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Discovery of Antifungal Constituents from the Miao Medicinal Plant *Isodon Flavidus*

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ABSTRACT

***Ethnopharmacological relevance**:* Leigong Mountain is an area in the Southwest of China where there is a high incidence rate of athlete's foot, but the Miao people, a Chinese minority who reside in this mountainous area have suffered less from this disease due to their use of the herbal medicine *Isodon flavidus* (Hand.-Mazz.) H. Hara.

***Aim of the study**:* The present study is to identify the active chemical constituents responsible for antifungal effects of the folk medicine plant.

***Materials and methods**:* The natural compounds were separated from the methanol extract of the twigs and leaves of *I. flavidus* by phytochemical study using chromatographic methods, and their chemical structures were determined by analysis of the spectroscopic data including 1D and 2D NMR spectra. The absolute configuration of fladin A (**1**) was further confirmed by X-ray crystallographic analysis. The compounds were evaluated for their antifungal activity against the athlete's foot fungus *Trichophyton rubrum*. They were further evaluated for their antimicrobial and anti-biofilm activity against the dental pathogens *Streptococcus mutans*, *Porphyromonas gingivalis* and *Candida albicans*.

***Results**:* Phytochemical and biological studies of *I. flavidus* led to the discovery of two antifungal compounds, fladin A (**1**) and lophanic acid (**2**). Fladin A (**1**) is a novel diterpene with an unprecedented cyclic ether group formed between C-4 and C-9. Lophanic acid (**2**) displayed inhibition activity against the athlete's foot fungus *Trichophyton rubrum* with an MIC value of 7.8 μg/mL, and fladin A (**1**) also showed inhibition activity against the fungus with a MIC value of 62.5 μg/mL.

***Conclusions**:* Our identification of two antifungal compounds provided strong evidence for the Miao people to use *I. flavidus* as a medicinal plant for treatment of athlete's foot disease. The very different chemical structures of the active compounds from those in the market presents them as potential antifungal lead compounds for follow-up study.

Chemical compounds studied in this article

Fladin A; Lophanic acid (PubChem CID: 101787485)

Keywords

Antifungal activity; Fladin A; *Isodon flavidus*; Isolation; Lophanic acid; Structure identification

1. Introduction

Tinea pedis, known as athlete's foot, is a skin disease that causes foot infections of about 15% of the global population (Bell-Syer et al., 2012; Hawkins and Smidt, 2014). The disease is contagious and infects the feet and the interdigital areas. It is most commonly caused by the fungus *Trichophyton rubrum* in a tropical or subtropical region where a high humidity may allow the fungus to thrive. Other pathogenic fungi, such as *Epidermophyton floccosum* and *T. interdigitale* may cause similar skin infection symptoms on the feet. The incidence rate of tinea pedis is very high in southern and southwestern China. In some areas, up to 50% of the population is found to be infected by the skin disease (Chen et al., 2012; Yang and Wang, 1998).

In 2010, we launched a research program to explore the medicinal plants used by Chinese minorities in Guizhou Province, a subtropical area in the southwest of China. Most rural areas of Guizhou are economically underdeveloped, and their medical care heavily relies on folk medicines passed on from generation to generation. As part of the ethnomedicinal program (Jiang et al., 2000; Li et al., 2015; Zou et al., 2011, 2012; Xiang et al., 2004), we have researched Miao traditional medicine in the Leigong Mountain region, the highest mountain in southeastern Guizhou with an average elevation of about 1800 meters. The mountainous region covers a total area of 47,300 hectares, and is one of the designated national forest parks in China. The ecosystem in the district is kept largely intact. The region is home to Miao people, a Chinese minority. As part of the ecosystem, the Miao have co-existed and prospered with this natural habitat for thousands of years. During this long period of history, the Miao have accumulated extensive experience with medications and have formulated their own medical system in which the use of medicinal plants from Leigong Mountain plays an indispensable role (Bao and Ran, 1999; Fan et al., 2002; Chen et al., 1992).

