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Chemical constituents from fermented broth and mycelium of the basidiomycete *Lacrymaria velutina*

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ABSTRACT. Lacrymarone (**1**), a novel diterpenoid, together with seven known compounds **2-8** were isolated from the fermented broth and mycelium of *Lacrymaria velutina*, an edible fungus collected in Taiwan. The structures of **1-8** were elucidated by analyzing their spectroscopic data. Further biological tests revealed that the co-crystal of **7** and **8** exhibited a moderate inducible nitric oxide synthase (iNOS) inhibitory activity, and with an IC_{50} value of 59.54 ± 3.74 μM.

Keywords: Basidiomycete; Inducible nitric oxide synthase (iNOS); *Lacrymaria velutina*; Lacrymarone.

INTRODUCTION

Fungal natural products have long been an important source of compounds for new drug development (Singh et al., 2000). During our preliminary bioactivity screening of fungal fermentation extracts, *Lacrymaria velutina*, a basidiomycete, was found to exhibit a significant inducible nitric oxide synthase (iNOS) inhibitory activity without any cytotoxicity at a concentration of 100 μg/ml, the inhibition being 97.9 ± 0.8%. This finding prompted us to investigate the active principles from this fungus, and led to the isolation and identification of a novel diterpenoid along with seven known chemical entities. This paper deals with the characterizations of these compounds and their iNOS inhibitory activities on RAW 264.7 cell line.

MATERIALS AND METHODS

General experimental procedures

Optical rotations were measured on a JASCO DIP-1000 digital polarimeter (JASCO, Kyoto, Japan). ^{1}H- and ^{13}C-NMR were acquired on a Bruker DMX-500 SB spectrometer (Bruker, Ettlingen, Germany). High resolution and low resolution mass spectra were obtained using a ThermoQuest Finnigan MAT 95XL spectrometer (ThermoQuest Finnigan, Bremen, Germany) and VG Platform Electrospray ESI/MS (VG Organic, Altrincham, UK), respectively. Infrared spectra were recorded on a JASCO FT/IR 4100 spectrometer (JASCO, Tokyo, Japan). Ultraviolet spectra were measured on a Helios α spectrophotometer (Thermo Scientific, Waltham, MA, USA). Column chromatography was carried out with Sephadex LH-20 gel (Amersham Biosciences, Uppsala, Sweden). Pre-coated Si gel plates (Si 60 F_{254}, 0.2 mm, Merck, Darmstadt, Germany) were used for analytical TLC.

Fermentation of *Lacrymaria velutina*

Lacrymaria velutina (strain No. 96110901 from Taipei, Taiwan) collected, isolated, and identified by one of us (YMJ), was inoculated into 1-1 Erlenmeyer flasks containing 10 g Bacto™ Malt Extract (Becton, Dickinson and Company, Sparks, MD, USA) and 500 ml deionized water. The fermentation was conducted at 25-30°C for 30 days.

Extraction and isolation

The filtered fermented broth (153 l) of *L. velutina* was partitioned three times with 50 l recycled ethyl acetate, then concentrated in vacuum to dryness (1.7 g). Subsequently, this residue was re-dissolved in 25 ml of MeOH, and applied onto a Sephadex LH-20 column (3 cm i.d. × 65 cm) eluted by MeOH with a flow rate of 2.5 ml/min. Each subfraction (18 ml) collected was checked for its compositions by TLC using isopropanol/isooctane (2:8, v/v) or EtOAc/acetic acid/H_2O (85:10:10, v/v/v) for development, and observation under UV 254 nm was used in the detection of compounds with similar chromophores.

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Subsequently, subfractions were combined into seven portions I~VII. The portion II (subfr. 11~13) was purified by HPLC on a semi-preparative reversed-phase column (BDS Hypersil C_{18}, 10 × 250 mm, Thermo Hypersil-Keystone, Bellefonte, CA, USA) with acetonitrile/H_2O (3:2, v/v) as eluent, 2 ml/min, afforded **1** (3.2 mg). HPLC of the same portion on the same column with acetonitrile/H_2O (1:9, v/v) as eluent, 2 ml/min, gave **2** (11.4 mg). The portion III (subfr. 14~15) was purified by HPLC on the same column with MeOH/H_2O (1:4, v/v) as eluent, 2 ml/min, obtained **3** (21.2 mg). The portion V (subfr. 18~19) was purified by HPLC on a semi-preparative reversed-phase column (BIOSIL PRO-ODS-U, 10 × 250 mm, Biotic Chemical Co., Taipei, Taiwan) with MeOH/H_2O (9:1, v/v) containing 0.1% trifluoroacetic acid as eluent, 2 ml/min, afforded **4** (12.5 mg), **5** (17.2 mg) and **6** (17.2 mg). The dried mycelium (20.5 g) of *L. velutina* was extracted three times with 2 l MeOH, then partitioned using *n*-hexane to obtain a yellow residue (1.6 g), which was re-crystallized from *n*-hexane to afford a mixture of **7** and **8** (1:1, 105.8 mg).

