Regioselective Oxidation of (+)- α -Longipinene by *Aspergillus niger*

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Abstract: The regioselective oxidation of (+)- α -longipinene (**1**) with sesquiterpen hydrocarbon was investigated using *Aspergillus niger* (NBRC 4414) as a biocatalyst. Compound **1** was converted to three new terpenoids, (+)-(5*S*)-5,12-dihydroxy- α -longipinene (**2**), (-)-(5*R*)-5,12-dihydroxy- α -longipinene (**3**), and (+)-12-hydroxy- α -longipinen-5-one (**4**). These structures were determined by NMR, IR, specific rotation and mass spectral studies.

Key words: (+)- α -longipinene, (+)-(5*S*)-5,12-dihydroxy- α -longipinene, (-)-(5*R*)-5,12-dihydroxy- α -longipinene, (+)-12-hydroxy- α -longipinen-5-one, *Aspergillus niger*

1 INTRODUCTION

Microbial transformation is the biologically synthetic process, using enzymes in the living body as biocatalysts. The characters of biotransformation are as follows: regio- and stereo-selective reaction under mild condition and producing optically active compounds. These points suggested that the biotransformation is advantageous method for production of the natural terpenoids.

We have been studying the biotransformation of terpenoids by fungi to produce new biologically active terpenoids. In our previous papers, some terpenoids, (+)-fenchone¹⁾, 1, 4-cineole²⁾, (-)- α -bisabolol³⁾, (-)-*cis*-rose oxide⁴⁾, (+)-*trans*-rose oxide⁴⁾ were transformed into novel terpenoids, which is stereo selective oxidation by *Aspergillus niger*. We were interested in biotransformation of longifolene skeleton by microorganism., Longifolenes exist in essential oil of conifer species⁵⁾ and which has antioxidant⁶⁾, anti-inflammatory⁷⁾ activity and unique flavor. As in previous studies, we reported that biotransformation of (+)-cycloisolongifolol which is a sesquiterpene alcohol with a longifolene skeleton by *Glomerella cingulata*⁸⁾.

(+)- α -Longipinene (**1**) is a sesquiterpene hydrocarbon with a longifolene skeleton. In natural product, **1** was contained in the essential oil of *Hypericum perforatum* L.⁹⁾. We expected to produce new biologically active terpenoids by the biotransformation of **1** by *A. niger*, and interested in metabolic process of **1** by microorganism. In this paper,

regioselective oxidation of **1** was described by *A. niger*.

2 Experimental

2.1 Material

α -Longipinene used for substance was purchased from Fluka (Tokyo, Japan).

2.2 Gas chromatography (GC)

A Agilent technologies 6890N gas chromatograph equipped with a flame ionization detector, an HP-5 capillary column (30 m length, 0.25 mm i.d.), and a split injection of 20:1 were used. Helium at a flow rate of 1 mL/min was used as a carrier gas. The oven temperature was programmed from 90 to 270°C at 4°C/min. The injector and detector temperature were 270°C and 280°C respectively. System software control and data analysis were performed with GC Chemstation software (Agilent Technologies Inc.).

2.3 Gas chromatography-mass spectrometry (GC/MS)

A Agilent-technologies 5973N gas chromatograph equipped with a split injector HP-5MS capillary column (30 m length, 0.25 mm i.d.) was combined by direct coupling to a Agilent-technologies 5973N mass spectrometer. The same temperature program as just described for GC was used. Helium at 1 mL/min was used as a carrier gas. The elec-

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tron impact (EI) energy was 70eV, and the ion source temperature was 280°C.

2.4 Gas chromatography-preparative fraction collector (GC/PFC)

A Agilent technologies 6890N gas chromatograph was used. The preparative unit is equipped with a 7683B Series injector (Agilent technologies), a flame ionization detector (FID), and a preparative fraction collector (PFC) (GERSTEL, Germany). PFC consists of a PFC oven, a coolant and seven glass tubes (six sample traps and one waste trap) for compound trapping. The GC oven temperature was programmed from 90 to 270°C at a rate of 7°C/min. Switching and transfer line temperature were 250°C. Volumes of 2 µL (sample concentration of 10 mg/mL) were injected splitless 700 times with an autosampler 7683B Series injector equipped with 10 µL syringe. One percent of the effluent passed to the FID and the remaining 99% were directed to the PFC and collected in 600 µL traps each filled with dichloromethane at a temperature of -10°C.

