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1 **Discovery of Antifungal Constituents from the Miao Medicinal Plant**

2 ***Isodon Flavidus***

3

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23

1 ABSTRACT

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

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

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

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

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

23 Chemical compounds studied in this article

24 Fladin A; Lophanic acid (PubChem CID: 101787485)

25 Keywords

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

1 **1. Introduction**

2

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

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

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

40

41

42 **2. Materials and methods**

43

44 *2.1. General Experimental Procedures*

1
2 Optical rotation was measured with a Rudolph digital polarimeter. UV datum was obtained on
3 a HP8453 spectrophotometer. VECTOR22 spectrophotometer was used for scanning IR
4 spectroscopy with KBr pellets. 1D and 2D NMR spectra were recorded on Bruker AM-400,
5 DRX-500 and INOVA-400 spectrometers. Unless otherwise specified, chemical shifts (δ) were
6 expressed in ppm with reference to the solvent signals. High-Resolution Secondary Ion Mass
7 Spectrometry (HR-SIMS) was performed on a VG Autospec-3000 spectrometer under 70 eV.
8 Column chromatography was performed with silica gel (200-300 mesh; Qingdao Marine Chemical,
9 Inc., Qingdao, People's Republic of China). Fractions were monitored by TLC (mobile phase:
10 $\text{CHCl}_3/\text{MeOH}$ 4:1) and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4
11 in EtOH. All solvents including petroleum ether (60-90 °C) were distilled prior to use.
12

13 2.2. Plant material

14

15 The plant materials (twigs and leaves) of *Isodon flavidus* were collected in Leishan, Guizhou
16 Province, China, in September, 2012. The voucher specimen was identified by Professor Deyuan
17 Chen of the Guiyang College of Traditional Chinese Medicine, and deposited at Guiyang College
18 of Traditional Chinese Medicine with the number of the voucher specimen as No.20120903.
19

20 2.3. Extraction and isolation

21

22 The air-dried powder of the twigs and leaves of *I. flavidus* (5.5 kg) was percolated with 95%
23 MeOH at room temperature (3×10 L), and the crude extract (556 g) was subjected to silica gel
24 chromatography ($\text{CHCl}_3/\text{MeOH}$, from 10/1 to 0/1, v/v) to give six fractions (A-F). Fraction C
25 showed to be the most active fraction against the tinea pedis fungus *T. rubrum* with an MIC value at
26 $31.25 \mu\text{g/mL}$, and was thus selected for further separation of the antifungal compounds. The
27 fraction was subjected to separation of a silica gel column and Sephadex LH-20 column to afford
28 fladin A (**1**) (28 mg) and lophanic acid (**2**) (16.5 g).
29

30 2.4. Structure elucidation of fladin A (**1**) and lophanic acid (**2**)

31

32 Fladin A (**1**): Colorless crystals (MeOH), m.p. 159-161 °C. $[\alpha]_D^{25} +14.9^\circ$ (*c* 3.22, MeOH). UV
33 (CDCl_3) λ_{max} (log ϵ) 245 (1.44) nm. CD (MeOH) ($[\theta]_{215} +76.7$). IR (KBr) ν_{max} 3087, 3006, 2919,
34 1738, 1638, 1451, 1389, 1169, 1145, 1037 cm^{-1} . ^1H and ^{13}C NMR data, see Table 1. EIMS m/z 318
35 $[\text{M}]^+$ (100%). HRSIMS m/z : 318.2208 $[\text{M}]^+$ (calcd for $\text{C}_{20}\text{H}_{30}\text{O}_3$, 318.2195). The spectral data
36 determined fladin A (**1**) as a new compound, and the absolute structure was determined by X-ray
37 crystallography.