In our field trips to Leigong Mountain, we discovered that although the area has a high incidence rate of tinea pedis due to the high humidity, the local Miao have suffered less from this disease. We further learned from Miao healers that, to counter the fungus based athlete's foot disease, the Miao used an herbal plant that could effectively defend against the disease. The plant, called "Oh Ga Liang" in Miao's dialect, has been known for generations in the Miao villages of Leigong Mountain. To achieve optimal treatment of the disease, the Miao often apply the smashed fresh leaves of an herbaceous plant on the infected areas. Through visiting more than 40 tinea pedis patients, we have learned that the plant has good curative effect with no side effects upon application of the smashed leaves on the infected skins. The medicinal plant was identified as *I. flavidus*, which is mainly distributed in Yunnan and Guizhou provinces of China (ECFG, 1986). The plant species was investigated previously by two research groups, which led to the identification of isopirmarane diterpenoids, *ent*-kaurane diterpenoids along with a few other types of compounds such as flavonoids and steroids (Li et al., 2014; Zhao et al., 2014; Zhao et al., 1998). However, no biologically active compounds have been reported from this plant species. We thus carried out the present study in order to determine the antifungal constituents of the plant.

2. Materials and methods

2.1. General Experimental Procedures

Optical rotation was measured with a Rudolph digital polarimeter. UV datum was obtained on a HP8453 spectrophotometer. VECTOR22 spectrophotometer was used for scanning IR spectroscopy with KBr pellets. 1D and 2D NMR spectra were recorded on Bruker AM-400, DRX-500 and INOVA-400 spectrometers. Unless otherwise specified, chemical shifts (δ) were expressed in ppm with reference to the solvent signals. High-Resolution Secondary Ion Mass Spectrometry (HR-SIMS) was performed on a VG Autospec-3000 spectrometer under 70 eV. Column chromatography was performed with silica gel (200-300 mesh; Qingdao Marine Chemical, Inc., Qingdao, People's Republic of China). Fractions were monitored by TLC (mobile phase: $CHCl_3$/MeOH 4:1) and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in EtOH. All solvents including petroleum ether (60-90 ℃) were distilled prior to use.

2.2. Plant material

The plant materials (twigs and leaves) of *Isodon flavidus* were collected in Leishan, Guizhou Province, China, in September, 2012. The voucher specimen was identified by Professor Deyuan Chen of the Guiyang College of Traditional Chinese Medicine, and deposited at Guiyang College of Traditional Chinese Medicine with the number of the voucher specimen as No.20120903.

2.3. Extraction and isolation

The air-dried powder of the twigs and leaves of *I. flavidus* (5.5 kg) was percolated with 95% MeOH at room temperature (3 × 10 L), and the crude extract (556 g) was subjected to silica gel chromatography ($CHCl_3$/MeOH, from 10/1 to 0/1, v/v) to give six fractions (A-F). Fraction C showed to be the most active fraction against the tinea pedis fungus *T. rubrum* with an MIC value at 31.25 μg/mL, and was thus selected for further separation of the antifungal compounds. The fraction was subjected to separation of a silica gel column and Sephadex LH-20 column to afford fladin A (**1**) (28 mg) and lophanic acid (**2**) (16.5 g).

*2.4. Structure elucidation of fladin A (**1**) and lophanic acid (**2**)*

Fladin A (**1**): Colorless crystals (MeOH), m.p. 159-161 °C. $[\alpha]^{25}_{D}$ +14.9° (*c* 3.22, MeOH). UV ($CDCl_3$) λ_{max} (log ε) 245 (1.44) nm. CD (MeOH) ($[\theta]_{215}$ +76.7). IR (KBr) ν_{max} 3087, 3006, 2919, 1738, 1638, 1451, 1389, 1169, 1145, 1037 cm^{-1}. ^1H and ^{13}C NMR data, see Table 1. EIMS *m/z* 318 $[M]^+$ (100%). HRSIMS *m/z*: 318.2208 $[M]^+$ (calcd for $C_{20}H_{30}O_3$, 318.2195). The spectral data determined fladin A (**1**) as a new compound, and the absolute structure was determined by X-ray crystallography.

