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Antifungal Diterpenoids of *Pseudolarix kaempferi*, and Their Structure–Activity Relationship Study

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Abstract—The in vitro antifungal activities of 19 structurally diversified analogues of pseudolaric acids tested against the major pathogenic fungus *Candida albicans* has led to the establishment of a very clear structure–activity relationship of pseudolaric acids derivatives. Pseudolaric acid A was first found to be a potent antifungal component comparable with pseudolaric acid B. Among the tested 19 diterpenoids, pseudolaric acids A_2 (**1**), B_2 (**3**), B_3 (**4**) and methyl pseudolarate A_2 (**2**) are new isolates of the root bark of *Pseudolarix kaempferi*, and their structures were elucidated mainly by 2D-NMR techniques and chemical methods. Compounds **12**–**19** were first semi-synthesized by efficient routines from pseudolaric acid B.

Introduction

The root and trunk barks of *Pseudolarix kaempferi* Gordon (Pinaceae) distributes in the south China, known as 'Tu-Jin-Pi' in traditional Chinese medicine, have been used to treat skin diseases caused by fungal infections. The isolation of a number of pseudolaric acids analogous from the bark[1] and a few of cytotoxic triterpenoids from the seeds[2] has been reported. A bioassay-directed fractionation allowed to the isolation of pseudolaric acid B as the major antifungal principle of this plant.[3] Pseudolaric acid B, the best antifungal agent isolated from higher plants, showed potent antifugal activities against *Candida* and *Torulopsis* species. However, the further application of pseudolaric acid B as antifungal drug has not been progressed due largely to the inherent shortages, such as low solubility, latent structural instability and low biological availability, which made the application more difficult and gave low biological response in the in vivo tests. The promising in vitro antifungal activity of pseudolaric acid B and its potential value as an antifungal drug had driven us to conduct this study. There has been very limited study on structure–activity relationship (SAR) of antifungal pseudolaric acids analogous,[3] largely due to the protracted and complex nature of their structures for modification and synthesis. Fortunately, a series of pseudolaric acids derivatives with structurally diversified changes isolated from *P. kaempferi* in our laboratory[1h] has made the SAR study possible. Recently, another four new pseudolaric acids derivatives, pseudolaric acids A_2 (**1**), B_2 (**3**), B_3 (**4**) and methyl pseudolarate A_2 (**2**), have been further isolated from the same material. Pseudolaric acids B_2 and B_3 are novel degraded derivatives of pseudolaric acid B with shorten and saturated side chain. The structures of four new compounds **1**–**4** were elucidated mainly by spectral data and chemical evidences. For the SAR study, 19 compounds were involved, in which compounds **1**–**11** are naturally occurrence and compounds **12**–**19** are newly semi-synthesized ones by efficient routines. Except for only a few minor natural pseudolaric acid derivatives were not available, the most of the natural derivatives were applied in this SAR study (Fig. 1).

It is well known that fungal infection is one of the major factors causing the death of patients with immunocompromised problems, especially for the AIDS suffers. The most common pathogenic culprit *Candida albicans* causing *candidiasis* in AIDS patients was chosen to conduct our study. The antifungal MICs of compounds **1**–**19** against *C. albicans* are tested. Pseudolaric acid A is

