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Short communication

Biotransformation of pseudolaric acid B by *Chaetomium globosum*

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ABSTRACT

Pseudolaric acid B (**1**) is a natural product with potent antifungal activity. We discovered that pseudolaric acid B did not kill but only suppress the growth of the filamentous fungus *Chaetomium globosum*. It was proposed that pseudolaric acid B was converted to metabolites with decreased antifungal activities. In this study, a scaled-up biotransformation of pseudolaric acid B by *C. globosum* produced five metabolites, including three new compounds, pseudolaric acid I (**2**), pseudolaric acid B 18-oyl-alanine (**4**) and pseudolaric acid B 18-oyl-serine (**6**), together with two known compounds, pseudolaric acid F (**3**) and pseudolaric acid B 18-oyl-glycine (**5**). The structures were characterized by NMR and MS spectroscopy. The major biotransformation reaction was conjugation with amino acids. None of the metabolites showed inhibitory effects on the growth of *Candida albicans*. The results suggested that biotransformation might be a detoxification process for fungi to resist antifungal drugs.

1. Introduction

Pseudolaric acid B (**1**) is a diterpene isolated from the medicinal plant *Pseudolarix kaempferi* Gordon (Pinaceae family) [1]. Pharmacological studies have demonstrated that **1** displays potent biological activities, including antifungal, anticancer, and antifertility [2–8]. Particularly, **1** is considered as a promising antifungal drug candidate. In our research on its antifungal mechanism, we discovered that **1** did not kill certain filamentous fungi, but only inhibited their growth. Similar phenomenon had been reported by Aleu et al. who revealed that structural modification through biotransformation was a detoxification mechanism for fungi to resist antifungal drugs [9]. Bearing this in mind, we tried to find out whether pseudolaric acid B could be metabolized by the filamentous fungi.

In this work, the biotransformation of pseudolaric acid B by *C. globosum* IFFI 2445 was studied for the first time. Antifungal activities of the biotransformed metabolites against *Candida albicans* were also evaluated.

2. Methods

2.1. General

Melting points were determined on an X-4 apparatus and were uncorrected. Optical rotations were measured with a Rudolph Autopol III polarimeter. UV spectra were recorded on a TU-1901 UV-Vis spectrophotometer. IR spectra were obtained on a NEXUS-470 FT-IR spectrophotometer. NMR spectra were recorded on Bruker Avance III spectrometer (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR) in $CDCl_3$ at room temperature with TMS as the internal standard. High-resolution mass spectra (HR-MS) were obtained on a Bruker Apex IV FT-MS spectrometer. Chemical solvents for column chromatography were analytical grade or higher.

2.2. Chemicals

Pseudolaric acid B (**1**) was isolated as white amorphous powder from the root bark of *Pseudolarix kaempferi* by the authors [1]. The purity was above 95% determined by HPLC–DAD analysis.

2.3. Microorganism and culture medium

The fungal strains were purchased from China General Microbiological Culture Collection Center (Beijing, China). A total of twelve strains were used for the screening test, including *Absidia coerulea* AS 3.3389, *Alternaria alternata* AS 3.4578, *Aspergillus niger* AS 3.795, *C. globosum* IFFI 2445, *Crebrothecium ashbyii* ACCC 2114, *Cunninghamella elegans* AS 3.1207, *Fusarium avenaceum* (Corda ex Fries) Saccardo AS 3.4594, *Gibberella fujikuroi* var. *fujikuroi* AS 3.4748, *Mucor spinosus* van Tieghem AS 3.3450, *Rhizopus chinensis* IFFI 3043, *Saccharomyces cerevisiae* ACCC 2168, and *Syncephalastrum racemosum* AS 3.264. Fermentations were carried out in potato medium consisting of 100 g of potato extract, 20 g of glucose, and 1000 mL of distilled water. The media were sterilized in an autoclave at 121 °C and 1.06 kg/cm^2 for 30 min.

2.4. Preliminary screening tests

The fungi (3 days old) were inoculated from agar slants, and were incubated in 250 mL Erlenmeyer flasks containing 100 mL of liquid medium at 25 °C on a rotary shaker (120 rpm). It was allowed to grow for 48 h until the exponential phase was reached, when 2 mg of pseudolaric acid B (**1**, 2 mg/mL in ethanol) was added. After five days of incubation, the cultures were pooled and filtered, and extracted with an equal volume of ethyl acetate. The extract was evaporated to dryness in a

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Table 1
^{1}H NMR spectral data of pseudolaric acid B (**1**) and metabolites **2**, **4** and **6**.