Lacrymarone (1)

R_f= 0.45 on Si-60 F_{254} plate using isopropanol/isooctane (2:8, v/v) for development; Light yellow oil; $[\alpha]^{18}_{D}$: +10.4 (*c* 0.55, pyridine); UV (MeOH): λ_{max} (log ε) = 208 (3.7), 251 (3.7) nm; IR (ZnSe): ν_{max} = 3370 (-OH), 2929, 1715 (C=O), 1638 (C=C), 1458, 1255, 1181, 1035 cm^{-1}; ^{1}H-NMR data: see Table 1; ^{13}C-NMR data: see Table 1; ESI-MS: *m/z* = 339 $[M + Na]^+$; HR-ESI-MS: *m/z* = 339.1933 $[M + Na]^+$; calcd for $C_{20}H_{28}O_3Na^+$: 339.1936.

Table 1. ^{1}H- and ^{13}C-NMR data of **1** (pyridine, 500 MHz for ^{1}H and 125 MHz for ^{13}C).

No.	^{13}C	^{1}H	HMBC (H→C)	COSY (H↔H)	NOESY (H↔H)
1	203.7				
2a	41.9	2.14 (1H, dd, *J* = 11.8, 18.1 Hz)	1, 3, 4, 18	2b, 3	2b, 3, 19
2b	41.9	2.40 (1H, dd, *J* = 7.3, 18.1 Hz)	1,3, 4, 5	2a, 3	2a,17, 18, 19
3	46.2	1.59 (1H, m)	18	2a, 2b, 18	2a, 9b
4	46.4				
5	145.5				
6	134.6	6.85 (1H, s)	1, 4, 7		15a, 15b, 16
7	80.5				
8	48.6				
9a	40.4	1.66 (1H, dd, *J* = 8.1, 13.0 Hz)	4, 7, 11	9b, 10	9b, 10, 11b
9b	40.4	1.82 (1H, dd, *J* = 2.2, 13.0 Hz)	4, 11, 16	9a, 10	3, 9a, 16
10	81.5	4.45 (1H, dd, *J* = 2.2, 8.1 Hz)	5, 7	9a, 9b	9a, 17, 20
11a	34.4	1.28 (1H, m)		11b	11b, 12b, 16
11b	34.4	1.96 (1H, m)	8, 12, 16	11a	9a, 11a
12a	23.6	2.01 (1H, m)		12b, 13	13, 15a, 15b
12b	23.6	2.09 (1H, m)		12a, 13	11a, 13, 16
13	126.3	6.37 (1H, brd, *J* = 4.1 Hz)		12a, 12b	12a, 12b, 15a, 15b
14	137.1				
15a	63.1	4.53 (1H, d, *J* = 13.7 Hz)		15b	6, 12a, 13
15b	63.1	4.78 (1H, d, *J* = 13.7 Hz)		15a	6, 12a, 13
16	19.4	0.89 (3H, s)	7, 8, 9, 11		6, 9b, 11a, 12b
17	18.9	1.25 (3H, s)	3, 4, 5, 10		2b, 10, 18, 20
18	29.8	1.54 (1H, m)	3, 4	3, 19, 20	2b, 17, 19, 20
19	22.4	0.81 (3H, d, *J* = 6.3 Hz)	3, 18, 20	18	2a, 2b
20	22.2	0.78 (3H, d, *J* = 6.3 Hz)	3, 18, 19	18	10, 17

Nitrite measurement and cell viability assay

The methods were essentially the same as reported previously (Wang et al., 2007; Lee et al., 2008). To assess the effects on LPS-induced NO production, crude extracts of fermented broth of *L. velutina*, compounds **1-8**, two positive controls-N^{ω}-nitro-L-arginine (L-NNA, a non-selective NOS inhibitor) and aminoguanidine (a specific inhibitor of iNOS)—or vehicle (0.1%, DMSO) were added in the presence of LPS (200 ng/ml) to the RAW 264.7 cells. Both inhibitors were purchased from Sigma-Aldrich Chemical Co. and the purity of each compound was more than 98%.

