Microbial Metabolism of (+)-Cycloisolongifol-5β-ol

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Microbial transformation of a cyclic sesquiterpene, (+)-cycloisolongifol-5β-ol (**1**), was carried out with the fungus *Cunninghamella elegans*, resulting in three new metabolites, cycloisolongifol-3β, 5β-diol (**2**), cycloisolongifol-5β-ol-11-one (**3**), and cycloisolongifol-3β, 5β, 11α-triol (**4**). The structures of new compounds were deduced on the basis of spectroscopic evidences.

Key words: Microbial Transformation, *Cunninghamella elegans*, Cycloisolongifol-3β,5β-diol, Cycloisolongifol-5β-ol-11-one, Cycloisolongifol-3β,5β,11α-triol

Introduction

The sesquiterpene (+)-cycloisolongifol-5β-ol (**1**) was synthesized by exposing longifolene to a strong proton or Lewis acid which leads to a molecular rearrangement to an isomeric tricyclic hydrocarbon, (-)-isolongifolene, which is further converted into cycloisolongifol-5-ol (**1**) [1]. Compound **1** has a great commercial importance as it is used in a number of perfumery materials. Longifolene and its derivatives are potent tyrosinase inhibitors and are added in certain cosmetics and dermatological preparation to suppress melanin production [2].

Microorganisms are able to transform a wide variety of organic compounds, such as terpenes, hydrocarbons, alkaloids, steroids, antibiotics and amino acids into their metabolites [1]. Many compounds with therapeutic properties and industrial interests are obtained by microbial transformation [3].

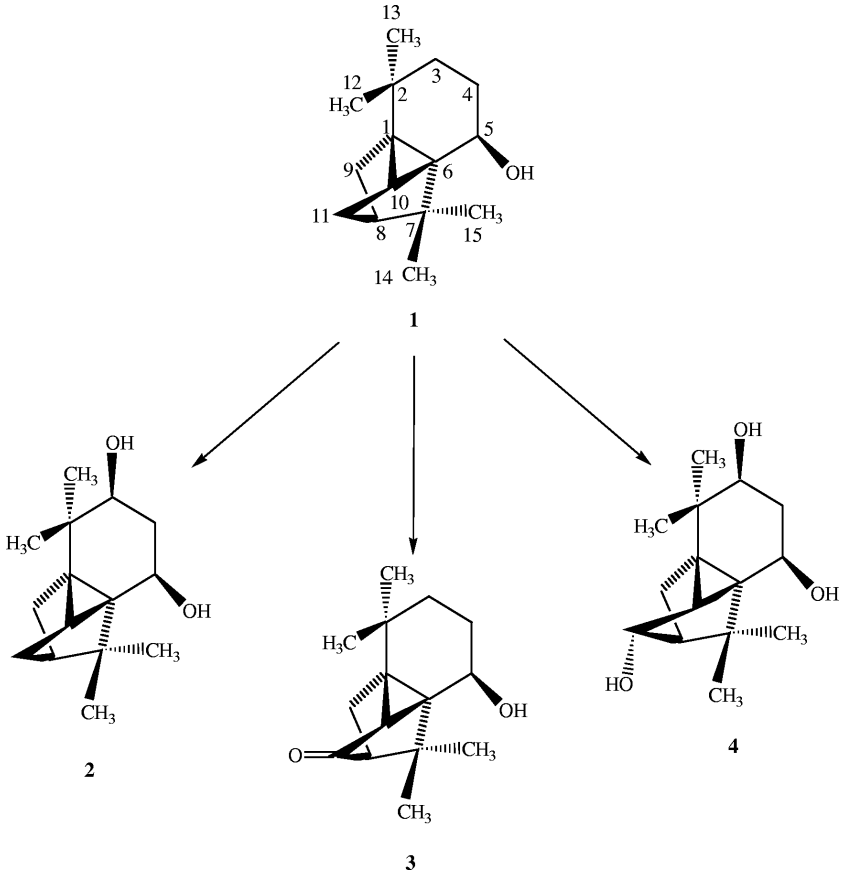
The bridged ring systems are suitable substrates for the study of the effect of the structures on the regiochemistry of the microbial hydroxylation. For example, microbiological hydroxylation of the sesquiterpenoid patchoulol by *Mucor plumbeus* yielded 5α,9α-dihydroxylated products [4].

In continuation of our studies on biotransformation of bioactive compounds [5 – 15], we have recently investigated the biotransformation of a complex bridged ring compound (+)-cycloisolongifol-5β-ol (**1**) with various fungal strains. New metabolites, cycloisolongifol-3β,5β-diol (**2**), cycloisolongifol-5β-ol-11-one (**3**) and cycloisolongifol-3β,5β,11α-triol (**4**) were obtained by incubation of compound **1** with *Cunninghamella elegans* (NRRL 1392) (Scheme 1). The structures of the new compounds were elucidated on the basis of spectroscopic techniques.