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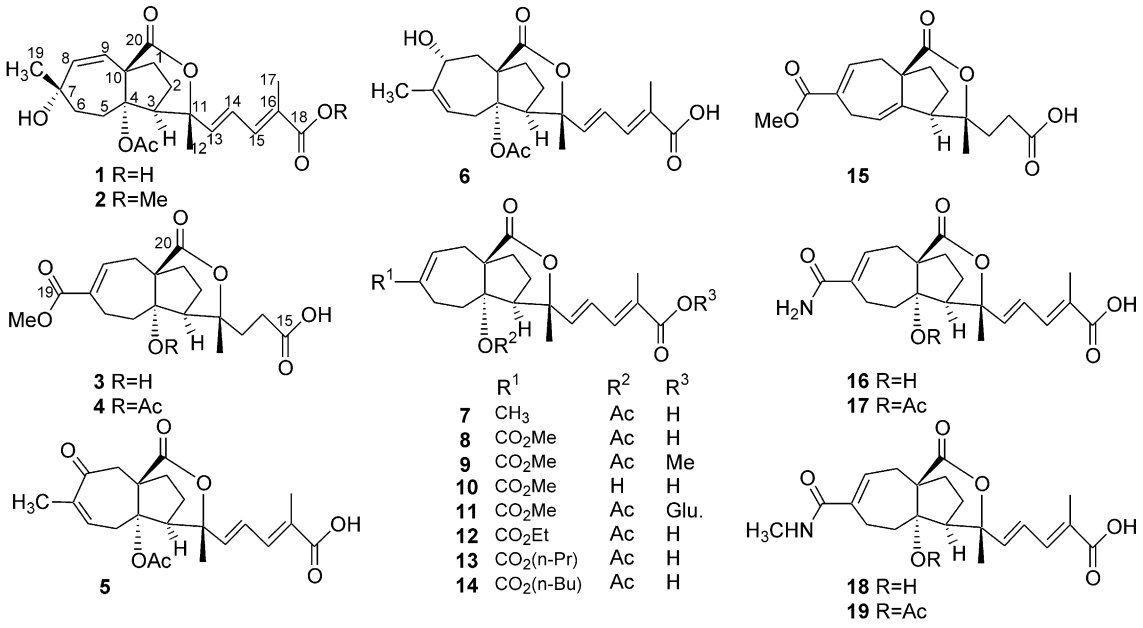


Figure 1.

first found to be a potent antifungal comparable with pseudolaric acid B. Besides the verification of the former observation that the free carboxylic acid group in the side chain and the C-4 acetyl group are necessary for antifungal activity,[3] some significant new findings of SAR study were obtained. We report herein the isolation of four new diterpenoids, pseudolaric acids A_2 (**1**), B_2 (**3**), B_3 (**4**) and methyl pseudolarate A_2 (**2**), and the in vitro antifungal activities of 19 pseudolaric acids analogues against *C. albicans* and their SAR study.

Results and Discussion

Isolation of compounds 1–11

Compounds **1**–**11** were successfully isolated from the ethanolic extract of the root bark of *P. kaempferi* by extensive column chromatography (the details see experimental).

Structural elucidation of new compounds 1–4

Psudolaric acid A_2 (**1**) was obtained as a quadratic crystal (in acetone). Its molecular formula was determined by HR-EIMS (*m*/*z* 386.1710, $C_{22}H_{26}O_6$ corresponding to $[M-H_2O]^+$, calcd 386.1729) and the negative mode ESI-MS at *m*/*z* 403 $[M-H]^-$. The ^1H NMR (Table 2) revealed the presence of five olefinic protons at δ 7.11, 6.38, 6.25, 5.66, and 5.17; two angular methyls at δ 1.06 and 1.58 (each 3H, s); a vinylic methyl at δ 1.83 and a methyl of acetyl at δ 2.07. Two typical ABq olefinic proton signals at δ 5.66 (d, $J=12.6$ Hz) and δ 5.17 (d, $J=12.6$ Hz) suggested the occurrence of a disubstituted double bond. All 22 carbons signals were resolved in the ^{13}C NMR spectrum corresponding to 22 atoms in the molecule, and were distinguished as methyls, secondary carbons, tertiary carbons and quaternary carbons by DEPT techniques.

A direct comparison of the spectral data of **1** with those of pseudolaric acid A (**7**)[1a,1b] which was also obtained from this plant, indicates that **1** is an analogue of pseudolaric acid A, two compounds possess the same side chain, and the only structural changes are that compound **1** has one more hydroxyl group linked to a sp^3 quaternary carbon and a disubstituted double bond instead of the Δ^7 trisubstituted double bond in the seven-membered ring. The assignments of the one more hydroxyl group and the location of disubstituted double bond therefore become to the main issues for the structure elucidation of **1**. All the protons were allocated to the corresponding carbons by the correlations appeared in the HMQC spectrum. An excellent HMBC spectrum was performed as shown in Figure 2a to outline the structure of **1**. The sp^3 quaternary carbon signal at δ 72.6 was assigned to C-7 bearing a hydroxyl judged from the strong correlation with the CH_3-19 at δ 1.06.

