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Hydroxylation of the sesterterpene leucosceptrine by the fungus *Rhizopus stolonifer*

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Abstract

The microbial transformation of leucosceptrine (**1**), the first member of class leucosesterterpenes, by *Rhizopus stolonifer* afforded two metabolites, 1α-hydroxyleucosceptrine (**2**), and 8α-hydroxyleucosceptrine (**3**).

Keywords: Leucosceptrine; Sesterterpene; Fungal transformation; *Rhizopus stolonifer*; 1α-Hydroxyleucosceptrine; 8α-Hydroxyleucosceptrine

1. Introduction

Selective functionalization by chemical methods at unactivated carbon atoms has been a major challenge in organic synthesis, and thus microbiological methods have frequently been used for this purpose (Fraga et al., 1996). Biotransformations involve the use of enzymes or microorganisms to perform chemical reactions in which the starting substances and products are of comparable chemical complexity.

Leucosceptrine (**1**), $C_{25}H_{36}O_7$, the first member of a new class of sesterterpene named as leucosesterterpenes, was isolated from a medicinal plant *Leucosceptrum canum* Smith, belonging to the family Lamiaceae, by our research group (Choudhary et al., 2004a,b). *L. canum*, locally known as Bhusure (Hooker, 1983) is traditionally used as an insecticidal agent in remote areas of Nepal. Compound **1** has exhibited prolylendopeptidase (PEP) inhibitory activity ($IC_{50} = 80$ μM) in a mechanism-based assay (Choudhary et al., 2004a,b). The novel structure of leucosceptrine (**1**) stimulated us to carry out microbiological transformation on this compound by employing *Rhizopus stolonifer*.

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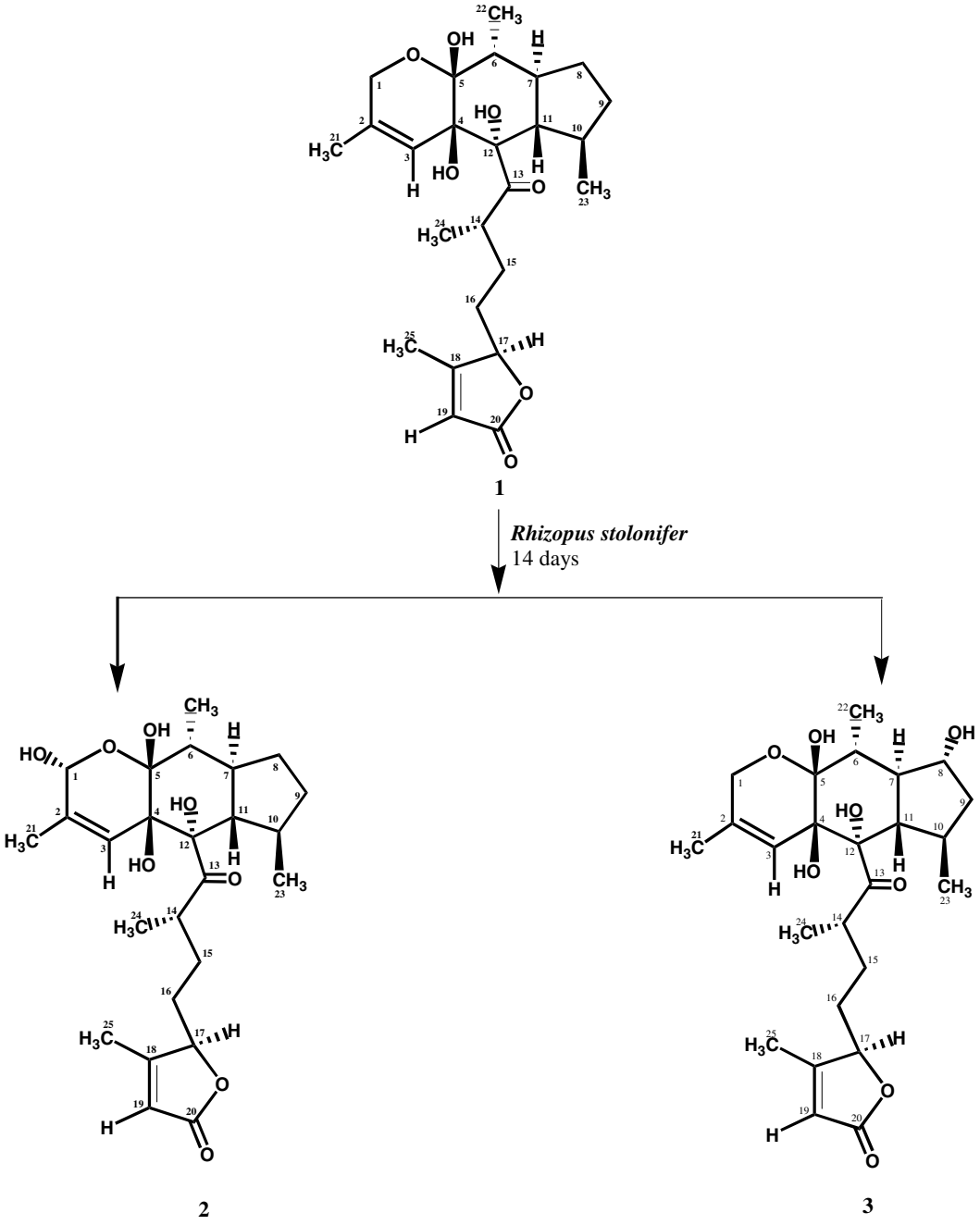
2. Results and discussion

