]^+$ (3), 220 $[M-H_2O]^+$ (14), 202 $[M-2H_2]^+$ (5), 187 $[M-2H_2O-CH_3]^+$ (6), 164 (42), 147 (33), 124 (90), 123 (50), 109 (40), 82 (32), 59 (62), 43 (100) and 41 (62). ^{1}H NMR (250 MHz, $CDCl_3$, δ ppm, J = Hz); 4.73 (1 H, m, H-2), 5.49 (1 H, br dd, 4, H-3), 1.17 (3 H, s, H-12*), 1.18 (3 H, s, H-13*), 0.99 (3 H, s, H-14) and 1.66 (3 H, s, H-15) (* = assignments are interchangeable).

3.6.3. Metabolite **5***, (-)eudesma-4(15)-en-3β,11-diol*

Yellow oil, $\alpha[D]^{25}$, −20.0 ($CHCl_3$; c. 0.05), IR $\nu_{max}^{cm^{-1}}$; 3410, 2960, 2850, 1640, 1465, 1450, 1370, 1260, 1140, 1040, 900 and 730. EI-MS, 70 eV, m/z (relative intensity); 220 $[M-H_2O]^+$ (6), 202 $[M-2H_2O]^+$ (4), 187 $[M-2H_2O-CH_3]^+$ (7), 180 (12), 162 (14), 147 (32), 133 (15), 119 (15), 105 (20), 91 (18), 59 (100), and 43 (45). ^{1}H NMR (250 MHz, $CDCl_3$, δ ppm, J = Hz); 4.32 (1 H, br t, 4, H-3), 1.19 (6 H, s, H-12 and H-13), 0.67 (3 H, s, H-14), 462 (1 H, d, 1, H-15) and 4.94 (3 H, d, 1, H-15′).

3.6.4. Metabolite **6***, (+)eudesma-4-en-2β,11-diol*

Needles, m.p. 62–63 °C, $\alpha[D]^{25}$, +28.6 ($CHCl_3$; c. 1.50), IR $\nu_{max}^{cm^{-1}}$; 3400, 3090, 2980, 2940, 2840, 1640, 1470, 1450, 1380, 1040, 880 and 700. EI-MS, 70 eV, m/z (relative intensity); 238 $[M]^+$ (10), 220 $[M-H_2O]^+$ (8), 205 $[M-H_2O-CH_3]^+$ (28), 190 $[M-H_2O-2CH_3]^+$ (10), 177 (100), 159 (10), 147 (15), 138 (20), 119 (12), 105 (22), 91 (18), 59 (60), 43 (87) and 41 (40). ^{1}H NMR (250 MHz, $CDCl_3$, δ ppm, J = Hz); 3.99 (1 H, br t, 4, H-3), 1.19 (6 H, s, H-12 and H-13), 1.06 (3 H, s, H-14) and 1.71 (3 H, s, H-15).

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