Microbial Transformation of a β- and γ-Eudesmols Mixture

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Z. Naturforsch. **57c,** 654–659 (2002); received January 2/February 21, 2002

Eudesmol, *Gibberella suabinetti,* Biotransformation

Beta- and *gamma*-eudesmols mixture was microbiologically transformed by *Gibberella suabinetti* ATCC 20193. Seven different eudesmanoidal metabolites (**3–9**) were isolated and their structures were elucidated by the different spectroscopic techniques. These metabolites are: eudesma-4-en-11-ol-3-one (carissone), eudesma-3-en-2β, 11-diol, eudesma-4-en-3β,11-diol, eudesma-4(15)-en-8,11-diol, eudesma-4(15)-en-2α,11-diol (pterocarpol), 1(3)cyclo-eudesma-4(15)-en-11,12-diol and eudesma-4-en-11,15-diol.

Introduction

Chemical derivatizations using microbial enzymes has many advantages, which include high catalytic activity and high regio- and stereo-specificity (Kieslich, 1976). A semi-crude mixture of *beta-* and *gamma*-eudesmols was obtained during our search for antifungal agents (Maatooq and Hoffmann, 1996). Eudesmols are sesquiterpene alcohols which could serve as precursors for many biologically active derivatives *e.g.* santonin, argentone and panellon (Maatooq *et al.,* 1996; Pergosin *et al.,* 1972; Sundin *et al.,* 1993). Alpha-eudesmol is a Ca(2+) channel blocker and neurogenic vasodilator, which is useful for treatment of neurogenic inflammation in trigemino-vascular system such as migraine (Asakura *et al.,* 2000 a). It attenuates post-ischemic brain injury by reducing the extra cellular glutamate (Asakura *et al.,* 2000 b). *Beta*-eudesmol is an antidote for intoxication from organophosphorous anti-choline esterase agents (Chiou *et al.,* 1995). It could be used as anti-epileptic (Chiou *et al.,* 1997), neuromuscular blocker (Kimura *et al.,* 1994; 1995) or in treatment of peptic ulcer (Nogami *et al.,* 1986).

This article describes the use of microorganisms in preparation of *beta-* and *gamma*-eudesmols derivatives, which may have more promising biological activity. Fermentation, isolation and structural assignments of the microbial metabolites are described.

Results and Discussion

For the biotransformation of a mixture of β- and γ-eudesmols, several microbes were subjected to screening studies. *Gibberella suabinetti* ATCC 20193 demonstrated the best results and was able to convert β- and γ-eudesmols mixture into several metabolites. Scale up of this biotransformation reaction afforded the isolation of seven metabolites (**3–9**).

The spectroscopic data for metabolite **3** were found to be identical to those of eudesma-4-en-11-ol-3-one; carissone (Maatooq *et al.,* 1996; Achenbach *et al.,* 1985).

The spectroscopic data of metabolite **4** and **5** were found to be identical to eudesma-3-en-2β,11-diol(2-β-hydroxy-α-eudesmol) and eudesma-4-en-3β,11-diol, respectively, produced from the same mixture by microbial transformation using *Rhizopus stolonifer* ATCC 6227 (Maatooq and Hoffmann, 2002).

The ^{13}C-NMR and ^{1}H-NMR spectra of **6** indicated the presence of a β-eudesmol metabolite, since two carbon signals at δ 108.3 and 148.4 and two doublets of proton signals at δ 4.55 and 4.81 (J = 1.5 Hz, each) were observed. The carbon signal at δ 68.2 is correlated to a multiplet proton signal at δ 3.85 was assigned to a new hydroxymethine group. The location of this hydroxymethine group was concluded to be at position-8, based on featuring of its proton signal as multiplet (only, position-2 or 8 can do that), alongwith its ^{13}C-NMR chemical shift value (Atta-Ur-Rahman and Ahmad, 1992). The appearance of C-5, C-7, C-9,