Results and Discussion

Screening scale experiments have shown that fungus *Cunninghamella elegans* was capable of converting compound **1** ($C_{15}H_{24}O$) into hydroxylated products. Incubation of compound **1** with *C. elegans* resulted in the formation of three metabolites. The structures of new metabolites were deduced through a comparative spectroscopic study with the substrate **1**.

Compound **2** was obtained as a white crystalline solid. The high resolution EI mass spectrum (HREIMS) of metabolite **2** displayed the molecular ion peak at $m/z = 236.1769$, corresponding to the formula $C_{15}H_{24}O_2$ (calcd. 236.1776), indicating one more oxygen, in comparison to compound **1**. The IR spectrum showed an absorption at 3380 cm^{-1} due to the presence of a hydroxyl group. The ^{1}H NMR spectrum of **2** showed an additional signal at $\delta = 4.53$ (br. s), due to the presence of a newly introduced hydroxyl group (see Table 1). The ^{13}C NMR spectrum of compound **2** (broad-band decoupled and DEPT) showed 15 carbon signals, including four methyl, three methylene, four methine and four quaternary carbons (see Table 2). An additional methine carbon signal at $\delta = 76.7$, as compared to **1**, indicated the presence of a hydroxyl group. The position of newly introduced OH was deduced by the 2D-NMR techniques. In the HMBC spectrum, 3-H



Scheme 1. Microbial transformation of compound **1** by *Cunninghamella elegans*.

Table 1. ^{1}H NMR chemical shift assignments[#] of (+)-cycloisolongifol-5β-ol (**1**) and its metabolites **2 – 4** (400 MHz, $CDCl_3$).

H	1	2	3	4
3	1.39 m	4.53 brs	1.43 m	4.22 dd (J = 5.3, 3.2)
4	1.58 m	2.52 dd (J = 5.5, 5.5)	1.26 m	2.509 m
5	4.48 t (J = 3.9)	4.46 t (J = 4.0)	4.39 t (J = 4.2)	4.41 dd (J = 5.7, 2.0)
8	1.03 dt (J = 4.4, 4.3)	1.84 m	2.00 m	1.55 m
9	1.75 br. d (J = 10.0)	1.34 m	1.86 m	1.62 m
10	1.50 br. d (J = 10.4)	1.85 t (J = 4.4)	2.08 s	2.36 d (J = 10.5)
11	1.38 m	1.61 m	–	3.42 dd (J = 11.6, 3.7)
12	0.95 s	0.89 s	0.97 s	0.91 s
13	0.96 s	0.94 s	1.06 s	0.85 s
14	1.08 s	1.13 s	1.19 s	1.05 s
15	0.86 s	1.07 s	1.12 s	0.99 s

[#] δ-Values are given in ppm (coupling constants, J in Hz).

Table 2. ^{13}C NMR chemical shift assignments[#] (δ) of (+)-cycloisolongifol-5β-ol (**1**) and its metabolites **2 – 4** (100 MHz, $CDCl_3$).

C	1	2	3	4
1	28.4 (C)	30.1 (C)	28.9 (C)	33.2 (C)
2	38.6 (C)	38.7 (C)	32.5 (C)	32.3 (C)
3	32.6 (CH_2)	76.7 (CH)	31.4 (CH_2)	74.6 (CH)
4	32.9 (CH_2)	29.3 (CH_2)	32.7 (CH_2)	39.1 (CH_2)
5	62.2 (CH)	62.7 (CH)	71.8 (CH)	62.2 (CH)
6	44.5 (C)	42.6 (C)	44.5 (C)	43.0 (C)
7	35.5 (C)	42.7 (C)	44.3 (C)	35.5 (C)
8	42.0 (CH)	48.9 (CH)	42.2 (CH)	48.6 (CH)
9	34.0 (CH_2)	35.4 (CH_2)	34.4 (CH_2)	34.0 (CH_2)
10	19.1 (CH)	18.7 (CH)	18.3 (CH)	26.0 (CH)
11	30.6 (CH_2)	30.7 (CH_2)	207.1 (C)	71.3 (CH)
12	26.9 (CH_3)	20.4 (CH_3)	32.7 (CH_3)	25.6 (CH_3)
13	28.8 (CH_3)	26.7 (CH_3)	28.0 (CH_3)	27.2 (CH_3)
14	21.6 (CH_3)	21.9 (CH_3)	21.8 (CH_3)	22.1 (CH_3)
15	21.6 (CH_3)	22.5 (CH_3)	21.6 (CH_3)	21.4 (CH_3)

[#] Carbon multiplicities were determined by DEPT experiments.