38 Lophanic acid (**2**): Colorless crystals (MeOH), m.p. 169-171 °C. $[\alpha]_D^{25} +112.9^\circ$ (*c* 1.05,
39 MeOH). ^1H NMR (pyridine- d_5 , 400 MHz): δ_{H} 2.93 (1H, brd, $J=13.2$, H-1 β), 2.86 (1H, m, H-6 β),
40 1.51 (1H, dd, $J=12.8$, 2.4, H-5 α), 1.49 (1H, brd, $J=11.2$, H-3 β), 1.15/1.17 (each 3H, d, $J=6.0$,
41 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),
42 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),
43 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
44 (C-17), 32.4 (C-18), 20.8 (C-19), 178.2 (C-20). EIMS m/z : 320 $[\text{M}]^+$ (0.2%), 302 $[\text{M}-\text{H}_2\text{O}]^+$ (66%),

1 274 [M-H₂O-CO]⁺ (38%), 257 [M-H₂O-COOH]⁺ (100%), 241 (19%), 231 (43%), 213 (53%), 202
 2 (18%), 187 (40%), 172 (17%), 161 (15%), 145 (18%), 131 (31%), 119 (34%), 101 (22%), 91 (28%),
 3 69 (21%), 43 (26%). The spectral data of lophanic acid (**2**) are in a good agreement with those
 4 reported for the diterpene previously from *I. lophanthoides* (Wang et al., 1995).

5
 6 **Table 1**

7 ¹H and ¹³C NMR data of **1** (CD₃OD, 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) 2.41 ddd (15.3, 11.9, 5.9)	31.0 t	11	1.54 m 1.58 ddd (14.5, 12.5, 4.9)	24.9 t
2	1.33 ddd (13.9, 11.9, 5.9) 2.04 brddd (13.7, 12.1, 4.3)	31.0 t	12	1.45 m 1.50 brtd (12.6, 3.9)	33.9 t
3		176.6 s	13		36.1 s
4		82.1 s	14	2.31 brdd (15.5, 2.3) 2.16 brdq (15.4, 3.5)	41.1 t
5	1.87 brs	49.3 d	15	5.62 ddd (17.7, 10.9, 0.8)	145.0 d
6	2.08 m 2.29 m	27.8 t	16	4.98 dd (10.9, 1.5) 4.93 dd (17.6, 1.5)	112.8 t
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

8 ^a Multiplicities in parentheses represent: s (singlet), brs (broad singlet), dd (doublet of doublet), brdd
 9 (broad doublet of doublet), ddd (doublet of doublet of doublet), brddd (broad doublet of doublet of doublet),
 10 brtd (broad triplet of doublet), brdq (broad doublet of quartet), and m (multiplet).

11 ^b Multiplicities in parentheses represent: s (quaternary carbon), d (CH), t (CH₂), and q (CH₃).

12 ^c The NMR assignments may be interchangeable between the two carbons.

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 14 **2.5. Single crystal X-ray data and structure of fladin A (**1**)**

15
 16 Crystal data of **1** (from MeOH): space group *P2₁2₁2₁*, C₂₀H₃₀O₃×2, M = 636.88, *a* =
 17 9.80360(10) Å, *b* = 12.52530 (10) Å, *c* = 29.2836(3) Å, $\alpha = \beta = \gamma = 90.00^\circ$, V = 3595.82(6) Å³, d =
 18 1.176g/cm³, Z = 4. A crystal of dimensions 0.20× 0.30×0.55 mm³ was used for measurements on a
 19 APEX DUO diffractometer with a graphite monochromator (ω - κ scans, $2\theta_{\text{max}} = 138.74^\circ$), Cu K α
 20 radiation. The total number of independent reflections measured was 16469, of which 6169 were
 21 observed ($|F|^2 \cong 2\sigma|F|^2$). The crystal structure was solved and refined by the direct method
 22 SHELXS-97 (Sheldrick, G. M. University of Gottingen: Gottingen, Germany, 1997), expanded
 23 using difference Fourier techniques and full-matrix least-squares calculations. Final indices: R1
 24 =0.0364, wR2 = 0.0911 ($w = 1/\sigma|F|^2$), s = 1.064.