0939–5075/2002/0700–0654 $ 06.00

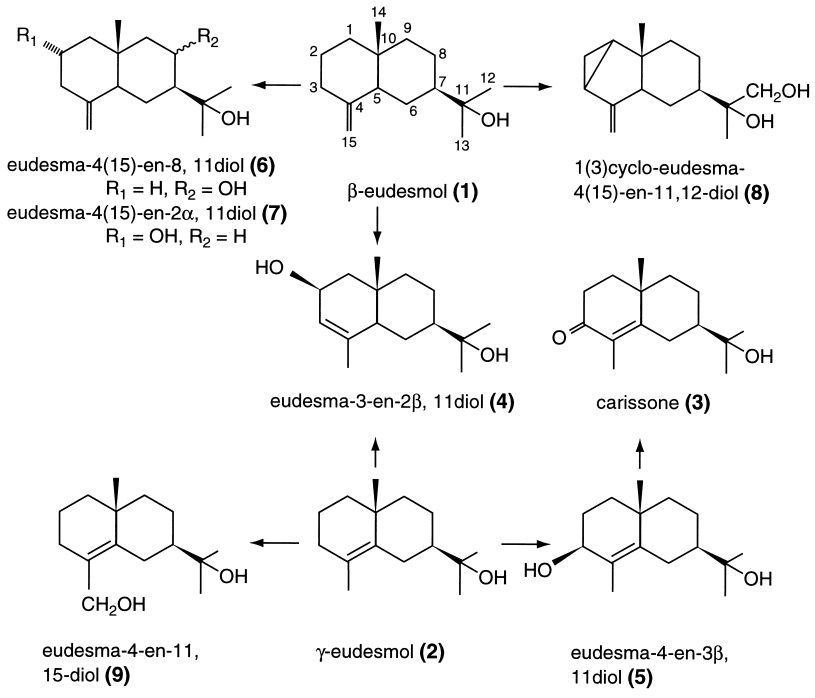

Fig. 1.

C-12 and C-13 each as a pair of signals gave more support for the presence of a hydroxyl group at position-8 and refer to a possible epimerization at this position. The mass spectrum of **6** gave *m/z* 223 rather than 238 which was assigned to $[M-CH_3]^+$, and indicated that **6** possesses the empirical formula $C_{15}H_{26}O_2$. These clues concluded that metabolite **6** is a mixture of the 8α and β-isomers of eudesma-4(15)-en-8,11-diol.

Metabolite **7** displayed ^{1}H-, ^{13}C-NMR and mass spectral data in full agreement with those reported for eudesma-4(15)-en-2α, 11-diol; pterocarpol (Atta-Ur-Rahman and Ahmad, 1992; Nasini and Piozzi, 1981; Bahl *et al.,* 1968).

Metabolite **8** was isolated after its acetylation. The two doublets at δ 4.56 and 4.69 (J = 1 Hz, each) in the proton spectrum and the two carbon signals at δ 106.8 and 152.8 in the carbon spectrum, alongwith the DEPT experiment indicated the likely presence of a β-eudesmol metabolite. The GC-EI-MS gave *m/z* 278 analyzed for $C_{17}H_{26}O_3$, which implies the presence of five unsaturation equivalents. Four of these equivalents are attributed to an acetate carbonyl, a double bond at 4(15)-position (β-eudesmol type) and two rings. The fifth one has to be due to the formation of a new cyclopropyl ring system, formed by a new linkage between positions-1 and 3. This finding was supported by the DEPT results, where six

Table I. ^{13}C-NMR data of β- and γ-eudesmols and their metabolites*.

C#	**1**	**2**	**4**	**5**	**6**	**8**$^+$	**9**$^+$
1	41.1	40.3	32.5	41.6	46.8	27.0	39.7
2	23.5	19.2	74.5	36.1	22.3	25.8	24.8
3	36.9	33.2	126.8	72.7	41.2	24.0	29.6
4	151.1	124.5	136.7	126.8	148.4	152.8	124.0
5	49.4	134.9	36.9	139.4	51.0, 51.3	53.0	142.9
6	25.0	26.4	27.3	28.8	25.1	26.4	27.4
7	49.8	50.6	46.9	50.5	49.5, 49.7	53.1	50.8
8	22.4	23.3	22.5	23.1	68.2	24.6	23.2
9	41.9	42.3	31.7	26.5	26.6, 26.9	41.7	41.8
10	35.9	34.5	31.1	35.1	35.6	38.3	34.9
11	72.9	72.8	72.9	71.4	73.3	80.9	72.6
12^{++}	27.2	27.2	27.5	27.1	27.0, 27.5	74.4	26.4
13^{++}	27.2	26.9	27.6	27.2	27.2, 27.7	24.0	26.5
14	16.3	24.7	19.2	15.0	17.7	12.3	18.3
15	105.3	19.2	22.6	24.6	108.3	106.8	64.8
Others	–	–	–	–	–	171.4	171.3
	–	–	–	–	–	20.9	21.1

* At 62.5 MHz, using $CDCl_3$ as a solvent (except **11** in acetone-d_6), TMS is the internal standard and the chemical shifts (δ) are expressed in ppm.
$^+$ As acetate. $^{++}$ Assignment may be interchangeable.