2.5 Pre-culture of *Aspergillus niger*

Spores of *A. niger*, which had been preserved at low temperature were inoculated into sterilized culture medium (1.5% saccharose, 1.5% glucose, 0.5% polypeptone, 0.05% MgSO₄·7H₂O, 0.05% KCl, 0.1% K₂HPO₄, 0.001% FeSO₄·7H₂O and distilled water, pH 7.2) in a shaking flask at 27°C for 1 day.

2.6 Time-course experiment

Pre-cultured *A. niger* were transferred into a culture medium (20 mL in a 50 mL Petri dish) and incubated for 1 day (until mycelia occupied 60–80% of the surface area of a culture medium) under the stationary culture. After the growth of *A. niger*, 1 (0.3 mg/mL) was added to the medium and the culture continued for 7 days, then sated with NaCl and then extracted with EtOAc. The extract was analyzed by GC. The ratios between the substrate 1 and metabolic products were determined based on the peak areas of GC and GC-MS.

2.7 Isolation of metabolites

(+)- α -Longipinene was incubated with *A. niger* as described above for 7 days. After the biotransformation for 7 days, culture medium and mycelia were separated by filtration. The medium was salted out with NaCl and extracted with EtOAc. The mycelia were also extracted with EtOAc. The EtOAc extracts were mixed and dried over Na₂SO₄ and the solvent was evaporated to yield the crude extract (832 mg). The extract was chromatographed on silica-300 columns with hexane-EtOAc; compound 1 (320 mg) was recovered. Mixture of metabolites 2 and 3 (125 mg), and 4 (90 mg) were isolated. Compounds 2 and 3 were separated to 0.8 mg respectively by GC/PFC¹⁰⁾.

2.8 (+)-(5S)-5,12-dihydroxy- α -longipinene (2)

Colorless oil; EIMS m/z (rel. int.): 236(M⁺, 2), 218(3), 203(6), 148(48), 105(80), 105(78), 91(100), 79(73), 43(70); HR-EIMS: m/z 236.1795 [M]⁺, calcd. for C₁₅H₂₂O₂ 236.1776; IR (film) $\nu_{\max}$ cm⁻¹: 3407, 2962, 2930; ¹H and ¹³C NMR as shown Table 1 and 2.

2.9 (–)-(5R)-5,12-dihydroxy- α -longipinene (3)

Colorless oil; EIMS m/z (rel. int.): 236(M⁺, 3), 218(4), 203(6), 148(44), 105(80), 105(80), 91(100), 79(78), 43(65); HR-EIMS: m/z 236.1795 [M]⁺, calcd. for C₁₅H₂₂O₂ 236.1776; IR (film) $\nu_{\max}$ cm⁻¹: 3407, 2962, 2930; ¹H and ¹³C NMR as shown Table 1 and 2.

2.10 (+)-12-hydroxy- α -longipinene-5-one (4)

Colorless oil; [α]_D^{26.0} +6.2° (c 1.0, CHCl₃); HR-EIMS: m/z 234.1615 [M]⁺, calcd. for C₁₅H₂₂O₂ 234.1620; EIMS m/z (rel. int.): 234(M⁺, 2), 216(3), 198(58), 145(26), 125(70), 105(75), 91(100), 77(59), 41(45); IR (film) $\nu_{\max}$ cm⁻¹: 3446, 2966, 1701; ¹H and ¹³C NMR as shown Table 1 and 2.

3 RESULTS AND DISCUSSION

Biotransformation of (+)- α -longipinene (1) by *A. niger* was investigated. For time-course experiments, a small amount of 1 was incubated with *A. niger* 7 days. Compound 1 was transformed to three metabolites, which were detected by TLC, GC and GC-MS analysis. These products were not detected on TLC, GC and GC-MS analysis of culture of *A. niger* to which no substrate was fed. The conversion rate of metabolite 2, 3 and 4 was 18%, 15% and 22% respectively for 7 days.

In order to isolate the metabolites a large-scale incubation of 1 with *A. niger* was carried out, and the culture was extracted as described in the experimental. Metabolites were isolated from the EtOAc extract and separated by silica-300 columns mixture of metabolites 2 and 3. The mixture was separated to metabolites 2 and 3 by GC/PFC. These structure were determined by spectral data.