Position	**1**	**2**	**4**	**6**
1a	1.82	2.05	1.83	1.80
1b	1.79	1.91	1.79	1.78
2a	1.83	2.09	1.79	1.82
2b	1.73	1.90	1.76	1.80
3	3.31 d (4.4)	3.54 d (4.8)	3.31 d (3.3)	3.30 d (3.6)
5a	3.08 dd (16.0, 6.4)	2.73	3.09 dd (14.5, 5.6)	3.09 dd (15.5, 6.3)
5b	1.74	2.18	1.75	1.74
6a	2.89 dd (16.0, 6.4)	2.81 dd (11.0, 6.0)	2.90 dd (14.5, 5.6)	2.89 dd (15.5, 6.3)
6b	2.17	2.06	2.14	2.16
8	7.20	7.19	7.21	7.20
9a	2.76 dd (15.1, 8.8)	2.74	2.76 dd (15.0, 8.9)	2.75 dd (15.0, 8.9)
9b	2.61	2.60	2.63	2.62
13	5.93 d (15.0)	6.25 d (15.5)	5.89 d (15.0)	5.90 d (15.0)
14	6.60 dd (15.0, 11.5)	6.60 dd (15.5, 11.6)	6.52 dd (15.0, 11.5)	6.56 dd (15.0, 11.5)
15	7.27 d (11.5)	7.24	7.01 d (11.5)	7.01 d (11.5)
1′	–	–	4.64 brs	4.67 brs
2′a	–	–	1.52 d (6.0)	4.12
2′b	–	–	–	3.95
12-CH_3	1.60 s	1.47 s	1.59 s	1.59 s
17-CH_3	1.96 s	1.98 s	2.00 s	1.99 s
CH_3CO	2.13 s	2.14 s	2.14 s	2.13 s
CH_3O	3.72 s	3.71 s	3.73 s	3.73 s
NH	–	–	6.45 brs	5.83 brs

Note: Chemical shifts are displayed in δ (ppm) recorded in $CDCl_3$ at 400 MHz; coupling constants in Hz are given in parentheses. Those peaks whose multiplicities were not designated are multiplets (m).

rotary evaporator under reduced pressure, and the residue was dissolved in 1 mL of methanol and filtered through 0.45 μm micropore membranes for HPLC analysis. Culture controls consisted of fermentation blank in which microorganisms were grown without substrate but were fed with the same amount of ethanol. Substrate controls contained the sterile fermentation medium with the same amount of substrate.

2.5. HPLC analysis

Samples were analyzed on an Agilent 1100 series HPLC instrument equipped with a quaternary pump, a diode-array detector, an autosampler, and a column compartment. An Agilent Zorbax SB C18 column (4.6 mm × 250 mm, 5 μm) was used. The mobile phase consisted of methanol (A) and water containing 0.01% (v/v) formic acid (B). The following gradient elution program was used: 45–60% A for 0–25 min, 60–70% A for 25–40 min, and 70–80% A for 40–50 min. The flow rate was 1.0 mL/min; the detection wavelength was 262 nm; column temperature was 30 °C.

2.6. Preparative HPLC conditions

Reversed-phase preparative HPLC was performed on a TSP P100 SpectraSeries HPLC system (Thermo, USA) connected to a UV detector, using a Zorbax SB C_{18} column (250 mm × 10.0 mm, 5 μm; Agilent).

2.7. Preparative-scale biotransformation of pseudolaric acid B (*1*)

The fermentation procedure was carried out in 1000 mL Erlenmeyer flasks containing 400 mL of medium on rotary shakers (120 rpm, 25 °C). Pseudolaric acid B (**1**, 800 mg) was dissolved in ethanol to make a concentration of 10 mg/mL. After 48 h pre-culture, each flask was added with 1 mL of the substrate solution. The fermentation continued for 7 days. Then the culture supernatant was extracted with ethyl acetate (1:1, v/v) for four times to obtain a crude extract (4.0 g).