methylene groups and four methine groups were found. The location of the cyclopropyl ring can not be else where, since the presence of the upfield three groups of proton signals at δ 0.68, 0.91 and 1.04 integrated for 1H, 2H and 1H, respectively, require this location only. The ^{1}H-NMR spectrum showed two doublets at δ 3.75 and 3.87 (J = 12 Hz each), correlated with the carbon signal at δ 74.4 (DEPT), indicated the presence of a new terminal hydroxymethylene group. The location of this group was concluded to be at position-12 or 13, since position-11 carbon signal was shifted downfield to δ 80.9. These clues are good evidences for the unique structure of metabolite **8** to be 1(3)-cycloeudesma-4(15)-en-11,12-diol.

Metabolite **9** was obtained as acetate. The GC-EI-MS gave *m/z* 220, assigned for $[M-HOAc]^+$, which indicated a molecular formula of $C_{17}H_{28}O_3$. The proton and carbon spectra proved the likely presence of a γ-eudesmol metabolite, because two quaternary olefinic carbon signals were observed at δ 124.0 and 142.9 and assigned for C-4 and C-5, respectively. However, only three methyl proton signals were observed at δ 1.03, 1.15 and 1.16 assigned to positions-12, 13 and 14, respectively. This indicated the possible bioconversion of a methyl group, which appeared as two doublets at δ 4.45 and 4.63 (J = 11 Hz each) in the proton spectrum. These proton signals are linked to the carbon signal at δ 64.8 and were assigned to position-15. Therefore, the structure of **9** is 15-hydroxy γ-eudesmol (eudesma-4-en-11,15-diol).

In conclusion, seven different metabolites were isolated from the biotransformation reactions of β- and γ-eudesmols mixture (Fig. 1), by *Gibberella suabinetti* ATCC 20193 (**3–9**). The reaction products proved a superactivity of the hydroxylase, the dehydrogenase and the isomerase enzymes. The hydroxylation is a common feature, while cyclopropyl ring formations was evidenced, **8**. Metabolite **4** pointed to the activity of the isomerase enzyme and it could be produced either from β- or γ-eudesmol. Compounds **3**, **5** and **9** are γ-eudesmol metabolites while compounds **6**, **7** and **8** are β-eudesmol metabolites. Compounds **6**, **8** and **9** are newly reported metabolites.

Experimental

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 δ (ppm). DEPT (discriminate the carbon signals into CH_3, CH_2 and CHs, while the quaternaries are obscured) and HETCOR (direct C–H correlation) were measured on a Bruker WM 300 NMR spectrometer. EI-MS and GC-EI-MS were conducted on Hewlett Packard 5988 A, at 70 eV equipped with a Hewlett Packard RTE-6/VM data system and a Hewlett Packard 5890 GC, using a 25 meter HP-5 capillary column, 0.2 mm ID, film thickness 0.33 μm, cross-linked 5% phenylmethyl silicone, helium with head pressure of 18 psi (124.2 kPa), 1 μl injection, split ratio 1:50; injector 200°, detector 300°, temperature program was 70°, hold for 1 min, 20°/min^{-1} to 300°, 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, Fairfield, New Jersey).

Substrate material

β- and γ-eudesmols were isolated from the resin of *Parthenium argentatum* × *P. tomentosa* and were characterized by ^{1}H-, ^{13}C-NMR and mass spectrometry (Atta-Ur-Rahman and Ahmad, 1992; Elgamal and Wolf, 1987; Van Beek *et al.,* 1989). A semi-crude mixture of both materials was used in these biotransformation reactions.

Fermentation methods

Microbial cultures were grown according to the standard two-stage fermentation protocol (Betts *et al.,* 1972). Screening experiments were done, using 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° and 15 psi. After inoculation with the microbial slants, stage I cultures (in which the micro-

organism was transferred from the slants to the sterile medium) were incubated at 27° and 250 rpm for 72 h before being used to inoculate stage II culture flasks (in which, 10% inoculum volumes of stage I culture was used to inoculate another sterile medium and leave for 24 h to give this stage). Usually, 10% inoculum volumes are recommended. The eudesmols mixture, 10 mg in 0.1 ml of DMSO, was added to 24-hour-old stage II cultures, which were incubated again and sampled periodically for analysis.

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 $3000 \times g$ 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 $(CH_3)_2CO/CH_2Cl_2$, and visualized after spraying with 0.001% vanillin/H_2SO_4, followed by heating for 5–10 s with a heat gun. *Gibberella suabinetti* ATCC 20193 demonstrated the best results and was able to convert the eudesmols mixture into several metabolites.