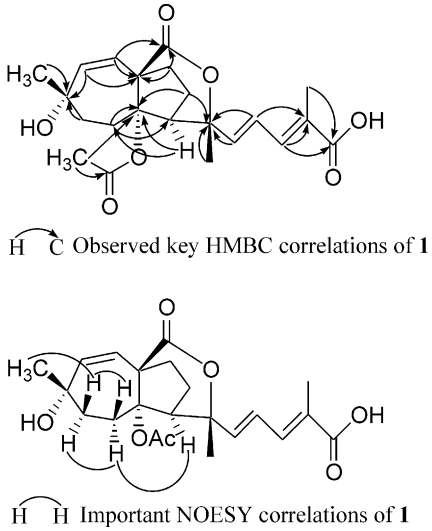


Figure 2.

One of the olefinic protons at δ 5.66 (d, $J = 12.6$ Hz) correlating with C-7, C-19 and C-10 was assigned to the H-8, suggesting the presence of Δ^8 double bond. The other olefinic proton at δ 5.17 (d, $J = 12.6$) coupled with H-8 was attributable to the H-9 by the correlations with C-7, C-10 and C-20, confirming the occurrence of Δ^8 double bond. The coupling constant between H-8 and H-9 ($J = 12.6$ Hz) indicated the double bond adopted a *Z*-geometry. The planar structure of **1** was assigned and confirmed by the typical EI–MS fragmentation patterns of pseudolaric acid A analogues as reported in the literature.[4]

The relative stereochemistry of the 7-CH_3 was fixed by the correlations observed in NOESY spectrum (Fig. 2b). The key NOE cross-peaks between the proton pairs H-3α/H-5α, H-5α/H-6α, H-5β/H-6β and H-6β/CH_3-7, clearly indicated the 7-CH_3 took a β-orientation. Therefore, the structure of psudolaric acid A_2 was established to be **1**.

Methyl pseudolarate A_2 (**2**) was isolated as a gum. The ^{1}H NMR (Table 2) of **2** exhibited five singlet methyls at δ 3.73, 2.07, 1.95, 1.68 and 1.21, and five olefinic protons at δ 7.21, 6.59, 6.26, 5.78, and 5.26. Its ^{13}C NMR (Table 1) displayed resonances of 23 carbon signals. The spectral data of **2** was very similar to that of pseudolaric acid A_2 (**1**), except for one more methoxyl group (δ_H 3.73, δ_C 52.0) in **2**. The down-field shifted proton signal of methoxyl group caused by the deshielding effect of carboxylic group was indicative of the presence of a 18-OMe, which was much like the case of a 18-OMe in methyl pseudolaric acid B.[1h,3] The structure of compound **2** was thus proposed to be the methyl ester of **1,** namely methyl pseudolarate A_2, and was confirmed by the methylation[1a] of pseudolaric acid A_2 (**1**) with CH_2N_2[5] to offer compound **2**.

Table 1. ^{13}C NMR (with DEPT) data of the new compounds **1–4**

Position	**1**[a]	**1**[b]	**2**[c]	**3**[d]	**4**[b]
1	34.6	34.7	35.5	35.1	33.6
2	24.3	24.4	24.9	19.7	22.0
3	49.1	49.1	50.1	54.2	49.8
4	88.4	88.6	89.3	80.1	90.6
5	28.4	28.4	29.6	22.0	30.8
6	35.6	35.7	36.2	33.7	20.3
7	72.6	74.3	73.7	134.0	134.4
8	141.8	140.2	141.8	143.1	141.8
9	122.4	122.9	123.2	27.3	27.9
10	57.6	57.7	58.5	54.7	55.2
11	84.0	84.0	84.5	82.7	82.5
12	28.4	28.2	28.3	26.2	26.3
13	145.0	144.2	145.7	37.2	36.9
14	121.2	121.8	121.9	28.6	28.4
15	136.5	138.5	137.7	175.4	177.0
16	128.6	128.0	128.5		
17	13.0	12.6	12.8		
18	169.4	169.9	168.9		
19	27.2	27.1	27.2	168.6	168.1
20	172.8	173.1	173.5	175.1	173.1
COMe	169.9	172.9	170.5		169.4
COMe	21.8	21.7	21.5		21.8
OMe			52.0	51.9	52.0

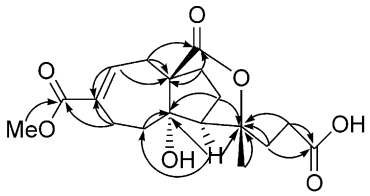
[a]Measured in DMSO-d_6.
[b]Measured in $CDCl_3$.
[c]Measured in acetone-d_6.
[d]Measured in $CDCl_3 + CD_3OD$.