Lophanic acid (**2**): Colorless crystals (MeOH), m.p. 169-171 °C. $[\alpha]^{25}_{D}$ +112.9° (*c* 1.05, MeOH). ^1H NMR (pyridine-d_5, 400 MHz): δ_H 2.93 (1H, brd, *J*=13.2, H-1β), 2.86 (1H, m, H-6β), 1.51 (1H, dd, *J*=12.8, 2.4, H-5α), 1.49 (1H, brd, *J*=11.2, H-3β), 1.15/1.17 (each 3H, d, *J*=6.0, CH_3-16/17), 0.97/1.09 (each 3H, s, CH_3-18/19). ^{13}C NMR (pyridine-d_5, 100 MHz) δ_C 35.2 (C-1), 20.9 (C-2), 42.6 (C-3), 34.2 (C-4), 52.8 (C-5), 18.8 (C-6), 32.6 (C-7), 129.7 (C-8), 131.2 (C-9), 49.1 (C-10), 23.0 (C-11), 33.7 (C-12), 71.1 (C-13), 42.5 (C-14), 34.1 (C-15), 17.4 (C-16), 17.2 (C-17), 32.4 (C-18), 20.8 (C-19), 178.2 (C-20). EIMS *m/z*: 320 $[M]^+$ (0.2%), 302 $[M-H_2O]^+$ (66%), 274 [M-H_2O-CO]$^+$ (38%), 257 [M-H_2O-COOH]$^+$ (100%), 241 (19%), 231 (43%), 213 (53%), 202 (18%), 187 (40%), 172 (17%), 161 (15%), 145 (18%), 131 (31%), 119 (34%), 101 (22%), 91 (28%), 69 (21%), 43 (26%. The spectral data of lophanic acid (**2**) are in a good agreement with those reported for the diterpene previously from *I. lophanthoides* (Wang et al., 1995).

Table 1
^{1}H and ^{13}C NMR data of **1** (CD_3OD, 500 and 125 MHz).

No	δ_H [Mult, J (Hz)][a]	δ_C (Mult.)[b]	No	δ_H [Mult, J (Hz)]	δ_C (Mult.)
1	2.25 ddd (15.3, 11.9, 4.3)	31.0 t	11	1.54 m	24.9 t
	2.41 ddd (15.3, 11.9, 5.9)			1.58 ddd (14.5, 12.5, 4.9)	
2	1.33 ddd (13.9, 11.9, 5.9)	31.0 t	12	1.45 m	33.9 t
	2.04 brddd (13.7, 12.1, 4.3)			1.50 brtd (12.6, 3.9)	
3		176.6 s	13		36.1 s
4		82.1 s	14	2.31 brdd (15.5, 2.3)	41.1 t
				2.16 brdq (15.4, 3.5)	
5	1.87 brs	49.3 d	15	5.62 ddd (17.7, 10.9, 0.8)	145.0 d
6	2.08 m	27.8 t	16	4.98 dd (10.9, 1.5)	112.8 t
	2.29 m			4.93 dd (17.6, 1.5)	
7	5.42 brs	123.8 d	17	0.95 s	29.2 q
8		137.4 s	18[c]	1.29 s	30.1 q
9		82.2 s	19[c]	1.28 s	26.4 q
10		46.2 s	20	1.14 s	21.6 q

[a] Multiplicities in parentheses represent: s (singlet), brs (broad singlet), dd (doublet of doublet), brdd (broad doublet of doublet), ddd (doublet of doublet of doublet), brddd (broad doublet of doublet of doublet), brtd (broad triplet of doublet), brdq (broad doublet of quartet), and m (multiplet).
[b] Multiplicities in parentheses represent: s (quaternary carbon), d (CH), t (CH_2), and q (CH_3).
[c] The NMR assignments may be interchangeable between the two carbons.

