



Microbial Transformation of (-)-Alloisolongifolene

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<http://dx.doi.org/10.13005/ojc/300304>

(Received: June 03, 2014; Accepted: August 09, 2014)

ABSTRACT

(-)-Alloisolongifolene (1) is a structural analogue of the naturally occurring longifolene. The microbial transformation of (-)-alloisolongifolene (1) by *Cunninghamella elegans* afforded two new metabolites, 3a-hydroxyalloisolongifolol (2) and 13-hydroxyalloisolongifolol (3). The structures of the metabolites were deduced on the basis of their spectral data, including 1D and 2D NMR, IR and HREIMS.

Key words: (-)-Alloisolongifolene; Microbial Transformation; *Cunninghamella elegans*; 3a-Hydroxyalloisolongifolol; 13-Hydroxyalloisolongifolol.

INTRODUCTION

Microbial transformation of terpenes has been an attractive method to produce new metabolites with enhanced biological activity¹. Insertion of a hydroxyl group in one step, with high regio- and stereo-selectivity, at an inactive terpenoid skeleton by microbial transformation remains unmatched by chemical means². Hydroxylation of terpenes is of importance to the flavor and fragrance industry in the search for new production methods and new compounds³. A large number of sesquiterpenes with a bridged tricyclic skeleton have been isolated from plants and fungi⁴, some of them have exhibited interesting biological properties⁵. However, only a

few reports about their fungal transformation have appeared⁶. Recently, biotransformation of various bridged sesquiterpenes has been described by Choudhary et al^{5,7,8}, who reported regio- and stereo-selective hydroxylation of (+)-cycloisolongifol-5b-ol, (-)-isolongifolol and (+)-isolongifolene-4-one by different fungi species.

(-)-Alloisolongifolene (1), a bridged tricyclic sesquiterpene, produced by acid-catalyzed isomerization of the naturally occurring longifolene^{9,10}.

Biotransformation of 1 has been reported by using enzymes from chicory roots (*Cichorium*

intybus) where the hydroxylation took place at an isopropylidene group at the allylic position ¹¹.

In the present study, we report that the microbial transformation of (-)-alloisolongifolene (**1**) using *Cunninghamella elegans* afforded two new hydroxylated metabolites (**2** and **3**).

MATERIAL AND METHODS

General experimental procedures

IR Spectra were recorded in CHCl₃ on an FTIR-8900 spectrophotometer. Optical rotations were measured on a JASCO DIP-360 digital polarimeter. UV Spectra were recorded in CHCl₃ solution on a Hitachi U-3200 spectrophotometer. The ¹H and ¹³C NMR spectra were recorded in CDCl₃ solution on a Bruker Avance-400 NMR at 400 and 100 MHz, respectively (*d* ppm, *J* in Hz). The EI and HREI MS were recorded on JEOL JMS 600H mass spectrometer. TLC was performed using precoated plates (Silica gel 60, PF₂₅₄, 0.2 mm, Merck). Compound **1** was purchased from Fluka.

Organism and media

Microbial culture, *Cunninghamella elegans* (NRRL 1392), was obtained from Northern Regional Research Laboratories (NRRL) and maintained on SDA and stored at 4 °C prior to use. The fermentation medium for *Cunninghamella elegans* (NRRL 1392), was prepared by dissolving the following ingredients in distilled water (3.0 L): glucose (30.0 g), glycerol (30.0 mL), peptone (15.0 g), yeast extract (15.0 g), KH₂PO₄ (15.0 g), and NaCl (15.0 g).

General fermentation and extraction procedure

The fermentation media thus obtained was distributed among 30 flasks of 250 mL capacity (100 mL each) and autoclaved. The fermentation was carried out according to a standard stage II protocol ^{12, 13}. Compound **1** (600 mg) was dissolved in 15 mL acetone and the solution was evenly distributed among the 30 flasks (20 mg/ 0.5 mL in each flask) containing 24 h culture of *Cunninghamella elegans* together with control flasks. Fermentation continued further for an additional 3 days on a rotatory shaker (200 rpm) at 29° C. During the fermentation, aliquots from flasks were taken daily and analyzed by TLC in order to determine the degree of transformation of the substrate. One control flask, without biomass (for

checking substrate stability), and one flask without exogenous substrate (for checking endogenous metabolite) were used. The culture media and mycelium were separated by filtration. The mycelium was washed with CH₂Cl₂ (1.0 L) and the filtrate was extracted with CH₂Cl₂ (3 × 3 L). The combined organic extracts was washed with brine, dried over anhydrous Na₂SO₄ and then evaporated under reduced pressure to yield a crude brown gum (2.6 g). Control flasks were also harvested and their contents tested by TLC to detect any biotransformed products. The crude residue (2.6 g) was subjected to column chromatography. Elution with petroleum ether and EtOAc yielded compounds **2** and **3** in 4.2%, and 2.8% yields, respectively.