Pseudolaric acid B_2 (**3**) was needle crystal (in acetone). Its molecular formula was established to be $C_{18}H_{24}O_7$ on the basis of HR-EIMS (*m*/*z* 352.1517, calcd 352.1522). ^{13}C NMR (Table 1) with DEPT experiments of **3** showed a total of 18 carbons signals corresponding to 18 carbon atoms. Two singlet methyls at δ 3.65 and 1.40 observed in the ^{1}H NMR (Table 2) suggested the presence of one methoxyl group and one methyl group, respectively. Comparison of the spectral data of **3** with those of pseudolaric acid C[3] (**10**), inferred **3** was most likely a degraded derivative of pseudolaric acid C with a shortened side chain. The molecular formula ($C_{18}H_{24}O_7$) of **3** revealed three carbon atoms less than that of pseudolaric acid C. Three carboxyls at δ 175.4, 175.1 and 168.6 were appeared in the ^{13}C NMR, except for the C-19 and C-20 carboxyl esters, there was one more carboxyl in the molecule. In the ^{1}H NMR and ^{13}C NMR spectra, no olefinic signal on the side chain was observed, suggesting the presence of a $-CH_2CH_2CO_2H$ side chain. The structure of pseudolaric acid B_2 was therefore elucidated to be **3**. The complete assignments of the proton and carbon signals were carried out by HMQC spectrum and comparison with the spectral data of pseudolaric acid C. The structure of **3** was further confirmed by an outstanding run of HMBC (Fig. 3).

Pseudolaric acid B_3 (**4**) was obtained as amorphous powder. The ^{1}H NMR (Table 2) and ^{13}C NMR (Table 1) spectra displayed the presence of an acetyl (δ_H 2.13 s, δ_C 21.8, 169.4), a methoxyl (δ_H 3.70 s, δ_C 52.0), a methyl (δ_H 1.50 s, δ_C 26.3) and a trisubstituted double bond (δ_H 7.17, δ_C 141.8 and 134.4). ^{13}C NMR and DEPT spectra exhibited the characteristic signals of four carboxyls (δ 177.0, 173.1, 169.4, and 168.1) and three sp^3 quaternary carbons at δ 90.6, 82.5 and 55.2. The spectral data of compound **4** was very similar with those of **3**, except for the presence of one more acetyl, suggesting that compound **4** was possibly an 4-acetyl derivative of **3**. Treatment of pseudolaric acid B_2 (**3**) with acetyl chloride at room temperature for overnight yielded the 4-acetyly derivative of **3** whose ^{1}H NMR and ^{13}C NMR spectral data were in good agreement with those of **4**. The structure of pseudolaric acid B_3 was unambiguously demonstrated to be **4**.

Semi-synthesis of compounds 12–19

Treatment of pseudolaric acid B (**8**) with ethanol, *n*-propanol and *n*-butanol respectively under alkaline condition to give the corresponding C-4 deacetylated



H C Observed key HMBC correlations of **3**

Figure 3.