($\delta = 4.53$) showed interactions with C-1 ($\delta = 30.1$) and C-12 ($\delta = 20.4$) indicating a new hydroxyl group at C-3. The configuration of the newly introduced OH

was assigned to be β- on the basis of NOESY correlations between 3α-H ($\delta = 4.53$) and 15α-H ($\delta = 1.07$) and 13α-H ($\delta = 0.94$). Thus the structure of the new metabolite was deduced as cycloisolongifol-3β,5β-diol (**2**).

Compound **3** was obtained as a colorless crystalline compound. The HREIMS of metabolite **3** displayed the M^+ peak at $m/z = 234.1646$, corresponding to the molecular formula $C_{15}H_{22}O_2$ (calcd. 234.1619), indicating one more oxygen and two hydrogens less in comparison to compound **1**. The IR spectrum showed an absorption at 1661 cm^{-1} which indicated the presence of a carbonyl group at a bridge in ring fused system. The ^{1}H NMR spectrum of **3** indicated the formation of a ketonic derivative, through the disappearance of 11-H_2 signal. The ^{13}C NMR spectrum of compound **3** (broad-band and DEPT) showed 15 carbon signals, including four methyl, three methylene, three methine and five quaternary carbons. A quaternary carbon signal at $\delta = 207.0$ and the disappearance of C-11 methylene carbon further indicated the oxidation of C-11. In the HMBC spectrum, 10-H ($\delta = 2.08$) showed interaction with C-11 ($\delta = 207.0$), confirming a ketonic carbonyl group at C-11. Thus the structure of the new metabolite was deduced as cycloisolongifol-5β-ol-11-one (**3**).

Compound **4** was obtained as a white crystalline solid. The HREIMS of metabolite **4** displayed the M^+ peak at $m/z = 252.1725$, 32 amu higher than that of compound **1** corresponding to the formula $C_{15}H_{24}O_3$ (calcd. 252.1675), indicating the presence of two more oxygen atoms. The IR spectrum showed an absorption at 3376 cm^{-1} which indicated the presence of a hydroxyl group. The ^{1}H NMR spectrum showed two additional methine proton signals, at $\delta = 4.22$ (dd, $J = 5.3$ Hz, $J = 3.2$ Hz) and $\delta = 3.42$ (dd, $J = 11.6$ Hz, $J = 3.7$ Hz), indicating the presence of two more hydroxyl groups. The ^{13}C NMR spectrum of compound **4** (broad-band decoupled and DEPT) showed 15 carbon signals indicating four methyl, two methylene, five methine, and four quaternary carbons. Appearance of two additional methine carbons at $\delta = 74.6$ and $\delta = 71.3$, in comparison to **1**, further supported two OH-bearing methine carbons. In the HMBC spectrum, 3-H ($\delta = 4.22$) showed correlations with C-13 ($\delta = 27.2$) and C-4 ($\delta = 39.1$), whereas in the COSY-45° spectrum, 11-H ($\delta = 3.42$) showed homonuclear interaction with 10-H ($\delta = 2.36$). These observations pointed to the presence of hydroxyl groups at C-3 and C-11. In the NOESY spectrum, 3-H ($\delta = 4.22$) showed interaction with 13α-H_3 ($\delta = 0.85$), whereas 11-H ($\delta = 3.42$) showed an interaction with 14β-H_3 ($\delta = 1.05$). The structure of the new metabolite was thus deduced as cycloisolongifol-3β,5β,11α-triol (**4**).

Experimental Section

Melting points were determined on a Büchi 535 melting point apparatus. Optical rotations were measured on a Jasco DIP–360 digital polarimeter. IR spectra were recorded in $CHCl_3$ on a FTIR-8900 spectrophotometer. UV spectra were recorded in MeOH on a Hitachi U-3200 spectrophotometer. The ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ solutions on a Bruker Avance 400 NMR at 400 and 100 MHz, respectively. Chemical shifts (δ) were recorded in ppm, relative to $SiMe_4$ as an internal standard, while the coupling constants (J) were measured in Hz. The EIMS and HREIMS were measured on a Jeol JMS-600H mass spectrometer. Thin layer chromatography (TLC) was performed on silica gel precoated plates (PF_{254}, 20×20, 0.25 mm, E. Merck). Compound **1** was purchased from Fluka. The compounds were detected on TLC with the help of vanillin spray reagent.

Organisms and culture media

The fungal culture was purchased from *Northern Regional Research Laboratories* (NRRL), Japan. A stock culture was maintained at 4 °C on agar slants (Sabouraud dextrose agar). The culture medium for *Cunninghamella elegans* (NRRL 1392) was prepared by mixing the following ingredients into distilled H_2O (4.0 l): glucose (40.0 g), peptone (20.0 g), yeast extract (20.0 g), KH_2PO_4 (20.0 g), and NaCl (20.0 g).