Compounds 2 and 3 were determined from the following MS, IR and NMR data. The IR spectrum showed the presence of a hydroxyl group (IR: 3407 cm⁻¹). ¹H and ¹³C NMR showed, relationship between 2 and 3 were diastereomer. NMR signals were similar to that of the substrate, except for the existence of new methine and methylene group, and disappearance of a methylene and methyl group. About the ¹H NMR evidence for the presence of two methyl group. Compounds 2 and 3 were confirmed by assignment of the NMR spectra using two dimensional techniques (COSY, HMQC and HMBC). In the characteristic HMBC spectrum correlation were observed of (i) H-12/C-9 and C-10, (ii) H-5/C-6, C-12, 14 and 15. Therefore, compounds 2 and 3 were

Table 1 ^1H NMR spectral data of compounds **1-4** (δ TMS, in CDCl_3 , at 700MHz)^a.

Carbon no.	1	2	3	4
1	2.08-2.10 (1H, <i>m</i>)	2.08-2.09 (1H, <i>m</i>)	2.34-2.36 (1H, <i>m</i>)	2.15-2.18 (1H, <i>m</i>)
2				
3	1.54-1.66 (2H, <i>m</i>)	1.63-1.64 (1H, <i>m</i>) 1.75-1.78 (1H, <i>m</i>)	1.65-1.66 (1H, <i>m</i>) 1.78-1.80 (1H, <i>m</i>)	2.75 (1H, <i>ddd</i> , 2.0, 5.4, 11.5) 2.54 (1H, <i>ddd</i> , 2.3, 5.1, 11.5)
4	1.54-1.66 (2H, <i>m</i>)	2.28-2.29 (1H, <i>m</i>)	2.31-2.33 (1H, <i>m</i>)	1.75-1.82 (2H, <i>m</i>)
5	1.34-1.36 (1H, <i>m</i>) 2.00-2.02 (1H, <i>m</i>).	3.49 (1H, <i>dt</i> , 1.5, 11.3)	3.51 (1H, <i>dt</i> , 1.5, 11.3)	
6				
7	1.45 (1H, <i>brs</i>)	1.54 (1H, <i>brs</i>)	1.57 (1H, <i>brs</i>)	1.60 (1H, <i>brs</i>)
8	1.98-2.01 (1H, <i>m</i>)	2.34 (1H, <i>brd</i> , 4.5)	2.14 (1H, <i>brd</i> , 4.5)	2.42-2.44 (1H, <i>m</i>)
9				
10	5.17 (1H, <i>m</i>)	5.44 (1H, <i>tt</i> , 1.8, 2.8)	5.47 (1H, <i>tt</i> , 1.8, 2.8)	5.52 (1H, <i>tt</i> , 1.8, 2.8)
11	2.22 (2H, <i>brd</i> , 4.0)	1.67-1.68 (1H, <i>m</i>) 1.89-1.92 (1H, <i>m</i>)	1.67-1.68 (1H, <i>m</i>) 1.93-1.94 (1H, <i>m</i>)	2.38 (2H, <i>brs</i>)
12	1.67 (3H, <i>s</i>)	3.98-3.99 (2H, <i>m</i>)	4.00-4.01 (2H, <i>m</i>)	4.04 (2H, <i>brs</i>)
13	0.84 (3H, <i>s</i>)	0.86 (3H, <i>s</i>)	0.91 (3H, <i>s</i>)	0.91 (3H, <i>s</i>)
14	0.83 ^b (3H, <i>s</i>)	0.83 ^b (3H, <i>s</i>)	0.87 ^b (3H, <i>s</i>)	1.03 ^b (3H, <i>s</i>)
15	0.90 ^b (3H, <i>s</i>)	0.94 ^b (3H, <i>s</i>)	0.97 ^b (3H, <i>s</i>)	1.13 ^b (3H, <i>s</i>)

^aMultiplicity and *J* (in parentheses) in Hz.^bThese values can be interchanged.**Table 2** ^{13}C NMR spectral data for compounds **1-4** (δ TMS, in CDCl_3 at 175 MHz)^a.