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

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 28 **2.6. Evaluation of biological activities**

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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 with 10 µL sample solutions, in triplicate, in 96-well tissue culture plate at 37°C in a humidified atmosphere of 5% CO₂ 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*, *Porphyrromonas 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 5×10^7 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 OD₆₀₀ = 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

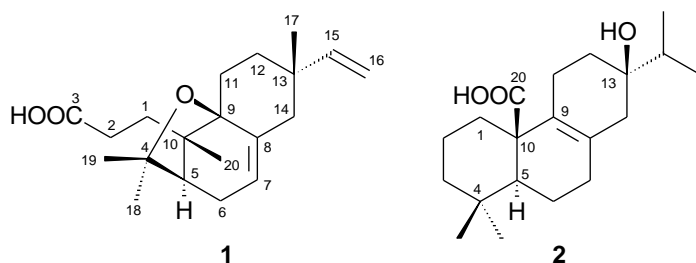
1 violet was 'released' by adding 50 μ L 33% acetic acid and incubating for 15 min on a shaking
2 platform. 25 μ L was transferred to a new 96-well plate, diluted 1:1 with 25 μ L water and the OD
3 read at 550 nm. Decreases in biofilm were calculated by comparing the OD₅₅₀ of the test wells with
4 the OD₅₅₀ of control wells that were untreated with the test agents.

6 3. Results and discussion

8 3.1. Isolation of fladin A (1) and lophanic acid (2)

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

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

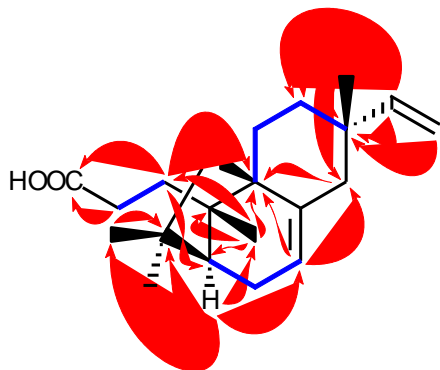


27 **Fig. 1.** Chemical structures of fladin A (1) and lophanic acid (2).

28 3.2. Elucidation of the chemical structure of fladin A (1)

29 Fladin A (1) is a novel diterpenoid with an unprecedented cyclic ether group formed between
30 C4 and C9. The formation of the five-membered tetrahydrofuran ring has resulted in flipped
31 configuration of C9 in comparison with the isopimarane diterpenes. The compound was obtained as
32 colorless crystals. Its molecular formula was determined to be C₂₀H₃₀O₃ on the basis of analysis of
33 its NMR spectroscopic data, which was verified by HR-EIMS data at m/z 318.2208 [M]⁺ (calcd
34 318.2195). The IR absorption at 1738 cm⁻¹ indicated the presence of a carbonyl group. The ¹H,
35 ¹³C and DEPT NMR spectra (Table 1) showed 20 carbons, characterized as four methyl carbons [δ _H
36 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,
37 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
38 and 145.0], one trisubstituted carbon-carbon double bond [δ _H 5.42 (1H, brs); δ _C 123.8 and 137.4],

1 six methylene carbons, one methine carbon [δ_{H} 1.87 (1H, brs); δ_{C} 49.3], a carbonyl carbon (δ_{C}
2 176.6), two oxy-tertiary carbons (δ_{C} 82.1 and 82.2), and two quaternary carbons (δ_{C} 36.1 and 46.2).
3 On the basis of these data and chemotaxonomic considerations, **1** was determined to be a
4 diterpenoid.
5



6
7 **Fig.2.** Key COSY (— in blue) and HMBC (→ in red) correlations for **1**.
8

9 In the HMBC spectrum (Fig. 2), the presence of the correlations from the vinyl methine proton
10 at δ_{H} 5.62 (H-15) to C-12, C-13, and C-17, from the vinyl methylene protons (H₂-16) at δ_{H} 4.98 and
11 4.93 to C-13 and C-15, and from the methyl proton at δ_{H} 0.95 (CH₃-17) to C-12, C-14, and C-15
12 assigned the vinyl group at C-13.