*2.5. Single crystal X-ray data and structure of fladin A (**1**)*

Crystal data of **1** (from MeOH): space group $P2_12_12_1$, $C_{20}H_{30}O_3\times 2$, M = 636.88, a = 9.80360(10) Å, b = 12.52530 (10) Å, c = 29.2836(3) Å, $\alpha = \beta = \gamma$ = 90.00°, V = 3595.82(6) Å^3, d = 1.176g/cm^3, Z = 4.Acrystalof dimensions 0.20× 0.30×0.55 mm^3 was used for measurements on a APEX DUO diffractometer with a graphite monochromator (ω-κ scans, 2θmax = 138.74°), Cu $K\alpha$ radiation. The total number of independent reflections measured was 16469, of which 6169 were observed ($|F|^2 \geqq 2\sigma|F|^2$). The crystal structure was solved and refined by the direct method SHELXS-97 (Sheldrick, G. M. University of Gottingen: Gottingen, Germany, 1997), expanded using difference Fourier techniques and full-matrix least-squares calculations. Final indices: R1 =0.0364, wR2 = 0.0911 ($w = 1/\sigma|F|^2$), s = 1.064.

The data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

2.6. Evaluation of biological activities

2.6.1. Cytotoxicity assay

The assay was conducted based on the slightly modified protocols reported previously used for the other cell lines (Jutiviboonsuk et al., 2005; Zhang et al., 2006). Briefly, human colon cancer (HCT116) cell line was purchased from the American Type Culture Collection (ATCC). HCT116 cells were maintained in MCCoy's 5a medium (ATCC30-2007). PSF (100 units/mL penicillin G, 100 μg/mL streptomycin sulfate, 250 ng/ml amphotericin B) was added. Medium was supplemented with 10% fetal bovine serum (FBS). Serial dilutions of the isolated compound were prepared using 10% aqueous DMSO as solvent. The 190 μL cell suspension (3×10^4 cells in 1 mL media) was incubated with10 μL sample solutions, in triplicate, in 96-well tissue culture plate at 37°C in a humidified atmosphere of 5% CO_2 in air for 72 hours. 10 μL 10% aqueous DMSO was used as control group. Then the cells were fixed to plastic substratum by the addition of 100 μL cold 20% aqueous trichloroacetic acid and washing with water after incubation at 4°C for 30 min. After staining cells with 100 μL of 0.4% sulforhodamine B in 1% aqueous AcOH for 30 min, unbound dye was removed by rinsing with 1% aqueous AcOH. The bound dye was solubilized with 200 μL 10 mM unbuffered Tris base, pH 10, and the optical density was measured at 515 nm using an ELISA plate reader. The average data were expressed as a percentage, relative to the control.

2.6.2. Evaluation of antifungal and antibacterial bioactivity

The antimicrobial assays against dental pathogens *Streptococcus mutans*, *Porphyromonas gingivalis* and *Candida albicans* as well as the athlete's foot fungus *Trichophyton rubrum* were carried out based the modified protocols previously reported (Zou et al., 2012). Briefly, inhibition of growth was assayed in 96-well microtiter plates. Each well contained 50 μL test agent serially diluted two-fold, 50 μL growth medium (BBL™ Brain Heart Infusion for *S. mutans*; Difco™ YM for *C. albicans* and *T. rubrum*, and for *P. gingivalis* Thioglycollate medium, Anaerobe Systems, Morgan Hill, CA), and 10 μL of an overnight culture representing approximately 5 x 10^7 CFU/mL. Wells inoculated with the particular microbial species but without the test agent served as positive controls; uninoculated wells with growth medium served as negative controls. The plates were incubated for 24 hours at 37°C. 20 μL from each well was then inoculated on blood agar plates and incubated for 48 hours at 37°C. The MIC (minimal inhibitory concentration) was defined as the lowest concentration that inhibited growth.

2.6.3. Biofilm breakdown assay

Biofilms were formed after 24 hours growth in 96 well plates using 10 μL from an overnight culture adjusted to OD_{600} = 0.1 and 90 μL growth medium appropriate for the species as noted above for the MIC assays. The planktonic phase was removed by inverting the plate over a waste tray (accompanied by a snap of the wrist to ensure complete emptying of contents) and the biofilm washed by submersion in water followed by emptying over a waste tray. Each test agent (5 μL) was added to 45 μL phosphate buffered saline (PBS) and incubated for 3 hours. The PBS was removed by inversion over a waste tray and the biofilm was washed three times by submersion. The biofilm was then stained for 10 minutes at room temperature with 100 μL 0.1% crystal violet in a modification of the crystal violet assay (O'Toole and Kolter, 1998). The crystal violet was removed to a waste tray and the biofilm washed three times by submersion. The plate was inverted and tapped on a paper towel to ensure removal of all liquid and air-dried for 10 min. The crystal violet was 'released' by adding 50 μL 33% acetic acid and incubating for 15 min on a shaking platform. 25μL was transferred to a new 96-well plate, diluted 1:1 with 25 μL water and the OD read at 550 nm. Decreases in biofilm were calculated by comparing the OD_{550} of the test wells with the OD_{550} of control wells that were untreated with the test agents.