Screening scale experiments have shown that the *R. stolonifer* was capable of converting compound **1** into polar metabolites **2**–**3**. Large scale fermentation was thus carried out to produce sufficient quantities of metabolites **2**–**3** for structure elucidation (Scheme 1). Two sets of controls were used to ensure the authenticity of metabolites. Metabolites were isolated from the culture medium by chloroform extraction. The residues obtained were fractionated by column chromatography. The PEP inhibitory activity of the metabolites could not be screened due to insufficient quantities after structure determination.

1α-Hydroxyleucosceptrine (**2**) was isolated as a colorless solid from the chloroform extract of culture broth. The compound **2** showed the strong IR absorptions at 3320 (OH), 1735 (C=O), and 1664 (CH=CH) cm^{-1}.

The FAB MS of compound **2** showed the $(M^+ - H)$ peak at *m/z* 463 ($C_{25}H_{36}O_8$), 16 amu higher than the substrate **1**. The HREI-MS spectrum showed an ion at *m/z* 446.3312 (calcd 446.3246) supporting the formula $C_{25}H_{34}O_7$ and representing a loss of H_2O from the M^+.

The ^1H NMR spectrum ($CDCl_3$) of compound **2** showed close resemblance with the substrate **1**. Five methyl signals,



Scheme 1. Metabolism of compound **1** by *Rhizopus stolonifer*.

including three secondary methyl groups were resonated at δ 1.15 (*d*, $J_{22,6} = 6.4$ Hz), 1.06 (*d*, $J_{23,10} = 6.8$ Hz), and 1.23 (*d*, $J_{24,14} = 7.1$ Hz), and assigned to the C-22, C-23, and C-24 methyl protons, while the tertiary methyl singlets at δ_H 1.75 and 2.04 were assigned to the C-21 and C-25 methyl protons, respectively.