13 The presence of the HMBC correlations of the alkenyl proton signal δ_{H} 5.42 (H-7) to C-5, C-9,
14 and C-14 determined the trisubstituted carbon-carbon double bond to be at C-7 and C-8. The
15 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
16 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
17 oxyquaternary carbons of δ_{C} 82.1 and 82.2 are C-4 and C-9, respectively. The fact that both
18 oxygenated carbon signals are significantly shifted downfield in comparison with those of normal
19 oxyquaternary carbon groups suggested an epoxide ring group formed between C-4 and C-9. When
20 the three rings [the epoxide, the six membered ring (ring B) formed by C-5, C-6, C-7, C-8, C-9, and
21 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
22 three double bonds (the carbonyl group and the two carbon-carbon double bonds) are considered,
23 no additional double-bond equivalent is required for the molecular formula (C₂₀H₃₀O₃). Ring A
24 (formed through C-1, C-2, C-3, C-4, C-5, and C-10) that exists in a normal three membered
25 diterpene is thus determined to be opened. No HMBC correlation was observed from H-5 to the
26 carbonyl carbon (C-3) indicating that the broken bond of open ring A occurred between C-3 and
27 C-4, which was further confirmed by the presence of the HMBC correlations of H₂-1 and H₂-2 to
28 the ¹³C NMR signal at δ_{C} 176.6.
29

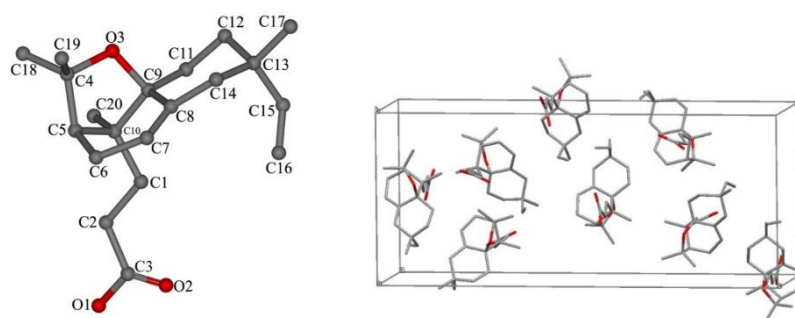
30 3.3. Determination of the absolute configuration of fladin A (**1**) 31

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

35 To determine the absolute configuration, **1** was crystallized in MeOH to afford a colorless

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

11



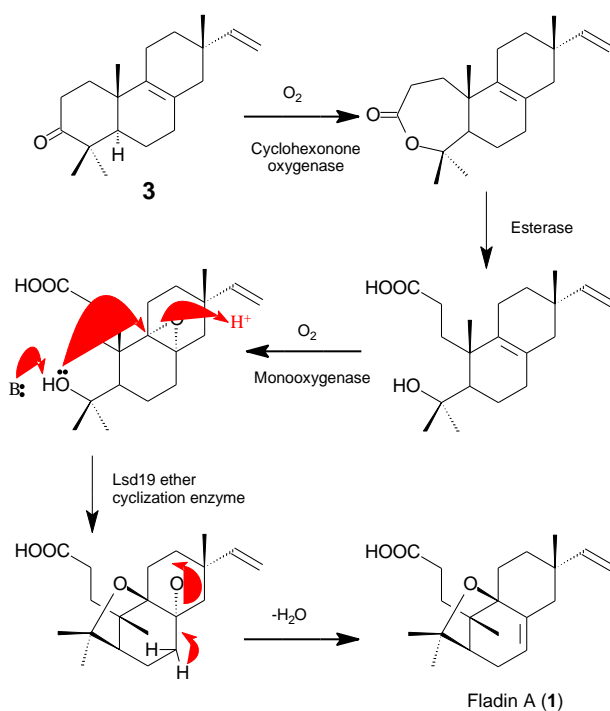
12

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

14

15 3.4. Plausible biogenetic pathway of fladin A (**1**)