Statistical analyses

Comparisons of the concentration and treatment effects were made using ANOVA, followed by *post hoc* comparisons using Newman-Keuls test as appropriate. The average IC_{50} was determined by data fitting with GraFit (Erithacus Software, Staines, UK).

RESULTS AND DISCUSSION

The fermented broth of *L. velutina* was partitioned initially using EtOAc to give a brown residue, which was subjected to Sephadex LH-20 column separation followed by HPLC purification to obtain one new diterpenoid (**1**) along with 5-hydroxymethylfurfural (**2**) (McNelis et al., 1994), *bis*-2,5-hydroxymethylfuran (**3**) (Fawcett et al., 1987), maltol (**4**) (Sun et al., 1995), succinic acid (**5**) (Abdel-Farid et al., 2007), and furan-2,5-diol (**6**) (Rappoporta et al., 2001). Meanwhile, the cultured mycelium of the same fungus was extracted with methanol, then partitioned using *n*-hexane to obtain a yellow residue, which was re-crystallized from *n*-hexane to afford a mixture of (22*E*,24*S*)-24-methyl-27-norcholesta-5,7,22-trien-3β-ol (**7**) and (22*E*,24*R*)-24-methyl-27-norcholesta-5,7,22-trien-3β-ol (**8**) (Itoh et al., 1983).

Compound **1** was isolated as light yellow oil, and its molecular formula, $C_{20}H_{28}O_3$, was established through analysis of its ^{13}C-NMR and HR-ESI-MS data. The IR spectrum of **1** exhibited the presence of a carbonyl group (1,715 cm^{-1}), a double bond (1,638 cm^{-1}) and a hydroxyl group (3,370 cm^{-1}). The interpretation of the ^{1}H-NMR of **1** supported by its HSQC assignments revealed signals for four methyl groups [δ_H 0.78 (3H, d, J = 6.3 Hz, H_3-20), δ_H 0.81 (3H, d, J = 6.3 Hz, H_3-19), δ_H 0.89 (3H, s, H_3-16) and δ_H 1.25 (3H, s, H_3-17)], five methylene groups [δ_H 1.28 (1H, m, H-11a), δ_H 1.96 (1H, m, H-11b), δ_H 1.66 (1H, dd, J = 8.1, 13.0 Hz, H-9a), δ_H 1.82 (1H, dd, J = 2.2, 13.0 Hz, H-9b), δ_H 2.01, 2.09 (each 1H, m, H_2-12), δ_H 2.14 (1H, dd, J = 11.8, 18.1 Hz, H-2a), δ_H 2.40 (1H, dd, J = 7.3, 18.1 Hz, H-2b), δ_H 4.53, 4.78 (each 1H, d, J = 13.7 Hz, H_2-15)] and five methine protons [δ_H 1.54 (1H, m, H-18), δ_H 1.59 (1H, m, H-3), δ_H 4.45 (1H, dd, J = 2.2, 8.1 Hz, H-10), δ_H 6.37 (1H, brd, J = 4.1 Hz, H-13), δ_H 6.85 (1H, s, H-6)] (Table 1). The ^{13}C-NMR spectrum together with DEPT analysis displayed 20 signals including four methyl carbons, five methylene carbons, three methine carbons and three quaternary carbons in the aliphatic region, two methine carbons and two quaternary carbons in olefinic region (2 double bonds) as well as one carbonyl carbon (Table 1). On account of the molecular formula $C_{20}H_{28}O_3$, the double bond equivalence of **1** was seven including one carbonyl and two olefinic functionalities. Thus, there should be four rings in **1**. Analysis of COSY and HSQC spectral data allowed the assignments of three spin systems including an aliphatic chain [(H_3-19, -20)-(H-18)-(H-3)-(H_2-2)-], two two-resonance units [-(H-10)-(H_2-9)- and -(H_2-12)-(H-13)-] and five geminal coupled methylene functionalities [-(H_2-2)-, -(H_2-9)-, -(H_2-11)-, -(H_2-12)- and -(H_2-15)-]. In the HMBC spectrum, key long range proton-carbon correlations including H_3-20 (δ_H 0.78)/C-19 (δ_C 22.4), -18 (δ_C 29.8), -3 (δ_C 46.2), H_3-19 (δ_H 0.81)/C-20 (δ_C 22.2), -18 (δ_C 29.8), -3 (δ_C 46.2), H_3-17 (δ_H 1.25)/C-10 (δ_C 81.5), -5 (δ_C 145.5), -4 (δ_C 46.4), -3 (δ_C 46.2), H_3-16/C-11 (δ_C 34.4), -9 (δ_C 40.4), -8 (δ_C 48.6), -7 (δ_C 80.5), H-10 (δ_H 4.45)/C-5 (δ_C 145.5), H-6 (δ_H 6.85)/C-7 (δ_C 80.5), -4 (δ_C 46.4), -1 (δ_C 203.7) and H-2b (δ_H 2.40)/C-5 (δ_C 145.5), -4 (δ_C 46.4), -3 (δ_C 46.2), -1 (δ_C 203.7) established the major connectivities of the fragments deduced from COSY spectrum. All above assignments suggested that the plane structure of **1** seemed to be analogous to guanacastepenes, unique diterpenoids with a 5/7/6-fused ring system, isolated previously from an unidentified endophytic fungus (Brady et al., 2000, 2001). However, the conspicuous cross peak between carbinoyl H-10 (δ_H 4.45) and oxygenated quaternary C-7 (δ_C 80.5) in the HMBC spectrum of **1** suggested that C-10 and C-7 were bridged by an ether functionality, forming a different 5/6/5/6-fused ring skeleton of **1**. In the NOESY spectrum of **1,** key mutual correlations were listed as follows: H_3-20/H_3-17, H_3-17/H-10, H-10/H-9a and H-9b/H_3-16, which were further supported by the three dimensional molecular modeling under minimized energy condition (Figure 1). The relative configurations of the isopropyl group attached at C-3, H_3-17, ether linkage between C-7 and C-10, and H_3-16 were thus corroborated to be respective β, β, β and α oriented to fit the distinguishing features in NOESY spectrum (Table 1). Accordingly, **1** was characterized as the shown structure (Figure 2), and named lacrymarone.