The ^{13}C NMR spectrum showed the resonances for five methyl carbons, resonated at δ 17.1, 12.5, 21.2, 13.8, and 12.1, which were assigned to C-21, C-22, C-23, C-24, and C-25, respectively. Two trisubstituted double bond carbons were appeared at δ 142.0, 123.6, 168.3, and 117.2 and assigned to C-2, C-3, C-18, and C-19, respectively. Four methylene carbons were resonated at δ 35.3, 32.4, 28.1, and 26.3 due to C-8, C-9, C-15, and C-16, respectively. Six methine carbons were appeared at δ 104.2 (C-1), 46.7 (C-6), 47.7 (C-7), 35.3 (C-10), 57.4 (C-11), and 41.7 (C-14). Similarly six quaternary carbons, including two carbonyl carbons, were resonated at δ 75.2, 101.2, 86.5, 218.3, and 174.1, and were assigned to C-4, C-5, C-12, C-13, and C-20, respectively.

The main difference between compound **2** and the substrate **1** was the absence of oxymethylene protons and presence of a downfield proton signal at δ 5.82 in compound **2** which indicated that the C-2 oxymethylene was oxidized into an OH-containing methine. The presence of a downfield signal at δ 104.2 further supported the presence of a

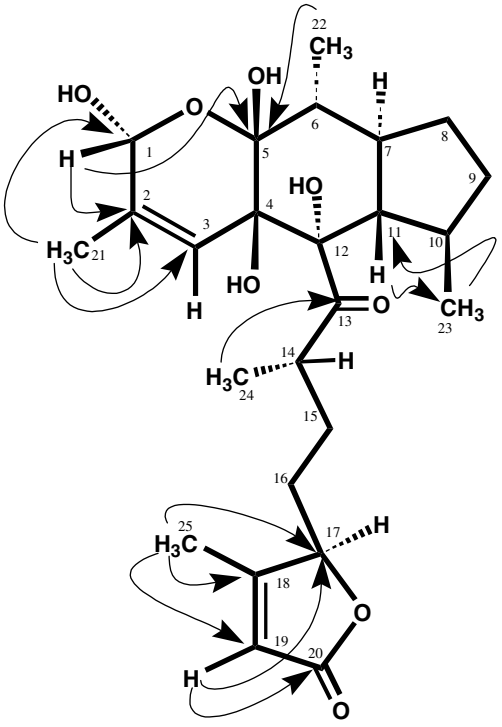


Fig. 1. Key HMBC correlations in compound **2**.

hydroxyl group at C-1 in compound **2**. The mass fragments at *m/z* 265, 247, 219, 108, and 110 further supported the presence of a hydroxyl group at C-1 in hemiacetal ring. In the HMBC spectrum (Fig. 1), H-1 was found to be coupled with C-2 (δ 142.0), C-3 (δ 123.6) and C-5 (δ 101.2). The Horeau's method (Horeau and Kagan, 1964) was employed to deduce the stereochemistry of the newly introduced hydroxyl group at C-1 in compound **2**. The sign of rotation of residual acid was negative (*R*), which indicated the stereochemistry of newly formed hydroxyl group as *S* (α). These observations supported the structure of compound **2** as 1α-hydroxyleucosceptrine.

The compound **3** was isolated as a colorless solid from the chloroform extract of broth of *R. stolonifer*. Compound **3** showed the strong IR absorptions at 3300 (OH), 1725 (C=O), and 1665 (C=C) cm^{-1}.

The FAB MS of compound **3** showed the $(M^+ - H)$ peak at *m/z* 463, 16 amu higher than the substrate **1**. The HREI-MS showed $(M^+ - H_2O)$ peak at *m/z* 446.3012 ($C_{25}H_{34}O_7$, calcd 446.2946).

The ^1H NMR spectrum ($CDCl_3$) of **3** showed close resemblance with the substrate **1**. The only difference being the appearance of a downfield methine proton at δ 4.29, geminal to an OH group.

The broad-band (BB) decoupled ^{13}C NMR spectrum ($CDCl_3$) of compound **3** indicated the presence of 25 carbons including 5 methyls, 4 methylene, 10 methine and 6 quaternary carbons. The ^{13}C NMR spectrum of **3** was distinctly similar to substrate **1**, with a downfield methine carbon at δ 72.1, indicating the introduction of a OH group.