Table 2. ^{1}H NMR data of the new compounds **1–4**

Position	1[a]	2[c]	3[d]	4[b]
1	1.96 (dd, 13.0, 2.3) 1.88 (m)	2.07 (dd, 13.0, 2.0) 1.97 (dd, 10.0, 4.1)	1.98 (m) 1.78 (m)	1.97 (m) 1.86 (m)
2	1.78 (dd, 11.7, 5.8) 1.65 (m)	1.89 (dd, 11.2, 5.4) 1.85 (m)	2.67 (m) 2.56 (m)	1.91 (m) 1.78 (m)
3	3.17 (d, 5.8)	3.30 (d, 5.7)	2.06 (d, 5.8)	3.22 (d, 5.3)
5	2.91 (14.3, 5.6) 1.88 (m)	3.07 (ddd, 14.7, 6.3, 1.9) 2.06 (m)	2.15 (m) 1.79 (m)	3.05 (dd, 14.1, 6.2) 1.70 (t, 12.8)
6	1.65 (m) 1.53 (dd, 13.8, 5.4)	1.83 (m) 1.71 (m)	1.81 (2H, m)	2.87 (dd, 15.5, 6.2) 2.14 (m)
8	5.66 (d, 12.6)	5.78 (dd, 12.5, 1.5)	7.14 (br.s,)	7.17 (m)
9	5.17 (d, 12.6)	5.26 (d, 12.5)	2.58 (br.d, 11.7) 2.53 (br.d, 11.7)	2.74 (dd, 15.1, 8.8) 2.60 (dd, 15.1, 2.3)
12	1.58 (3H, s)	1.68 (3H, s)	1.40 (3H, s)	1.50 (3H, s)
13	6.25 (d, 15.2)	6.26 (d, 15.1)	1.98 (m) 1.81 (m)	2.04 (m) 1.85 (m)
14	6.38 (dd, 15.2, 11.1)	6.59 (dd, 15.1, 11.4)	2.34 (2H, m)	2.52 (ddd, 16.3, 10.7, 5.2) 2.40 (ddd, 16.3, 10.7, 5.2)
15	7.11 (d, 11.1)	7.21 (dm, 11.4)		
17	1.83 (3H, s)	1.95 (3H, d, 1.2)		
19	1.06 (3H, s)	1.21 (3H, s)		
COMe	2.07 (3H, s)	2.07 (3H, s)		2.13 (3H, s)
OMe		3.73 (3H, s)	3.65 (3H, s)	3.70 (3H, s)

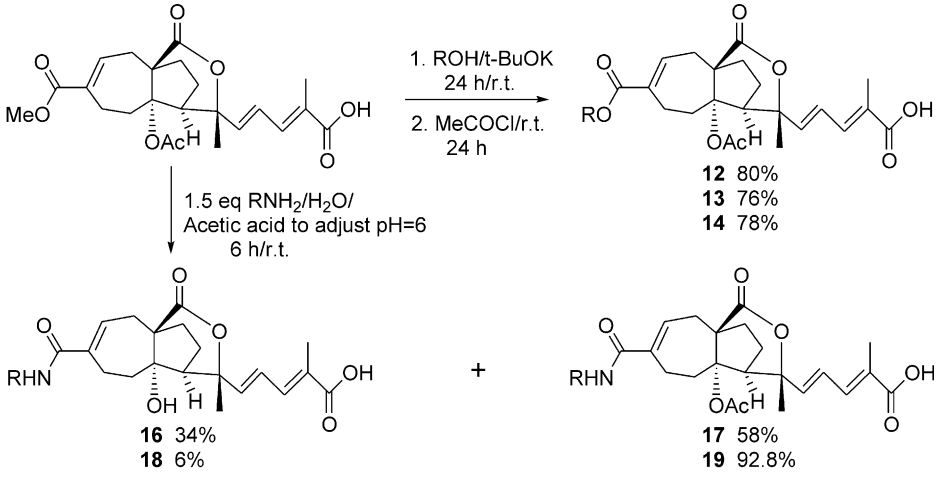
[a]Measured in DMSO-d_6.
[b]Measured in $CDCl_3$.
[c]Measured in acetone-d_6.
[d]Measured in $CDCl_3 + CD_3OD$.

compounds of **12–14**, which were then acetylated in one-pot reaction to offer compounds **12–14**, respectively, in good yield (Scheme 1). Dehydration of pseudolaric acid B_2 (**3**) with pyridine and thionyl chloride afforded compound **15**.[1a] Compounds **16–19** were obtained via the aminolysis of pseudolaric acid B in a buffer (acetic acid and amine, the pH of aqueous solution is about 6).

Antifungal evaluation and structure–activity relationship study

For antifungal activity evaluation and structure–activity relationship study, 19 compounds were applied in this practice, in which compounds **1–11** are naturally occurrence and compounds **12–19** are semi-synthesized ones. Compounds **1–4** are newly isolated novel chemical entities from the same material of *P. kaempferi*. The antifungal MICs of compounds **1–19** against *C. albicans* are listed in Table 3. The fungal strain *C. albicans* ATCC 1600 was from the American Type Culture Collection, and the antifungal activity tests were carried out by microdilution assay,[6] and amphotericin B was utilized as positive control.