2.8. Isolation of biotransformed metabolites

The crude residue was applied to a silica gel column (150 g, 200–300 mesh, ϕ3 × 25 cm) and eluted with chloroform–methanol to afford twelve fractions. Fr. 6 [chloroform–methanol (50:1, v/v), 128 mg] was subjected to preparative HPLC and eluted with methanol–water (55:45, v/v) to give **2** (15 mg). Fr. 7 [(30:1), 426 mg] was purified by preparative HPLC and eluted with methanol–water (45:55, v/v) to give **3** (8 mg). Fr. 8 [(10:1), 187 mg] was subjected to preparative HPLC and eluted with methanol–water (50:50, v/v) to give **4** (25 mg). Fr. 10 [(10:1), 255 mg] was subjected to preparative HPLC and eluted with acetonitrile–water (38:62, v/v) to give **5** (40 mg). Fr. 12 [(0:100), 1.5 g] was dissolved in methanol (10 mL) and eluted through a Sephadex LH-20 column to give ten fractions (Fr. T1-T10). Fr. T5 (130 mg) was subjected to preparative HPLC and eluted with methanol–water (45:55, v/v) to give **6** (6 mg).

Pseudolaric acid I (**2**): white powder (15 mg, 1.9% yield); m.p. 112–114 °C; $[\alpha]_D^{31}$ +57.9 (*c* 0.13, MeOH); UV (MeOH) λ_{max} nm: 199, 261 (log ε 4.54, 4.20); IR (KBr) ν_{max} cm^{-1}: 3451, 3000, 1710, 1641, 1440, 1384, 1243, 1198, 1018, 950; ^{1}H and ^{13}C NMR data, see Tables 1 and 2, respectively; HR-ESI-MS: *m/z* 450.2121 $[M+NH_4]^+$ (calcd. for $C_{23}H_{32}NO_8^+$; 450.2122).

Pseudolaric acid B 18-oyl-alanine (**4**): white powder (25 mg, 3.1% yield); m.p. 107–109 °C; $[\alpha]_D^{31}$ +7.5 (*c* 0.08, MeOH); UV (MeOH) λ_{max} nm: 199, 256 (log ε 4.51, 4.07); IR (KBr) ν_{max} cm^{-1}: 3398, 2920, 2851, 1739, 1646, 1527, 1445, 1374, 1207, 1176, 1074, 890; ^{1}H and ^{13}C NMR data, see Tables 1 and 2, respectively; HR-ESI-MS: *m/z* 502.2090 $[M-H]^-$ (calcd. for $C_{26}H_{32}NO_9^-$; 502.2083).

Pseudolaric acid B 18-oyl-serine (**6**): white powder (6 mg, 0.8% yield); m.p. 108–110 °C; $[\alpha]^{31}{}_D$ +72.2 (*c* 0.03, MeOH); UV (MeOH) λ_{max} nm: 199, 256 (log ε 4.48, 4.20); IR (KBr) ν_{max} cm^{-1}: 3396, 2919, 2850, 1739, 1648, 1527, 1464, 1370, 1243,

Table 2
^{13}C NMR spectral data of pseudolaric acid B (**1**) and metabolites **2**, **4** and **6**.

Position	**1**	**2**	**4**	**6**
1	33.3	34.0	33.3	33.3
2	24.3	21.7	24.3	24.3
3	49.2	49.4	49.3	49.3
4	90.1	90.1	90.4	90.1
5	30.7	31.2	30.7	30.7
6	20.1	20.0	20.1	20.1
7	134.5	134.7	134.5	134.5
8	141.7	141.6	141.7	141.7
9	27.7	27.5	27.8	27.7
10	55.2	55.7	55.3	55.3
11	83.7	83.4	83.9	84.0
12	28.4	29.3	28.5	28.4
13	144.5	146.4	144.0	143.7
14	121.7	124.1	121.2	121.2
15	138.7	139.1	135.0	134.7
16	127.8	127.6	129.5	129.4
17	12.6	12.5	12.9	12.9
18	169.4	172.0	169.7	170.6
19	168.0	168.1	168.0	168.2
20	173.1	172.9	173.3	173.4
1′	–	–	48.9	55.0
2′	–	–	17.8	62.0
3′	–	–	175.6	172.5
$CH_3\underline{C}O$	172.8	169.6	170.0	169.5
$\underline{C}H_3CO$	21.7	21.8	21.7	21.7
$\underline{C}H_3O$	52.0	52.1	52.1	52.1

Note: Chemical shifts are displayed in δ (ppm) recorded in $CDCl_3$ at 100 MHz.