3. Results and discussion

*3.1. Isolation of fladin A (**1**) and lophanic acid (**2**)*

Previous phytochemical studies showed that the plants in the *Isodon* genus (Lamiaceae) were rich in producing abundant bioactive diterpenoids, especially the *ent*-kaurane diterpenes with a four-membered ring system (Sun et al., 2006). Diterpenes with a three-membered ring system have also been discovered from *Isodon* plants including *I. flavidus* and *I. rubescens* (Zhao et al., 1998; Zou et al., 2011; Zou et al., 2012). Our initial bioassay data showed that the alcohol extract made from the leaves of *I. flavidus* displayed good inhibitory activity against the tinea pedis fungus *T. rubrum* as well as the oral fungus *Candida albicans* with MIC values at 62.5 μg/mL. The positive results further encouraged us to investigate the potentially potent antifungal compounds responsible for the antifungal activity of this plant.

The plant materials (twigs and leaves) of *I. flavidus* were thus re-collected from Leishan (in Leigong Mountain region) in September, 2012 to isolate the active compounds. Chromatographic separation of the methanol extract of the plant materials led to the isolation of two antimicrobial compounds, fladin A (**1**, 28 mg) and lophanic acid (**2**, 16.5 g) (Fig. 1).

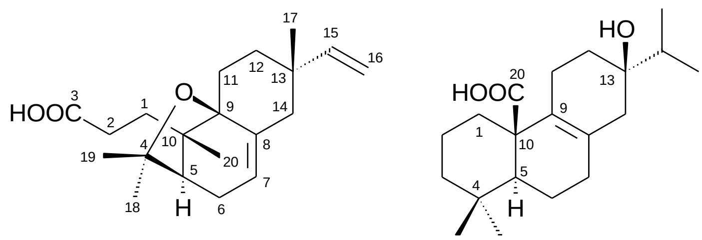

Fig. 1. Chemical structures of fladin A (**1**) and lophanic acid (**2**).

*3.2. Elucidation of the chemical structure of fladin A (**1**)*

Fladin A (**1**) is a novel diterpenoid with an unprecedented cyclic ether group formed between C4 and C9. The formation of the five-membered tetrahydrofuran ring has resulted in flipped configuration of C9 in comparison with the isopimarane diterpenes. The compound was obtained as colorless crystals. Its molecular formula was determined to be $C_{20}H_{30}O_3$ on the basis of analysis of its NMR spectroscopic data, which was verified by HR-EIMS data at *m/z* 318.2208 $[M]^+$ (calcd 318.2195). The IR absorption at 1738 cm^{-1} indicated the presence of a carbonyl group. The ^1H, ^{13}C and DEPT NMR spectra (Table 1) showed 20 carbons, characterized as four methyl carbons [δ_H 0.95, 1.14, 1.28, and 1.29 (each 3H, s); δ_C 21.6, 26.4, 29.2, and 30.1], a vinyl group [δ_H 5.62 (1H, ddd, *J*=17.7, 10.9, 0.8 Hz), 4.98 (1H, dd, *J*=10.9, 1.5 Hz), 4.93 (1H, dd, *J*=17.6, 1.5 Hz); δ_C 112.8 and 145.0], one trisubstituted carbon-carbon double bond [δ_H 5.42 (1H, brs); δ_C 123.8 and 137.4], six methylene carbons, one methine carbon [δ_H 1.87 (1H, brs); δ_C 49.3], a carbonyl carbon (δ_C 176.6), two oxy-tertiary carbons (δ_C 82.1 and 82.2), and two quaternary carbons (δ_C 36.1 and 46.2). On the basis of these data and chemotaxonomic considerations, **1** was determined to be a diterpenoid.