Carbon no.	1	2	3	4
1	46.3	41.4	43.2	41.4
2	39.7	39.5	39.7	39.9
3	37.0	37.0	36.6	37.1
4	21.7	34.1	33.8	36.7
5	77.2	77.2	76.9	215.8
6	32.6	43.2	41.4	46.8
7	58.8	58.6	58.7	56.5
8	39.8	40.4	38.6	42.9
9	147.9	150.9	150.7	149.7
10	117.0	118.7	118.9	118.9
11	34.1	30.4	30.7	32.8
12	22.8	65.7	65.8	65.5
13	23.5	18.2	18.3	23.0
14	27.7 ^b	23.3 ^b	23.5 ^b	22.8 ^b
15	27.9 ^b	26.4 ^b	27.4 ^b	24.1 ^b

^aChemical shifts in ppm, multiplicities were determined by the DEPT pulse sequence.^bThese values can be interchanged.

produced by hydroxylation at the C-5 and C-12 positions of **1**. From this, **2** and **3** were epimer in the position of C-5. In previous paper, conformation of the cycloheptane ring in longipinenes was investigated, which has four main conformations of the cycloheptane ring¹¹⁻¹³. When there is a hydroxyl group of the C-5 position in α or β configuration, it is presumptively determined that the compound has different conformation each by chemical shift of ^{13}C -NMR. In ^{13}C NMR, compound **2** had 41.4 ppm at C-1 position. The signal had shifted 4.9 ppm (from δ 46.3 to δ 41.4), which was δ -effect of non-bonding steric interactions, involving the atoms in the C-1 and C-14 positions (1,4-positions). Similarly, C-8 carbon of compound **3** had 38.6 ppm. The signal had shifted 1.2 ppm (from δ 39.8 to δ 38.6), which is upfield shifted by δ -effect of non-bonding steric interactions, involving the atoms in the C-8 and C-15 positions (1,4-positions). So, compounds **2** and **3** had classical chair and boat conformation of the cycloheptane ring respectively (Fig. 1). A hydroxyl group of compound **2** was α configuration, and the absolute configuration of C-5 was *S* form. The hydroxyl group of C-5 position of compound **3** was β configuration, and the absolute configuration was *R* form. The specific rotation of the mixture was $+1.2^\circ$ (c 1.0, CHCl_3). Abundance of compounds **2** and **3** in mixture were 6:5 from GC and ^1H -NMR. So, compound **2** has (+)-form, and compound **3** has (–)-form. From these data it was concluded that the structures of **2** and **3** are (+)-(5*S*)-5,12-dihy-

Fig. 1 Conformations of compounds 2 and 3.

Fig. 2 Biotransformation of 1 by *A. niger*.

droxy- α -longipinene and (-)-(5*R*)-5,12-dihydroxy- α -longipinene respectively, which are new compounds.

Compound 4 had a molecular formula C₁₅H₂₂O₃ from HR-EIMS: m/z 234.161 [M]⁺(calcd. for C₁₅H₂₂O₂ 234.1620). The IR and ¹³C NMR spectrum of 4 showed the presence of a hydroxyl group (IR: 3446 cm⁻¹, δ_c 65.5 (CH₂)) and a carbonyl group (IR: 1701 cm⁻¹, δ_c 215.8 ppm (C)). About the ¹H NMR evidence for the presence of two methyl group. This was confirmed by assignment of the NMR spectra using two dimensional techniques (COSY, HMQC and HMBC). In the characteristic HMBC spectrum correlation were observed of (i) H-12/C-9 and C-10, (ii) H-14 and H-15/C-5. Therefore, compound 4 was produced by oxolation at the C-5 position and hydroxylation at the C-12 position of 1. The specific rotation shows the (+)-form. From these data it was concluded that the structure of 4 is (+)-12-hydroxy- α -longipinen-5-one, which is a new compound.

In order to clarify the metabolic pathway in the biotrans-

formation of 1 by *A. niger*, compounds 2, 3 and 4 were incubated with *A. niger* for 7 days respectively. As a result, compounds 2 and 3 were bioconverted to compound 4 by *A. niger*, and compound 4 was not bioconverted to compounds 2 and 3 (Fig. 2).

In conclusion, compound 1 was regioselective oxidized to the C-5 position of the seven-membered ring and C-12 position of end of methyl group by *A. niger*. The reaction in C-5 position is oxidized to ketone, and an intermediate hydroxide is not stereo selective. In addition, reactive site was α -position of geminal dimethyl in the seven-membered ring. Reportedly, (-)-isolongifolol was biotransformation into 10 α -hydroxyisolongifolol and 9 α -hydroxyisolongifolol by *A. niger*¹⁴. This report showed reactive site was α and β -position of geminal dimethyl in the seven-membered ring. It is suggested that, *A. niger* has ability of oxidation at the α -position of geminal dimethyl on seven-membered ring in longifolene skeleton.

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