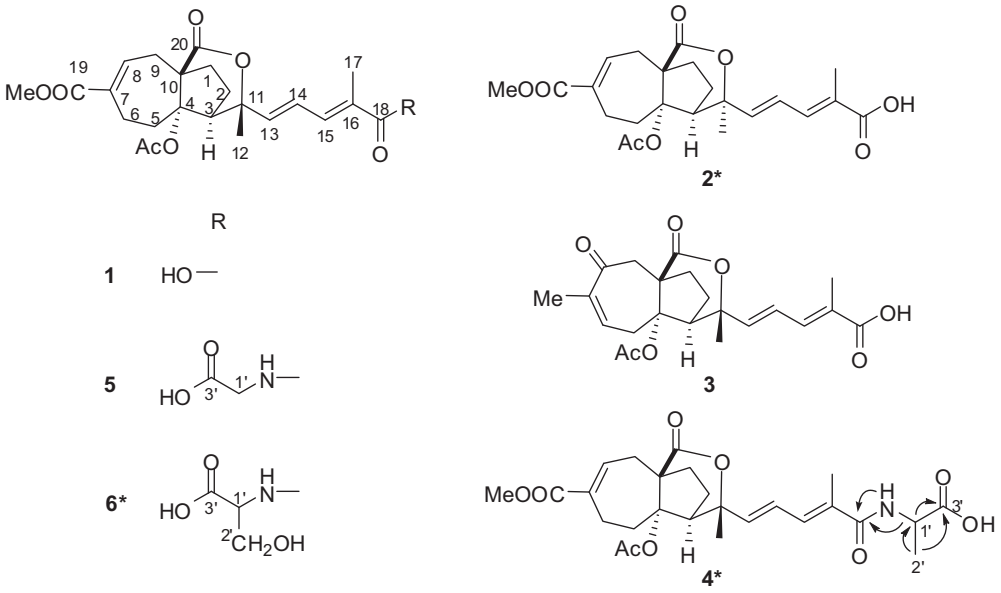

Fig. 1. Biotransformation of pseudolaric acid B (**1**) by *Chaetomium globosum* (* new compound). Arrows indicate ^{1}H–^{13}C HMBC correlations for compound **4**.

1168, 1073, 770; ^{1}H and ^{13}C NMR data, see Tables 1 and 2, respectively; HR-ESI-MS: *m/z* 520.2166 $[M+H]^+$ (calcd. for $C_{26}H_{34}NO_{10}^+$; 520.2177).

2.9. Antifungal assay

Compounds **1**–**6** were tested for antifungal activity using a modified NCCLS method [10]. *Candida albicans* ATCC10231 was grown overnight in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) at 37 °C, the cells were diluted to approximately 2.0×10^3 CFU/mL final concentration with fresh broth. The stock solution of compounds was prepared in DMSO at 20 mg/mL. The range of final concentration in the assay was 0.25–1000 μg/mL. The plates were incubated at 35 °C in a moist and dark chamber for 24 h. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of antifungal showing no visible growth.

3. Results and discussion

A total of 12 strains of filamentous fungi were screened for their capabilities to metabolize pseudolaric acid B (**1**). When pseudolaric acid B was added to the cultures, the growth of most strains slowed down or they even died. It was noteworthy that *C. globosum* IFFI 2445 and *Crebrothecium ashbyii* ACCC 2114 stopped growing for two days, and then the mycelia continued growing. By TLC analysis, we found that new metabolites were produced in the culture media. Substrate control and culture control were set to ensure that the detected products were true biocatalytic metabolites. *C. globosum* and *C. ashbyii* produced similar metabolites, and the efficiency of *C. globosum* was higher. Therefore, we conducted a preparative scale biotransformation reaction with *C. globosum*. After 7 days of incubation, five pure products were isolated from the culture supernatant. Their structures were respectively identified as pseudolaric acid I (**2**), pseudolaric acid F (**3**) [11], pseudolaric acid B 18-oyl-alanine (**4**), pseudolaric acid B 18-oyl-glycine (**5**) [4] and pseudolaric acid B 18-oyl-serine (**6**) on the basis of their NMR and MS spectroscopy (Fig. 1). The ^{1}H and ^{13}C NMR spectral data are given in Tables 1 and 2, respectively.

3.1. Structural elucidation by spectroscopic analyses

Compound **2** was obtained as a white power. Its molecular formula of $C_{23}H_{28}O_8$ was established from HR-ESI-MS ($[M+NH_4]^+$, found *m/z* 450.2121, calcd. 450.2122). Detailed analysis of its NMR spectra revealed that **2** had the same planar structure as **1**. In addition, their ^{1}H and ^{13}C NMR spectral data were almost the same. The only differences were H-2a (δ 1.83 in **1** vs δ 2.09 in **2**), H-3 (δ 3.31 in **1** vs δ 3.54 in **2**), H-12 (δ 1.60 in **1** vs δ 1.47 in **2**), and H-13 (δ 5.93 in **1** vs δ 6.25 in **2**). By analyzing a computer-generated 3D structure, we found the differences were due to the stereochemistry of C-12 (Fig. 2). The diagnostic NOE effects of H-12 with H-2a (δ 2.09) and H-3 (δ 3.54) further confirmed the α-configuration of C-12. Thus, the structure of **2** was established as pseudolaric acid I.