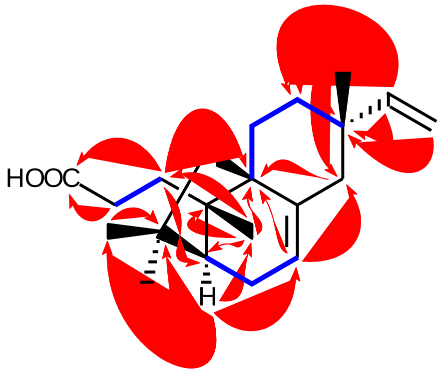

Fig.2. Key COSY (— in blue) and HMBC (→ in red) correlations for **1**.

In the HMBC spectrum (Fig. 2), the presence of the correlations from the vinyl methine proton at δ_H 5.62 (H-15) to C-12, C-13, and C-17, from the vinyl methylene protons (H_2-16) at δ_H 4.98 and 4.93 to C-13 and C-15, and from the methyl proton at δ_H 0.95 (CH_3-17) to C-12, C-14, and C-15 assigned the vinyl group at C-13.

The presence of the HMBC correlations of the alkenyl proton signal δ_H 5.42 (H-7) to C-5, C-9, and C-14 determined the trisubstituted carbon–carbon double bond to be at C-7 and C-8. The presence of the HMBC correlations from the proton at δ_H 1.87 (H-5) to C-4, C-7, C-10, C-18, and C-19, and from the methyl proton at δ_H 1.14 (H-20) to C-5, C-9, and C-10 indicated that the two oxyquaternary carbons of δ_C 82.1 and 82.2 are C-4 and C-9, respectively. The fact that both oxygenated carbon signals are significantly shifted downfield in comparison with those of normal oxyquaternary carbon groups suggested an epoxide ring group formed between C-4 and C-9. When the three rings [the epoxide, the six membered ring (ring B) formed by C-5, C-6, C-7, C-8, C-9, and C-10 and the six membered ring (ring C) formed by C-8, C-9, C-11, C-12, C-13, and C-14] and the three double bonds (the carbonyl group and the two carbon-carbon double bonds) are considered, no additional double-bond equivalent is required for the molecular formula ($C_{20}H_{30}O_3$). Ring A (formed through C-1, C-2, C-3, C-4, C-5, and C-10) that exists in a normal three membered diterpene is thus determined to be opened. No HMBC correlation was observed from H-5 to the carbonyl carbon (C-3) indicating that the broken bond of open ring A occurred between C-3 and C-4, which was further confirmed by the presence of the HMBC correlations of H_2-1 and H_2-2 to the ^{13}C NMR signal at δ_C 176.6.

*3.3. Determination of the absolute configuration of fladin A (**1**)*

The CD spectrum of **1** showed a positive Cotton effect ($[\theta]_{215}$ +76.7) indicative of the n→π exiton of the carboxylic acid group.The CD data, together with the optical rotation datum, determined **1** as an enantiomerically pure compound.

To determine the absolute configuration, **1** was crystallized in MeOH to afford a colorless crystal of the orthorhombic space group $P2_12_12_1$, which was analyzed by X-ray crystallography. Through structural refinement (Flack, 1983; Flack and Bernardinelli, 2008), the absolute configuration of **1** was authenticated by the measurement of the Flack parameter. In our study, the final refinement on the Cu Kα data of the crystal of **1** resulted in a Flack parameter of 0.03 (13), allowing an explicit assignment of the absolute structure as shown in Fig. 3 (Zou et al., 2012). In contrast to the absolute configuration of an isopimarane diterpene, the chiral center of C9 in **1** was inversed (Fig.4). The four chiral centers, C-5, C-9, C-10, C-13, were thus determined as *R*, *S*, *S*, *S*, respectively. Accordingly, the structure of **1** was established as 3-(4, 10, 10, 12-tetramethyl-4-vinyl-11-oxa-tricyclo [7.2.1.01, 6] dodec-6-en-12-yl)-propionic acid, and given the trivial name fladin A.