In our tests, pseudolaric acid A (**7**) was first found to be a very active compound against *C. albicans* at the MIC of 6.25 μg/mL. The antifungal activities of pseudolaric acid B against several fungal strains, including *C. albicans* were reported before.[1g,3] Pseudolaric acid B (**8**) displayed more potent activity at the MIC of 3.12 μg/mL, 2-fold stronger than pseudolaric acid A against *C. albicans* in our tests. Both pseudolaric acids A (**7**) and B (**8**) have nearly identical basic skeleton, the only struc-



Scheme 1.

Table 3. Antifungal activities of compounds **1–19**

Compd	*Candida albicans* ATCC1600 MIC (μg/mL)[a]	Compd	*Candida albicans* ATCC1600 MIC (μg/mL)[a]
1	>100	**11**	>100
2	>100	**12**	12.5
3	>100	**13**	25
4	>100	**14**	25
5	>100	**15**	>100
6	>100	**16**	>100
7	6.25	**17**	>100
8	3.12	**18**	>100
9	>100	**19**	>100
10	>100	Amphotericin B	0.39

[a]MIC was defined as the lowest concentration that inhibited visible growth; The MIC > 100 μg/mL was considered to be inactive; Amphotericin B was used as positive control.

tural difference is happened at the 7-position of the seven-membered ring, the former linked with a 7-Me and the latter connected with a 7-CO_2Me. Inspection of antifungal activities of compounds **1–19** against *C. albicans* immediately reveals that all the active compounds **7**, **8** and **12–14** are of amphipathic properties, and possess a hydrophobic domain of constrained-rings system and a hydrophilic domain of an unsaturated carboxylic acid chain (Fig. 4). Compounds **1**, **2**, **5** and **6**, structurally belonging to the subclass of pseudolaric acid A, showed inactive against *C. albicans* in this test. Compared with pseudolaric acid A (**7**), the structures of **1**, **2**, **5** and **6** have only slightly changes at seven-membered ring (**2** also methylated to form a 18-OMe), indicating that this domain with a Δ^7 double bond is crucial for the antifungal activity, any changes in the seven-membered ring, for example substituted with oxygenated functions at C-7/C-8/and double bond migration, will render the analogues inactive. Compounds **12–14** prepared from pseudolaric acid B by the replacement of 7-CO_2Me with 7-CO_2Et, 7-CO_2(*n*-Pr) and 7-CO_2(*n*-Bu) successively, showed remarkable attenuation of the antifungal activity (3–4-fold lower) compared with pseudolaric acid B. Compounds **16–19** semi-synthesized from pseudolaric acid B by inducting hydrophilic amido groups into the 7-position displayed inactive. Esterification or glucosylation of free acid group (C-18) of pseudolaric acid B is also detrimental to the inhibition, for example, in the case of compounds **9** and **11**. Removal of C-4-OAc of compound **8** to yield compound **10** by hydrolysis also renders the analogue inactive. Compounds **3** and **4** with shorten and saturated side chain exhibit inactive against *C. albicans*.

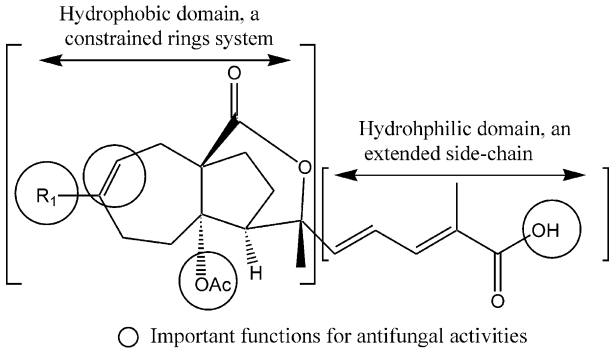


Figure 4.

The antifungal tests results of compounds **1–19** and their structural characteristics have clearly offered a structure–activity relationship, which are: (1) All the active antifungals **7**, **8** and **12–14** in the tested compounds are of amphipathic properties, and possess a hydrophobic domain of constrained-rings system and a hydrophilic domain of an unsaturated carboxylic acid chain. (2) Hydrophobic group R_1 at C-7 and the Δ^7 double bond are necessary for antifungal activity, for R_1, carboxylic methyl ester seems better than methyl, –CO_2Et, –CO_2(*n*-Pr) and –CO_2(*n*-Bu). (3) The length of the side chain/and the presence of conjugated double bonds in the side chain are essential for the antifungal activity, if the side chain becomes shortened/and losses the conjugated double bonds, the compound becomes less active. (4) Any structural changes in the seven-membered ring, for example Δ^7 double bond migration and oxygenation at C-7/C-8, compared with pseudolaric acids A and B, will render the analogues inactive. (5) The free carboxylic acid group in the side chain and C-4 acetyl group are necessary for antifungal activity.[3]