Fermentation of cycloisolongifol-5β-ol (**1**) *with* Cunninghamella elegans *and extraction conditions.* The fermentation medium thus obtained was distributed among 40 flasks of 250 ml capacity (100 ml in each) and autoclaved at 121 °C for 120 min. The fermentation was carried out according to a standard two-stage protocol [16]. The substrate **1** (400 mg) was dissolved in acetone (20 ml) and the resulting clear solution was evenly distributed among 40 flasks (10 mg/0.5 ml in each flask), containing 24-h-old stage II cultures, and fermentation was carried out for 12 d on a rotatory shaker (200 rpm) at 29 °C. During the fermentation period, aliquots from the culture were taken out daily and analyzed by TLC in order to determine the degree of transformation of substrate. In the experiment, one control flask without fungus (for checking substrate stability) and another flask without exogenous substrate (for checking on endogenous metabolites) were used. The culture media and mycelium were separated by filtration. The mycelium was washed with CH_2Cl_2 (2 l) and the filtrate was extracted with CH_2Cl_2 (3×2 l). The combined organic extracts were dried over anhydrous

Na_2SO_4, evaporated under reduced pressure and analyzed by TLC. Control flasks were also harvested and the content was compared with the test by TLC, to confirm the presence of biotransformed products. Fermentation was carried out for 12 d. After filtration, extraction and evaporation, a brown gum (1.32 g) was obtained which after repeated CC (EtOAc /Petroleum ether gradient) yielded: compound **2** (15.0 mg, 3.7% yield), compound **3** (16.0 mg, 4.0% yield) and compound **4** (12.0 mg, 3.0% yield) and unchanged compound **1**.

Cycloisolongifol-3β,5β-*diol* (**2**). M. p. 131 °C. – UV/vis (CH_2Cl_2): λ_{max} ($\lg\varepsilon$) = 202.2 nm (0.66). – $[\alpha] = -23°$ ($c = 0.1$, MeOH). – IR (film): $\nu = 3380, 3057, 2954, 2870, 1445, 1363$ cm^{-1} -^{1}H NMR (400 MHz, $CDCl_3$): Table 1. – $^{13}C\{^1H\}$NMR (100 MHz, $CDCl_3$): Table 2. – MS (EI, 70 ev): m/z (%) = 236 (10) [M$^+$], 218 (100) [M$^+$ $-H_2O$], 203 (21), 175 (36), 161 (27), 145 (57), 119 (50), 107 (60), 83 (13), 55 (100), -HREIMS $m/z = 236.1769$ (M^+, $C_{15}H_{24}O_2$; calcd. 236.1776).

Cycloisolongifol-5β-*ol-11-one* (**3**). M. p. 136 °C. – UV/vis (CH_2Cl_2): λ_{max} ($\lg\varepsilon$) = 210.0 nm (3.61). – $[\alpha]_D^{25} = -13°$ ($c = 0.1$, MeOH). – IR (film): $\nu = 3414, 3064, 2962, 2930, 2874, 1661, 1465, 1445, 1363$ cm^{-1}. – ^{1}H NMR (400 MHz, $CDCl_3$,): Table 1. – ^{13}C {H} NMR (100 MHz, $CDCl_3$): Table 2. – MS (EI, 70 ev): m/z (%) = 234.2 (56) [M$^+$], 219 (20.0) [M$^+$–Me], 205 (16), 190 (42), 163 (39), 147 (44), 119 (46), 105 (56), 91 (80), 55 (100.0). -HREIMS $m/z = 234.1646$ (M^+, $C_{15}H_{22}O_2$; calcd. 234.1619).

Cycloisolongifol-3β,5β,11α-*triol* (**4**). M. p. 142 °C. – UV/vis (CH_2Cl_2): λ_{max} ($\lg\varepsilon$) = 201.6 nm (0.87). – $[\alpha]_D^{25} = -11°$ ($c = 0.1$, MeOH). – IR (film): $\nu = 3376, 3064, 2962, 2930, 2874, 1661, 1465, 1445, 1365$ – ^{1}H NMR (400 MHz, $CDCl_3$,): Table 1. – $^{13}C\{H\}$NMR (100 MHz, $CDCl_3$): Table 2. – MS (EI, 70 ev): m/z (%) = 234 (56) [M$^+$], 219 (20.0) [M$^+$–Me], 205 (16), 90 (42), 163 (39), 147 (4), 119 (46), 105 (56), 91 (80), 55 (100), -HREIMS $m/z = 252.1725$ (M^+, $C_{15}H_{24}O_3$; calcd. 252.1675).

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