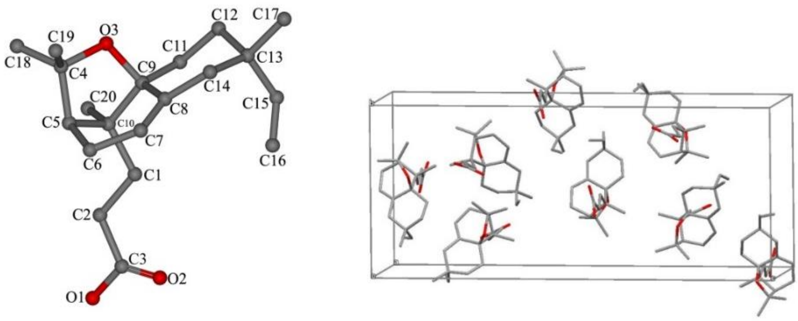

Fig. 3. X-ray crystallographic structure of **1**.

*3.4. Plausible biogenetic pathway of fladin A (**1**)*

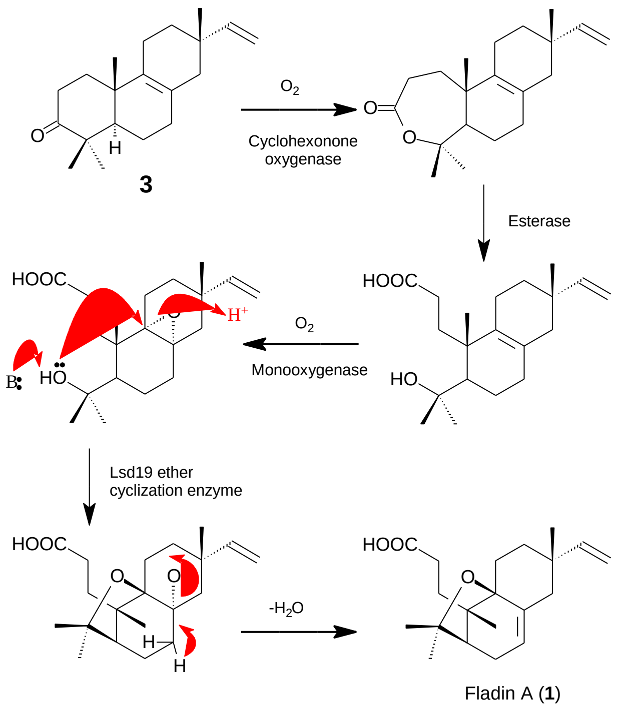

Fig.4. Proposed biogenetic pathway of **1**.

Fladin A (**1**) is a unique diterpenoid with a tetrahydrofuran group between C4 and C9. It may be derived from isopimarane diterpenes, which are found abundant in *Isodon* plants (Sun et al., 2006; Jiang et al., 2000; Zhang and Sun, 1989). A plausible biogenetic pathway for **1** originating from the precursor of the natural compound 11b-hydroxy-8, 15-isopimaradiene-3-one (**3**) is proposed (Fig.4) (Jiang et al., 2000; Kenmoku et al., 2004). The enzymatic Baeyer-Villager type oxidation of the ketone group in **3** inserts an oxygen atom between C-3 and C-4, leading to the formation of a seven-membered ring lactone (Abril et al., 1989), which is hydrolyzed by esterase to yield a diterpene carboxylic acid with a ring A opening. The double bond between C-8 and C-9 in the diterpene is transformed by monoxygenase to an epoxide, which is further converted to a tetrahydrofuran product via an epoxide-opening reaction catalyzed by the enzyme Lsd19 (Hotta et al., 2012). Loss of a H_2O molecule of the tetrahydrofuran product produces fladin A (**1**).