3a-Hydroxyalloisolongifolol (2) was obtained as an amorphous material. $[\alpha]_D^{25}$: +68 (*c* = 0.4, CHCl₃). UV (CHCl₃): λ_{max} (log ϵ) = 202 nm (1.8). IR (CHCl₃): ν_{max} = 3419, 2992 cm⁻¹. ¹H NMR (CDCl₃, 400 MHz): see Table 1. ¹³C NMR (CDCl₃, 100 MHz): see Table 2. MS (EI, 70 eV): *m/z* (%): 238 (7) [M⁺], 220 (14), 206 (6), 166 (38), 146 (28), 120 (100), 78 (74), 55 (28). MS (HREI): *m/z* = 238.1084 (C₁₅H₂₆O₂, calcd 238.1093).

13-Hydroxyalloisolongifolol (3) was obtained as an amorphous material. $[\alpha]_D^{25}$: +116 (*c* = 0.5, CHCl₃). UV (CHCl₃): λ_{max} (log ϵ) = 203 nm (2.2). IR (CHCl₃): ν_{max} = 3401, 2986 cm⁻¹. ¹H NMR (CDCl₃, 400 MHz): see Table 1. ¹³C NMR (CDCl₃, 100 MHz): see Table 2. MS (EI, 70 eV): *m/z* (%) = 238 (16) [M⁺], 220 (31), 208 (26), 166 (26), 152 (16), 120 (100), 78 (28), 55 (47). MS (HREI): *m/z* = 238.1074 (C₁₅H₂₆O₂, calcd 238.1086).

RESULTS AND DISCUSSION

To investigate the capacity of *Cunninghamella elegans* to metabolise compound **1**, a small-scale experiment was conducted. TLC of the crude chloroform extract indicated the presence of two new more polar metabolites. Subsequently, a large-scale fermentation was carried out to produce sufficient quantities of these metabolites for structure elucidation. Biotransformation of compound **1** with *Cunninghamella elegans* for 3 days yielded the new metabolites **2** and **3** (Scheme-1).

The HREIMS of compound **2** exhibited M⁺ at

m/z 238.1084, corresponding to the formula $C_{15}H_{26}O_2$ (calcd 238.1093), which is 34 a.m.u. higher than the substrate 1. The IR spectrum of compound 2 showed absorption at 3524 cm^{-1} indicating the presence of

hydroxyl groups. The ^1H NMR spectrum (Table 1) of compound 2 was quite different from the parent compound 1. The olefinic proton signals at δ 4.73 and 4.63 of 1 were absent, while three new additional