The mass fragment ions at *m/z* 265, 247 and 127 also supported the presence of an additional hydroxyl group in metabolite **3**. The methine proton resonated at δ_H 4.29 showed COSY 45° correlations with H-7 (δ 3.01) and H_2-9 (δ 1.19, 1.52), indicated the position of hydroxyl group at C-8 in ring C. The HMBC interactions between C-11 methine proton (2.19) and C-8 (δ_C 72.1), and between H-7 (δ 3.01) and C-8, further supported the assigned position of hydroxyl group at C-8 (Fig. 2). The C-9 methylene protons (δ 1.19, 1.52) also showed HMBC interactions with the C-8.

The relative stereochemistry in compound **3** were inferred from the cross peaks in NOESY spectrum (Fig. 2). The NOE correlations between H-6/H-8, H-8/H-11, and

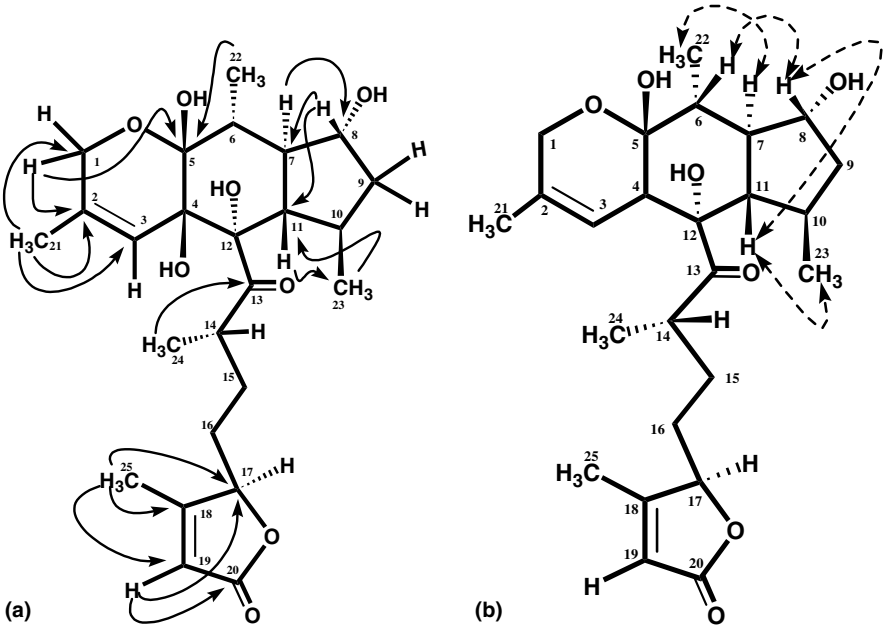


Fig. 2. (a) Key HMBC, and (b) NOESY correlations in compound **3**.

H_3-23/H-11 indicated that H-6, H-8, and H-11 were in β-configuration (see Fig. 2).

3. Experimental

3.1. General methods

The melting points were recorded on a micro melting point apparatus and are uncorrected. Optical rotations were measured on a digital polarimeter in methanol on a Jasco digital polarimeter (model DIP-3600). Ultraviolet spectra were recorded in methanol on a Hitachi UV 3200 spectrophotometer. Infrared spectra were recorded on a Jasco A-302 IR spectrophotometer. The mass spectra were recorded on a double focusing mass spectrometer. Accurate mass measurements were performed with FAB source using glycerol as matrix. The HREI-MS were recorded on a Jeol HX 110 mass spectrometer. The ^{1}H NMR spectra were recorded on 300 MHz, while ^{13}C NMR spectra were recorded on Bruker AMX-500 operating at 125 MHz using $CDCl_3$ as solvent. Methyl, methylene and methine carbons were distinguished by DEPT experiments. Homonuclear ^{1}H connectivities were determined by using the COSY experiment. One-bond ^{1}H–^{13}C connectivities were determined with HMQC gradient pulse factor selection. Two- and three-bond ^{1}H–^{13}C connectivities were determined by HMBC experiment. Chemical shifts were reported in δ (ppm) and coupling constants (J) were measured in Hz. Precoated TLC plates (silica gel) were used to check the purity of compounds, and ceric sulphate spraying reagent was used for the staining of compounds on TLC. All reagents used were of analytical grades.