*3.5. Antifungal activity of fladin A (**1**) and lophanic acid (**2**)*

Lophanic acid (**2**) was obtained as colorless crystals. The compound is a known abietane-type diterpenoid, which was reported previously from *I. lophanthoides* (Wang et al., 1995). The two isolated compounds (**1** and **2**) were evaluated for their cytotoxicity against the HCT116 colon human tumor cell line. No cytotoxicity was observed for the compounds against these cell lines at a concentration of 200 μg/mL, indicating the low toxicity of these compounds. The two compounds were then tested for their antifungal potential against the athlete's foot fungus *T. rubrum*. Lophanic acid (**2**) showed antifungal activity against *T. rubrum* with an MIC value of 7.8 μg/mL (Table 2). Considering the diterpenoid as a major constituent in *I. flavidus* (0.3 %) and its antifungal potency, lophanic acid (**2**) could well be a biologically active constituent for the plant *I. flavidus*, which has been used by Miao people as an herbal medicine for treatment of fungal infections of the skin of the foot. The new compound fladin A (**1**) also showed inhibition activity against *T. rubrum* with a MIC value of 62.5 μg/mL. The two isolates (**1** and **2**) were further evaluated for their antimicrobial and anti-biofilm potential against the dental pathogens *Streptococcus mutans*, *Porphyromonas gingivalis* and *Candida albicans*. In comparison with the athlete's foot fungus, both compounds showed less inhibitory activity against the dental fungus (*C. albicans*), indicating the strong selective inhibitory activity of the compounds toward the athlete's foot fungus. The compounds also displayed moderate inhibitory activity against the oral bacteria (*S. mutans* and *P. gingivalis*) (Table 2). Fladin A (**1**) and lophanic acid (**2**) were further found to be able to breakdown the formed biofilm of *C. albicans*. Fladin A (**1**) caused more than 30% breakdown of the biofilm at the concentrations of 1000 and 250 μg/mL, but lophanic acid (**2**) showed less biofilm breakdown activity with only about 26% breakdown of the biofilm at a concentration of 1000 μg/mL. No biofilm breakdown activity against the oral bacteria was observed for **1** and **2**. These results suggest that the compounds have preferential antifungal activity, and may be the basis for how the Miao have come to view *I. flavidus* as a medicinal plant that defends against athlete's foot disease. Since the compounds have very different chemical structures from those of the antifungal drugs in the market, they may present a unique antifungal mechanism of action. Further chemical and pharmacological studies including structural modification may advance these types of compounds for further development as unique antifungal drug candidates.

Table 2.

Antimicrobial activity of fladin A (**1**) and lophanic acid (**2**) against *S. mutans*, *P. gingivalis*, *C. albicans*, and *T. rubrum*.[a]

	Growth inhibition (MIC: μg/mL)				Biofilm breakdown (% at 1000/250/62.5 μg/mL)		
	Sm	*Pg*	*Ca*	*Tr*	*Sm*	*Pg*	*Ca*
Extract[b]	250	62.5	62.5	62.5	0/0/0	0/0/0	25.3/21.2/0
1	250	250	125	62.5	0/0/0	0/0/0	34.2/30.3/0
2	62.5	62.5	62.5	7.8	0/0/0	0/0/0	26.5/15,5/0
CHX[c]	9.4	4.7	9.4	–	0/0/0	0/0/0	0/0/0
CLT[d]	–	–	–	0.25	–	–	–

[a] *Sm*: *S. mutans*; *Pg*: *P. gingivalis*; *Ca*: *C. albicans*, *Tr*: *T. rubrum*.

[b] Methanol extract of the leaves of *I. flavidus*.

[c] Chlorhexidine.

[d] Clotrimazole.

4. Conclusions

In conclusion, we have isolated two antifungal compounds, fladin A (**1**) and lophanic acid (**2**) from *I. flavidus*. Fladin A (**1**) is a novel diterpene with an unprecedented carbon skeleton. The new compound fladin A (**1**) also showed inhibition activity against *T. rubrum* with a MIC value of 62.5 μg/mL. And Lophanic acid (**2**) displayed inhibition activity against the *T. rubrum* with an MIC value of 7.8 μg/mL. Lophanic acid is a completely different structure from the active pharmaceutical ingredient in market, revealing a great opportunity for follow-up study. Among the many scientific issues still to be addressed are determination of the mechanism of antifungal activity, the effect of structural modifications, and total synthesis, and related pharmacological experiments.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://xxx

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