Table 1: ^1H NMR chemical shift data of the compounds 1-3

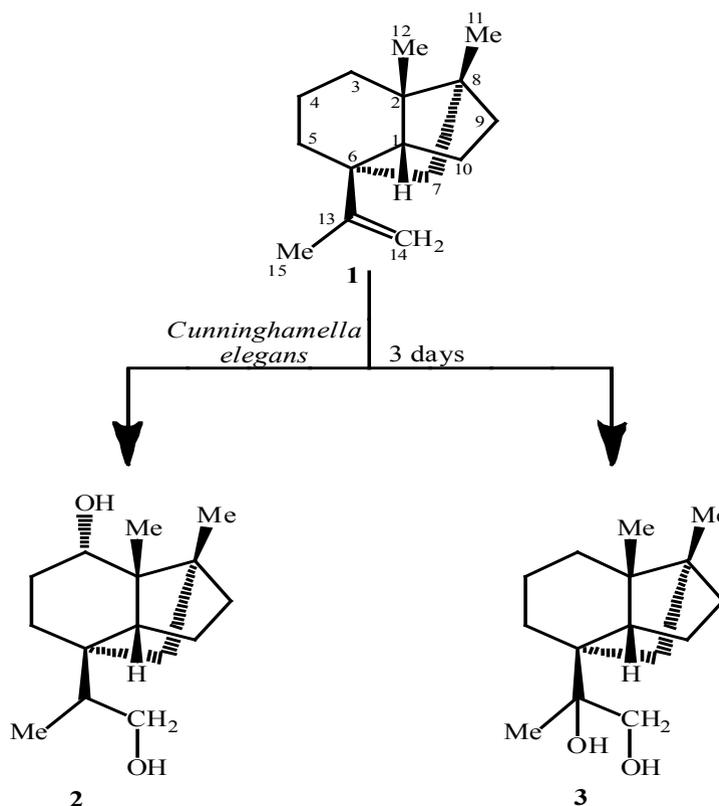
Position	$1\delta_{\text{H}}$ (J in Hz)	$2\delta_{\text{H}}$ (J in Hz)	$3\delta_{\text{H}}$ (J in Hz)
1	1.52, m	1.80, m	2.05, m
2	-	-	-
3	1.44; 1.12, m	3.57 (dd, $J = 15.1, 6.0$ Hz)	1.30; 1.18, m
4	1.57; 1.49, m	1.67; 1.04, m	1.54; 1.44, m
5	1.60; 1.40, m	1.62; 1.12, m	1.69; 1.37, m
6	-	-	-
7	1.53; 1.35, m	1.58; 0.82, m	1.50; 1.35, m
8	-	-	-
9	1.55; 1.47, m	1.59; 1.25, m	1.50; 1.44, m
10	1.59; 1.22, m	1.56; 0.97, m	1.48; 1.31, m
11	0.76, s	0.86, s	0.80, s
12	0.87, s	0.94, s	0.87, s
13	-	1.76, m	-
14	4.73; 4.63 (br s)	3.60 (dd, $J = 12.2, 6.0$ Hz); 3.38 (t, $J = 12.2$, Hz)	4.03; 3.26 (d, $J = 10.7$ Hz)
15	1.67, s	1.01 (d, $J = 6.8$ Hz)	1.09, s

Table 2: ^{13}C NMR Chemical shift data of the compounds 1-3

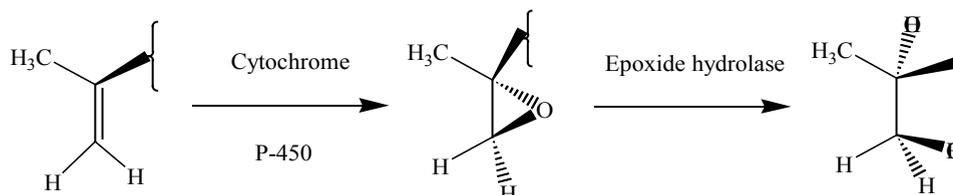
Position	$1d_{\text{C}}$	$2d_{\text{C}}$	$3d_{\text{C}}$
1	51.8, d	41.8, d	50.9, d
2	46.8, s	53.2, s	46.7, s
3	38.8, t	72.1, d	38.5, t
4	20.7, t	47.8, t	22.2, t
5	44.0, t	39.0, t	44.1, t
6	49.7, s	46.5, s	51.9, s
7	22.0, t	19.8, t	21.6, t
8	47.9, s	44.2, s	46.9, s
9	32.5, t	29.2, t	35.2, t
10	36.5, t	23.9, t	36.2, t
11	19.8, q	12.5, q	15.6, q
12	15.4, q	15.4, q	22.9, q
13	152.5, s	47.5, d	83.8, s
14	109.4, t	66.5, t	71.6, t
15	20.5, q	15.9, q	20.1, q

Carbon multiplicities were determined by DEPT experiment; s = quaternary, d = methine, t = methylene, q = methyl carbons.