16



17

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

19

1 Fladin A (**1**) is a unique diterpenoid with a tetrahydrofuran group between C4 and C9. It may
2 be derived from isopimarane diterpenes, which are found abundant in *Isodon* plants (Sun et al.,
3 2006; Jiang et al., 2000; Zhang and Sun, 1989). A plausible biogenetic pathway for **1** originating
4 from the precursor of the natural compound 11b-hydroxy-8, 15-isopimaradiene-3-one (**3**) is
5 proposed (Fig.4) (Jiang et al., 2000; Kenmoku et al., 2004). The enzymatic Baeyer-Villager type
6 oxidation of the ketone group in **3** inserts an oxygen atom between C-3 and C-4, leading to the
7 formation of a seven-membered ring lactone (Abril et al., 1989), which is hydrolyzed by esterase to
8 yield a diterpene carboxylic acid with a ring A opening. The double bond between C-8 and C-9 in
9 the diterpene is transformed by monooxygenase to an epoxide, which is further converted to a
10 tetrahydrofuran product via an epoxide-opening reaction catalyzed by the enzyme Lsd19 (Hotta et
11 al., 2012). Loss of a H₂O molecule of the tetrahydrofuran product produces fladin A (**1**).
12

13 3.5. Antifungal activity of fladin A (**1**) and lophanic acid (**2**) 14

15 Lophanic acid (**2**) was obtained as colorless crystals. The compound is a known abietane-type
16 diterpenoid, which was reported previously from *I. lophanthoides* (Wang et al., 1995). The two
17 isolated compounds (**1** and **2**) were evaluated for their cytotoxicity against the HCT116 colon
18 human tumor cell line. No cytotoxicity was observed for the compounds against these cell lines at a
19 concentration of 200 µg/mL, indicating the low toxicity of these compounds. The two compounds
20 were then tested for their antifungal potential against the athlete's foot fungus *T. rubrum*. Lophanic
21 acid (**2**) showed antifungal activity against *T. rubrum* with an MIC value of 7.8 µg/mL (Table 2).
22 Considering the diterpenoid as a major constituent in *I. flavidus* (0.3 %) and its antifungal potency,
23 lophanic acid (**2**) could well be a biologically active constituent for the plant *I. flavidus*, which has
24 been used by Miao people as an herbal medicine for treatment of fungal infections of the skin of the
25 foot. The new compound fladin A (**1**) also showed inhibition activity against *T. rubrum* with a MIC
26 value of 62.5 µg/mL. The two isolates (**1** and **2**) were further evaluated for their antimicrobial and
27 anti-biofilm potential against the dental pathogens *Streptococcus mutans*, *Porphyromonas*
28 *gingivalis* and *Candida albicans*. In comparison with the athlete's foot fungus, both compounds
29 showed less inhibitory activity against the dental fungus (*C. albicans*), indicating the strong
30 selective inhibitory activity of the compounds toward the athlete's foot fungus. The compounds
31 also displayed moderate inhibitory activity against the oral bacteria (*S. mutans* and *P. gingivalis*)
32 (Table 2). Fladin A (**1**) and lophanic acid (**2**) were further found to be able to breakdown the formed
33 biofilm of *C. albicans*. Fladin A (**1**) caused more than 30% breakdown of the biofilm at the
34 concentrations of 1000 and 250 µg/mL, but lophanic acid (**2**) showed less biofilm breakdown
35 activity with only about 26% breakdown of the biofilm at a concentration of 1000 µg/mL. No
36 biofilm breakdown activity against the oral bacteria was observed for **1** and **2**. These results suggest
37 that the compounds have preferential antifungal activity, and may be the basis for how the Miao
38 have come to view *I. flavidus* as a medicinal plant that defends against athlete's foot disease. Since
39 the compounds have very different chemical structures from those of the antifungal drugs in the
40 market, they may present a unique antifungal mechanism of action. Further chemical and
41 pharmacological studies including structural modification may advance these types of compounds
42 for further development as unique antifungal drug candidates.
43

44 **Table 2.**

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

	Growth inhibition (MIC: µg/mL)				Biofilm breakdown (% at 1000/250/62.5 µg/mL)		
	<i>Sm</i>	<i>Pg</i>	<i>Ca</i>	<i>Tr</i>	<i>Sm</i>	<i>Pg</i>	<i>Ca</i>
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.

3

4 **4. Conclusions**

5

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

15

16 **Conflict of interest**

17

18 The authors declare no conflict of interest.

19

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21

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27

28 **Appendix A. Supplementary material**

29

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

31

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