3.2. Organism

Cultures of *R. stolonifer* were obtained from the American Type Culture Collection (ATCC). All cultures were maintained on Sabouraud dextrose agar (SDA) slants and stored in a refrigerator at 4 °C prior to use.

3.3. Incubation of leucosceptrine (*1*)

R. stolonifer was grown in shake cultures at 25 °C in five conical flasks (250), each containing 100 mL of a sterile medium comprising (per dm^3) glucose (10 g), peptone (5 g), KH_2PO_4 (5 g), Yeast extract (5 g), Glycerol (10 mL), and NaCl (5 g). The media solution was adjusted to pH 7.0 before sterilization by autoclaving at 121°C for 15 min. Incubations were initiated by suspending the surface growth from slants in sterile medium and using the suspensions to inoculate stage I cultures. Cultures were incubated with shaking on a shaker. After two days of incubation in the above-described medium, stage I culture was used as inoculum for stage II culture.

Leucosceptrine (**1**) (76 mg), dissolved in 5 mL of DMSO, was uniformly distributed among five flasks (250), each containing 100 mL of a sterile medium comprising (per dm^3) glucose (10 g), peptone (5 g), KH_2PO_4 (5 g), Yeast extract (5 g), Glycerol (10 mL), and NaCl (5 g). Culture controls consisted of fermentation blanks in which microorganisms were grown under identical conditions but without the substrate. Substrate controls consisted of sterile medium containing the same amount of substrate and were incubated under the same conditions. After 14 days, the fermentation products were filtered, extracted with chloroform, and concentrated. The organic layer were *screened for biotransformation* by TLC methods.

3.4. Isolation of metabolites

The mycelium was filtered and the culture filtrate was extracted with $CHCl_3$. The extract was dried over Na_2SO_4 and the solvent evaporated to obtain a residue (122 mg). This material was chromatographed on a silica gel column with a pet. ether-$CHCl_3$ gradient, to afford 1α-hydroxyleucosceptrine (**2**) (5 mg), and 8α-hydroxyleucosceptrine (**3**) (2 mg).

3.4.1. 1α-Hydroxyleucosceptrine (*2*)

Colorless crystals, m.p. 154–157 °C, $R_f = 0.41$ (5% MeOH/$CHCl_3$), $[\alpha]_D^{25}$ 143° ($CHCl_3$, c 0.03), UV (MeOH) nm (log ε) λ_{max} 380 (2.78), 351 (2.76); λ_{min} nm 391 (1.06), 361 (2.16), 182 (2.56); IR ($CHCl_3$) ν_{max} 3456 (OH), 2935 and 2868 (CH), 1705 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see Table 1; FAB MS ($M^+ - 1$) m/z 463, HREI-MS m/z 446.3312 (calcd for $C_{25}H_{36}O_7$, m/z 446.3246), EI-MS m/z (rel. int. %): 446 (30), 265 (16), 247 (100), 219 (18), 181 (18), 153 (17), 139 (19), 125 (24), 110 (46), 97 (66), 83 (62).

3.4.2. 8α-Hydroxyleucosceptrine (*3*)

Colorless crystals, m.p. 145–147 °C, $R_f = 0.35$ (5% MeOH/$CHCl_3$), $[\alpha]_D^{25}$ 240° ($CHCl_3$, c 0.06); UV (MeOH) nm (log ε): λ_{max} 365 (2.78), 301 (2.76), λ_{min} 382 (1.79), 346 (2.02), 261 (2.16), IR ($CHCl_3$); ν_{max} 3456 (OH), 2935 and 2868 (CH), 1705 (C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see Table 1; FAB MS ($M^+ - 1$) m/z 463, HREI-MS m/z 446.3012 (calcd for $C_{25}H_{36}O_7$, m/z 446.2946), EI-MS m/z (rel. int. %): 446 (30), 265 (11), 247 (19), 181 (18), 155 (10), 153 (18), 127 (22), 125 (48), 110 (42), 97 (68), 92 (17), 83 (100).