Preparative scale conversion

Fourteen 2-liter stage-II cultures received 5.6 g of the eudesmols mixture in 28 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 3×4 liter of 10% MeOH/EtOAc. The extract was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to yield a crude dark orange oily residue of 6.9 g.

Isolation and purification of the metabolites

The crude extract of the biotransformation reaction (6.9 g) was loaded onto 500 g silica gel (63–200 μ) flash column. The elution solvent was $(CH_3)_2CO/C_6H_{14}$, one liter each of 5%, 10%, 15%, ..., 50% and 100%. Frs of 300 ml each were collected and analyzed by TLC. Similar frs were pooled together. This afforded four groups. Group (a) eluted with 5–15% $(CH_3)_2CO/C_6H_{14}$ gave 2.57 g of recovered substrates. Group (b) eluted with 15–25% $(CH_3)_2CO/C_6H_{14}$ afforded 1.45 g. Group (c) eluted with 25–30% $(CH_3)_2CO/C_6H_{14}$ gave 0.8 g and finally, group (d) eluted with 35–50% $(CH_3)_2CO/C_6H_{14}$ gave 0.6 g residue.

Group b (1.45 g) was subjected to medium pressure liquid chromatography, 140 g SiO_2, 15–25 μ, 2.6×46 cm. The eluting solvent was gradient by increasing the% of $EtOAc/C_6H_{14}$, 500 ml 20%, 500 ml 25%, and 1000 ml each of 30%, 35%, 40% and 50%. Twenty-five frs were collected (200 ml each). After solvent evaporation, all frs were tested by TLC on SiO_2 GF_{254} plates and 30% $EtOAc/C_6H_{14}$ as the solvent system (solvent-A). Similar frs were pooled together.

Fr 7 gave a yellow color, which changed to reddish-brown after spraying with vanillin/H_2SO_4 and heating for 10 s with the heat gun. After prep. TLC (210 mg) on 1-mm-thick silica gel plates using 40% $EtOAc/C_6H_{14}$ as a solvent, **3** (130 mg) was recovered as needles, $R_f = 0.22$ (solvent-A).

Frs 8–9 were treated same way as fr 7 to give 42 mg of **4** as a yellow oil, $R_f = 0.21$ (solvent-A).Frs 11–13 gave a gray color on TLC, which changed to reddish-brown after spraying and heating. It was treated the same way as fr 7 to give 40 mg of **5** and 48 mg of **6**, both as crystals with $R_f = 0.20$ and 0.18, respectively (solvent-A).

Frs 14–16 were treated the same way as fr 7, but using 50% $EtOAc/C_6H_{14}$ as a solvent system to afford 51 mg of **7** as a yellow oil, with $R_f = 0.12$ (solvent-A).

Frs 18–20 (180 mg) were subjected to acetylation and after the usual work-up, they provided 200 mg of crude acetate mixture. This mixture was purified by using multiple developments (3 times) on 1-mm-thick SiO_2 GF_{254} prep. TLC plates with 10% $EtOAc/C_6H_{14}$ as a solvent system. This afforded 27 mg of **8** acetate and 31 mg of **9** acetate, each as a yellow oil with $R_f = 0.26$ and 0.24 in the aforementioned solvent system.

Frs 21–22 (80 mg), group c (0.8 g) and group d (0.6 g) are still under investigations.

Metabolite **3***, [(+)eudesma-4-en-11-ol-3-one (carissone)]*

Needles m.p. 78°, IR, ORD, ^1H, ^{13}C-NMR and MS (Maatooq *et al.,*1996; Achenbach *et al.,* 1985).

Metabolite **4***, [(+)eudesma-3-en-2β,11-diol]*

Yellow oil, $\alpha[D]^{25}$, +32.88 ($CHCl_3$; c. 4.50), IR $\upsilon_{max}^{cm^{-1}}$; 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 (1H, *m*, H-2), 5.49 (1H, br *dd*, 4, H-3), 1.17 (3H, *s*, H-12*), 1.18 (3H, *s*, H-13*), 0.99 (3H, *s*, H-14) and 1.66 (3H, *s*, H-15) (* = assignments are interchangeable). The ^{13}C-NMR data of metabolites **4–6** and **8**, **9** acetate are listed in Table I.

Metabolite **5***, (+)eudesma-4-en-2β,11-diol]*

Needles, m.p. 62–63°, $\alpha[D]^{25}$, +28.6 ($CHCl_3$; c. 1.50), IR $\upsilon_{max}^{cm^{-1}}$; 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 (1H, br *t*, 4, H-3), 1.19 (6H, *s*, H-12 and H-13), 1.06 (3H, *s*, H-14) and 1.71 (3H, *s*, H-15).