Compound **4** was obtained as a white power. Its molecular formula of $C_{26}H_{33}NO_9$ was established from HR-ESI-MS ($[M-H]^-$, found *m/z* 502.2090, calcd. 502.2083). The IR spectrum showed absorption bands at 3398 and 1647 (amide) cm^{-1} indicating that an amide group was introduced into the molecule. Detailed analysis of 1D and 2D NMR spectra revealed that compound **4** was composed of two parts: partial structure **R** and the substrate **1**. In the partial structure **R**, the ^{1}H NMR spectrum displayed a broad one-proton singlet at δ 6.45, which disappeared by adding one drop of deuterated water. It should be a nitrogen-bearing proton. In the ^{13}C NMR spectrum, one methyl, one tertiary carbon and one carbonyl group appeared at δ 17.8, δ 48.9 and δ 175.6, respectively. The HMBC spectrum revealed the correlations of H-2′a (δ 1.52) with C-1′ (δ 48.9) and C-3′ (δ 175.6) (Fig. 1). Thus, the partial structure **R** was characterized as alanine. **R** was allowed to be linked to C-18 by the HMBC correlation between the amino hydrogen (δ 6.45) and C-18 (δ 169.7), as well as H-1′ (δ 4.46) and C-18 (δ 169.7). On the basis of

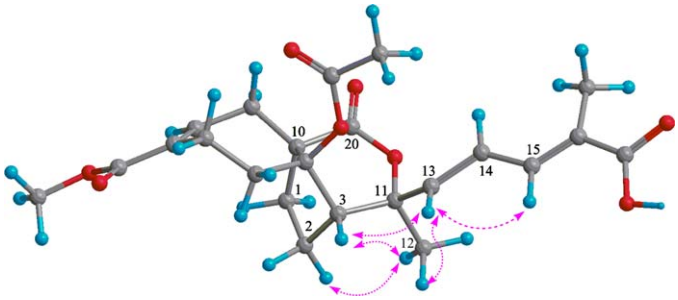

Fig. 2. Key NOE (H ↔ H) correlations for compound **2**. The 3D structure of compound **2** was obtained by ChemDraw 3D Ultra V 11.0 with MM2 forcefield calculations for energy minimization. Double-headed arrows indicated important NOE correlations for compound **2**.

Table 3
MIC values for compounds **1**–**6** on *Candida albicans* ATCC10231.

Compound	MIC (μg/mL)
1	2
2	>1000
3	>1000
4	>1000
5	>1000
6	250

Note: MIC, minimal inhibitory concentration.

the above analysis, compound **4** was identified as pseudolaric acid B 18-oyl-alanine.

Compound **6** was obtained as a white power. Its molecular formula of $C_{26}H_{33}NO_{10}$ was established from HR-ESI-MS ($[M+H]^+$, found *m/z* 520.2166, calcd. 520.2177). Similar to **4**, the IR spectrum showed absorption bands at 3398 and 1647 (amide) cm^{-1}, which indicated that **6** was also an amide derivative of **1**. ^1H and ^{13}C NMR data of the nitrogen containing group were consistent with those of serine [12]. According to the HMBC correlations between the amino hydrogen (δ 5.83) and C-16 (δ 129.4), the serine group should be linked to C-18. Thus, the structure of **6** was identified as pseudolaric acid B 18-oyl-serine.

3.2. Antifungal activity

The pure compounds **1**–**6** were evaluated for antifungal activity against *Candida albicans*, which is a commonly seen pathogenic fungus. The minimal inhibitory concentration (MIC) values are listed in Table 3. Unfortunately, none of the metabolites showed significant antifungal activity. The inhibitory effect of compound **5** against the growth of *C. globosum* was also tested. Compound **5** showed remarkably weaker effects than pseudolaric acid B (data not shown).

4. Conclusions

Incubation of pseudolaric acid B in *C. globosum* yielded three new and two known metabolites. The biotransformation reactions involved amidation at C-18, epimerization of C-12, and migration of the double bond. Bioconversion into metabolites with decreased antifungal activities could be the reason why *C. globosum* resists pseudolaric acid B. This study represents the first report on microbial transformation of pseudolaric acid B.

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