3.5. Horeau's procedure

Compound **2** (5 mg, ca. 0.01 mmol) was added to a solution of 2-phenyl butyric anhydride (0.1 mL) in 0.5 mL C_5H_5N. The resulting mixture was stirred overnight at room temperature. Distilled water (3.0 mL) was added and the reaction mixture allowed to stand for 30 min, 0.1 M NaOH was then added dropwise until the pH became 9 and the solution was then extracted with $CHCl_3$. The aqueous layer was acidified to pH 3 using 1.0 M HCl and the acidic layer was extracted with C_6H_6 (10 mL).

Table 1
^{1}H and ^{13}C NMR data of 1α-hydroxyleucosceptrine (**2**) and 8α-hydroxyleucosceptrine (**3**) in $CDCl_3$

Position	**2**			**3**		
	δ_H (*m*, *J* in Hz)[a]	δ_C[b]	Multiplicity	δ_H (*m*, *J* in Hz)[a]	δ_C[b]	Multiplicity
1	5.82 (*s*)	104.2	CH	3.99, 4.21 (*d*, 17.1)	64.7	CH_2
2	–	142.0	C	–	137.2	C
3	5.61 (*brs*)	123.6	CH	4.96 (*brs*)	121.3	CH
4	–	75.2	C	–	73.4	C
5	–	101.2	C	–	96.8	C
6	1.95 (*m*)	46.7	CH	2.10 (*m*)	41.6	CH
7	1.55 (*m*)	47.7	CH	3.01 (*m*)	42.6	CH
8	1.45, 1.91 (*m*)	35.3	CH_2	4.29 (*m*)	72.1	CH
9	1.23, 1.44 (*m*)	32.4	CH_2	1.19, 1.52 (*m*)	38.5	CH_2
10	1.67 (*m*)	35.3	CH	1.85 (*m*)	36.6	CH
11	1.55 (*m*)	57.4	CH	2.19 (*m*)	52.2	CH
12	–	86.5	C	–	82.2	C
13	–	218.3	C=O	–	221.2	C=O
14	3.01 (*m*)	41.7	CH	3.37 (*m*)	38.6	CH
15	1.25, 1.86 (*m*)	28.1	CH_2	1.21, 2.01 (*m*)	28.9	CH_2
16	1.15, 1.75 (*m*)	26.3	CH_2	1.15, 1.85 (*m*)	26.8	CH_2
17	4.81 (*brd*, 6.2)	84.5	CH	4.81 (*brd*, 6.7)	84.2	CH
18	–	168.3	C	–	167.7	C
19	5.94 (*m*)	117.2	CH	5.81 (*m*)	117.3	CH
20	–	174.1	C	–	181.6	C
21	1.80 (*brd*, 1.3)	17.1	CH_3	1.65 (*brs*)	14.7	CH_3
22	1.15 (*d*, 6.4)	12.5	CH_3	0.99 (*d*, 6.7)	10.8	CH_3
23	1.06 (*d*, 6.8)	21.2	CH_3	0.71 (*d*, 6.9)	17.7	CH_3
24	1.23 (*d*, 7.1)	13.8	CH_3	1.06 (*d*, 6.7)	13.7	CH_3
25	2.05 (*s*)	12.1	CH_3	2.05 (*s*)	13.4	CH_3

[a] ^{1}H NMR data, 300 MHz.
[b] ^{13}C NMR data, 125 MHz.

The benzene extract was evaporated to adjust the volume to 1.0 mL. The optical rotation of the resulting 2-phenyl butyric acid was found to be negative (*R*), indicating the "*S*" configuration at C-20 in compound **2**.

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