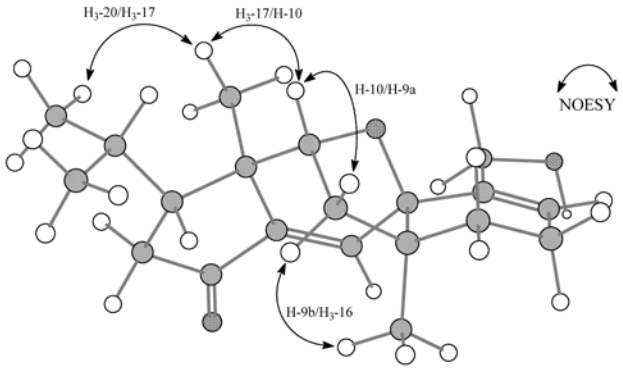

Figure 1. Computer-generated perspective drawing for **1**, which accommodate observed mutual key NOESY.

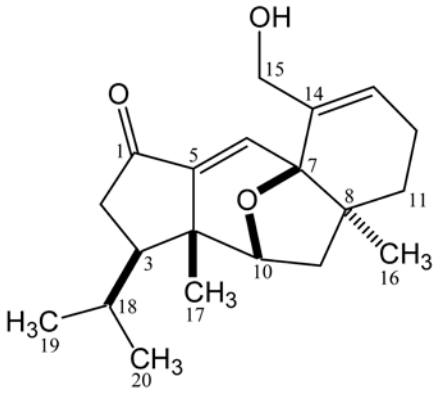

Figure 2. Chemical structure of **1**.

All the pure isolates **1**-**8** were examined for their effects on the LPS-induced NO production in RAW 264.7 cells. All the compounds exhibited no activity even at a concentration of 100 μg/ml except the mixture of **7** and **8.** The mixture of **7** and **8** exhibited a moderate iNOS inhibitory activity, and its IC_{50} value was calculated to be 59.54 ± 3.74 μM. Under the same conditions, the IC_{50} of aminoguanidine and L-NNA were 27.5 ± 0.4 μM and 145.5 ± 16.7 μM, respectively.

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擔子菌 *Lacrymaria velutina* 醱酵液及菌絲體之化學成分探究

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本研究自台灣採集的可食真菌株 *Lacrymaria velutina* 之醱酵液及菌絲體中，分離、純化出八個化合物，其中 lacrymarone (**1**) 爲一新穎的二萜類化合物。在化學結構上，藉由解析化合物 **1**–**8** 的各種光譜數據，其化學結構得以加以確認；在生物活性上，化合物 **7** 與 **8** 的共結晶 (co-crystal) 對於誘導型一氧化氮合成酵素 (inducible nitric oxide synthase, iNOS) 具中等強度的抑制作用，其半抑制濃度爲 59.54 ± 3.74 μM。

關鍵詞：擔子菌；*Lacrymaria velutina*；誘導型一氧化氮合成酵素；Lacrymarone。