Experimental

General

Optical rotations were measured on a Perkin–Elmer 341 polarimeter (Na filter, $\lambda = 589$ nm). IR spectra were recorded on a Perkin–Elmer 577 spectrameter with KBr disc. ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker AM-400 spectrometer with TMS as internal standard. EIMS (70 eV) was carried out on a Finnigan MAT 95 instrument. ESI-MS was recorded on a Finnigan LCQ^{DECA} Mass spectrometer. All solvents used were of analytical grade (Shanghai Chemical Plant). Silica gel (200–300 mesh) was used for column chromatography, and the pre-coated silica gel GF_{254} plate (Qingdao Haiyang Chemical Plant, Qingdao, People's Republic of China) was used for TLC. C_{18} reversed-phase silica gel (250 mesh, Merck) and MCI gel (CHP20P, 75–150 μ, Mitsubishi Chemical Industries

Ltd.) were also used for Column Chromatography. The purity checks for all the compounds **1**–**19** were carried out on a Waters 515HPLC pump with a Waters 2487 Dual λ Absorbance Detector, and the HPLC column was a Phenomenex Luna C18 silica gel (5 μm, 250 cm×4.60 mm). The mobile phases were 70–90% methanol in water with a flow rate of 1 mL/min, and column chamber was kept at around 20 °C for the analysis of all compounds.

Plant material

The root bark of *P. kaempferi* (Pinaceae) was collected in Jiangxi Province of the People's Republic China and identified by Professor Zeng-Tao Wang of Shanghai Traditional Chinese Medical University, where a voucher specimen has been deposited in the Herbarium (accession number TJP-1999-1Y).

Extraction and isolation

The dried root bark (10 kg) of *P. kaempferi* was ground and percolated with 95% ethanol. After removal of the ethanol under reduced pressure, 2 L of 5% $NaHCO_3$ solution was added to form a suspension and was immediately defatted with ethyl acetate. The aqueous solution was then acidified to about pH 6 with 5% hydrochloride and extracted with ethyl acetate to yield an acidic EtOAc fraction (78 g). The acidic EtOAc fraction was subjected to silica gel column chromatography using a gradient solvent system of petroleum–EtOAc (5:1 to 2:1), and the chloroform–methanol (10:1 to 0:1) to give seven major fractions (fr. 1 to fr. 7). The first fraction was chromatographied sequentially over MCI gel column (MeOH–water, 6:4 to 10:0), silica gel column (petroleum–EtOAc, 3:1 to 2:1), and C-18 reversed-phase silica gel column (aqueous methanol, 70%) to yield pseudolaric acids A_2 (**1**) (170 mg), F (**5**) (21.3 mg) and H (**6**) (33 mg), as well as methyl pseudolarate A_2 (**2**) (20 mg) and methyl pseudolarate B (**9**) (690 mg), respectively. Fraction 2 was recrystallized from petroleum-acetone to afford pseudolaric acid A (**7**) (630 mg). Fraction 3 was recrystallized from petroleum-acetone to afford pseudolaric acid B (**8**) (9.6 g). The residue was further purified on C-18 reversed-phase silica gel column (methanol–water, 5:5) to provide pseudolaric acid C (**10**) (53.8 mg). Fraction 4 was subjected to a MCI gel column (aqueous methanol, 60%), then a silica gel column (petroleum–EtOAc–formate, 2:1:0.1) to yield pseudolaric acid B_2 (**3**) (160 mg) and pseudolaric acid B_3 (**4**) (19.5 mg). Fraction 5 was purified on silica gel column eluted with a mixture of chloroform-methanol (10:1) to give pseudolaric acid B-*O*-β-D-glucopyranoside (**11**) (9.54 g).