Metabolite **6***, [(+)eudesma-4(15)-en-8,11-diol]*

Needles, m.p. 65–70°, $\alpha[D]^{25}$, +9.36 ($CHCl_3$; c. 4.5), IR $\upsilon_{max}^{cm^{-1}}$; 3400, 3090, 2930, 2840, 1640, 1470, 1450, 1370, 1270, 1040, 880 and 740. EI-MS, 70 eV, *m/z* (relative intensity); 223 $[M-CH_3]^+$ (3), 220 $[M-H_2O]^+$ (8), 202 $[M-2H_2O]^+$ (12), 187 $[M-2H_2O-CH_3]^+$ (15), 164 (12), 159 (30), 147 (23), 121 (18), 105 (24), 91 (20), 79 (20), 59 (100), 43 (55) and 41 (50). ^{1}H-NMR (250 MHz, $CDCl_3$, δ (ppm), *J* = Hz); 3.85 (1H, *m*, H-8), 1.20 (6H, *s*, H-12 and H-13), 0.69 (3H, *s*, H-14), 4.55 (1H, *d*, 1.5, H-15) and 4.81 (1H, *d*, 1.5, H-15′).

Metabolite **7***, [(+)eudesma-3-en-2β,11-diol, pterocarpol]*

Needles, m.p. 99–100° (100–102° [7]), $\alpha[D]^{25}$, +7.7 ($CHCl_3$; c. 0.10), IR, MS, ^{1}H-NMR and ^{13}C-NMR (Atta-Ur-Rahman and Ahmad, 1992; Nasini and Piozzi, 1981; Bahl *et al.,* 1968).

Metabolite **8***, [(+)1(3)-cycloeudesma-4(15)-en-11,12-diol acetate]*

Yellow oil, $\alpha[D]^{25}$, +14.8 ($CHCl_3$; c. 0.10), IR $\upsilon_{max}^{cm^{-1}}$; 3435, 3040, 2920, 2840, 1735, 1635, 1450, 1380, 1250, 1100, 1040, 980, 920, 880, 830 and 760. GC-EI-MS, 70 eV, *m/z* (relative intensity); 278 $[M]^+$ (2), 203 $[M-HOAc-CH_3]^+$ (9), 185 $[M-HOAc-H_2O-CH_3]^+$ (10), 160 (118), 145 (19), 105 (20), 91 (22), 79 (13), 55 (11) and 43 (100). ^{1}H-NMR (250 MHz, $CDCl_3$, δ (ppm), *J* = Hz); 0.68 (1H, *dd*, 10, 6, H-1), 0.91 (2H, *m*, H-2), 1.04 (1H, *dd*, 9, 5, H-3), 3.75 (1H, *d*, 12, H-12), 3.87 (1H, *d*, 12, H-12′), 1.27 (3H, *s*, H-13), 1.07 (3H, *s*, H-14), 4.56 (1H, *d*, 1, H-15), 4.69 (1H, *d*, 1, H-15′) and 2.04 (3H, *s*, H-acetate).

Metabolite **9***, [(+)eudesma-4-en-11, 15-diol acetate]*

Yellow oil, $\alpha[D]^{25}$, +55.2 ($CHCl_3$; c. 1.00), IR $\upsilon_{max}^{cm^{-1}}$; 3440, 2920, 2860, 1730, 1650, 1470, 1370, 1260, 1020, 950, 820 and 770. GC-EI-MS, 70 eV, *m/z* (relative intensity); 220 $[M-HOAc]^+$ (18), 205 $[M-HOAc-CH_3]^+$ (18), 187 $[M-HOAc-CH_3-H_2O]^+$ (57), 162 (30), 147 (44), 105 (32), 91 (32), 59 (55) and 43 (100). ^{1}H-NMR (250 MHz, $CDCl_3$, δ (ppm), *J* = Hz); 1.15 (3H, *s*, H-12*), 1.16 (3H, *s*, H-13*), 1.03 (3H, *s*, H-14), 4.45 (1H, *d*, 11, H-15), 4.63 (1H, *d*, 11, H-15′) and 2.02 (3H, *s*, H-acetate) (* = assignments are interchangeable).

Acknowledgements

The author is grateful to Prof. Dr. Joseph. J. Hoffmann, Bioresources Research Facility, Office of Arid Lands Studies, College of Agriculture, University of Arizona, USA, for his support and encouragement. This work is dedicated to the late colleague Dr. Sabry A. Awad (1954–1995).

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