signals appeared at δ 3.57 (1H, dd, $J_{3b,4a} = 15.1$ Hz, $J_{3b,4b} = 6.0$ Hz), 3.60 (1H, dd, $J_{14a,14b} = 12.2$ Hz, $J_{14,13} = 6.0$ Hz) and 3.38 (1H, t, $J = 12.2$ Hz), assigned for H-3 and the two vicinal protons attached to C-14, respectively. The ^{13}C -NMR and DEPT spectra of 2 (Table 2) confirmed anti-Markovnikov's hydration of the double bond. It showed the absence of the double bond signals, the presence of a CH at δ 47.5 and a downfield CH_2 at δ 66.5, assigned to C-13 and C-14, respectively. The downfield CH signal at δ 72.1, the absence of the methylene carbon at δ 38.8 and the downfield shifts of C-2 and C-4 signals (Table 2) indicated hydroxylation at C-3 of compound 2. COSY 45° and HMBC experiments supported these assignments. COSY 45° showed correlations between H-14 (δ 3.60, 3.38) and H-13 (1.76), and HMBC showed correlations between H-14 (δ 3.60, 3.38) and C-15 (δ 15.9), and C-13 (δ 47.5). The position of the second OH group at C-3 was confirmed by the HMBC correlations of H-3 (δ 3.57) with C-12 (δ 15.4), C-4 (δ 47.8), and C-2 (δ 53.2). The stereochemistry of the C-3 hydroxyl group was determined as *a* on the basis of NOESY correlations between H-3 (δ 3.57) and each of H-1 α (δ 1.80),



Scheme 1: Biotransformation of (-)-alloisolongifolene (1) with *Cunninghamella elegans*



Scheme 2: Proposed intermediate for the formation of compound 2

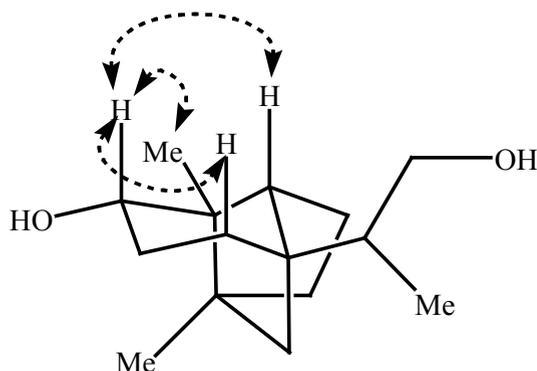


Fig. 1: Key NOESY correlations in compound 2

H-5 α (d 1.62) and Me-12 α (d 0.94). The structure of the new metabolite 2 was thus established as 3-hydroxyalloisolongifolol.

The HREI MS of metabolite 3 showed the M^+ at m/z 238.1074 ($C_{15}H_{26}O_2$, calcd 238.1086), similar to that of compound 2. The 1H NMR spectrum (Table 1) showed *AB* doublets at d 4.03 ($J_{14a,14b} = 10.7$ Hz) and 3.26 ($J_{14b,14a} = 10.7$ Hz), indicating the hydroxylation of the C-13/C-14 double-bond. The ^{13}C NMR spectrum (Table 2) of compound 3 was similar to that of compound 1, except for the absence of the double bond and the appearance of additional downfield OH bearing signals at d

71.6 (C-14), and 83.8 (C-13). The dihydroxylation of the double bond was further confirmed by the HMBC correlations between Me-15 (*d* 1.09) and C-13 (*d* 83.8), C-14 (*d* 71.6), and C-6 (*d* 51.9). Based on the above mentioned spectral data, the structure of the new metabolite **3** was deduced as 13-hydroxyalloisolongifolol.

It has been reported that the filamentous fungus *Cunninghamella elegans* contains enzymes with activities similar to those found in mammals, some of which are P-450 mono-oxygenase and epoxide hydrolase¹⁴. So we think that compound **2** may have formed from anti hydrolysis of the corresponding epoxide as shown in scheme 2.

Cunninghamella elegans fungus was able to bio-catalyze the hydroxylation of the bridged

sesquiterpenes, (-)-alloisolongifolene (**1**), in a regio and stereo-specific manner. It introduced a hydroxyl group at the 3a-position, and hydrate the double bond in an anti-Markovnikov's producing a new compound 3a-Hydroxyalloisolongifolol (**2**), in addition to oxidation of the double bond (C-13, C-14) to the corresponding diol to give the new compound 13-Hydroxyalloisolongifolol (**3**).

ACKNOWLEDGEMENTS

The authors acknowledge the Higher Education Commission, Islamabad, Pakistan for the financial support through Indigenous Ph. D. Fellowship Program, and acknowledge the assistance of Prof. Atta-ur-Rahman and Prof. Mohammad Iqbal Choudhary through out this work.

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