Pseudolaric acid A_2 (1). Quadratic crystal (acetone); $[\alpha]_D^{20}$ −29.7° (*c* 0.96, CH_3OH); IR (KBr) ν_{max} 3442, 2968, 1732, 1716, 1689 and 1645 cm^{-1}; ^1H NMR see Table 2; ^{13}C NMR see Table 1; ESIMS (negative) *m/z* 403 ($[M-H]^-$); EIMS *m/z* (rel. int.): 386 $[M-H_2O]^+$ (41), 344 $[M-AcOH]^+$ (14), 326 $[M-H_2O-AcOH]^+$ (46), 316 (15), 298 (33), 283 (12), 282 (15), 190 (17), 189 (40), 163 (100), 148 (47), 147 (65), 146 (73), 119 (99), 91 (57); HREIMS *m/z* 386.1710 $[M-H_2O]^+$ ($C_{22}H_{26}O_6$, calcd 386.1729).

Methylation of pseudolaric acid A_2 (1)

5 mg of pseudolaric acid A_2 (**1**) was dissolved in 1 mL methanol in a flask, and added 7 mL ethyl ether solution of CH_2N_2 at room temperature, and the mixture was then kept for 24 h. After removal solvent, about 5 mg methyl pseudolarate A_2 (**2**) was yielded.

Methyl pseudolarate A_2 (2). Yellow gum; $[\alpha]_D^{20}$ −23.8° (*c* 0.28, CH_3OH); IR (KBr) ν_{max} 3523, 3452, 2962, 2925, 1736, 1709 and 1639 cm^{-1}; ^1H NMR see Table 2; ^{13}C NMR see Table 1; EIMS *m/z* (rel. int.): 418 $[M]^+$ (1), 403 $[M-CH_3]^+$ (3), 387 (52), 386 $[M-CH_3OH]^+$ (100), 358 $[M-HOAc]^+$ (21), 344 (30), 330 (23), 327 (18), 326 (95), 312 (29), 189 (49), 163 (62), 147 (68), 146 (83), 145 (46), 119 (100), 91 (48); HREIMS *m/z* 418.1942 $[M]^+$ ($C_{23}H_{30}O_7$, calcd 418.1992).

Pseudolaric acid B_2 (3). Pale needle (acetone); $[\alpha]_D^{20}$ +67.1° (*c* 0.90, CH_3OH); IR (KBr) ν_{max} 3448, 3404, 2956, 1720, 1693 and 1639 cm^{-1}; ^1H NMR see Table 2; ^{13}C NMR see Table 1; EIMS *m/z* (rel. int.): 352 $[M]^+$ (1), 334 $[M-H_2O]^+$ (10), 302 (10), 194 (15), 193 (11), 149 (13), 140 (100), 95 (20); HREIMS *m/z* 352.1517 $[M]^+$ ($C_{18}H_{24}O_7$, calcd 352.1522).

Acetylation of 3

A mixture of 20 mg pseudolaric acid B_2 (**3**) and 2 mL acetyl chloride was stirred for 24 h at room temperature. After removal of the remaining acetyl chloride under reduced pressure, the residue was dissolved in water and extracted with ethyl acetate to give a major compound, which was then purified by silica gel chromatography eluted with petroleum–EtOAc–formate acid (2:1:0.1, by vol) to yield 12 mg pseudolaric acid B_3 (**4**).

Dehydration of 3

15 mg pseudolaric acid B_2 (**3**) was dissolved in 1 mL pyridine. The solution was cooled to minus 10 °C, and then 15 mg thionyl chloride was added dropwisely. The mixture was stirred at minus 2 °C for 1 h, and the reaction was quenched by addition of 5 mL icy water to form a suspension and extracted with EtOAc to give a yellow solid, which was then purified by silica gel column eluted with petroleum–EtOAc–formate acid (2:1:0.1, by vol) to yield 8.2 mg compound **15**. ^1H NMR ($CDCl_3$) δ (ppm): 1.25 (H_3-12, 3H, s), 1.86–1.72 (3H, m), 2.05 (m), 2.20 (m), 2.41 (d, 6.1), 2.44 (2H, m), 2.47 (dd, 13.2, 6.4), 2.98 (dd, 13.2, 7.8), 3.03 (br.d, 6.3), 3.34 (dd, 18.1, 7.8), 3.72 (OMe, 3H, s), 5.70 (H-5, dd, 7.9, 3.0), 7.24 (H-8, m).

Pseudolaric acid B_3 (4). White amorphous powder; $[\alpha]_D^{20}$ +69.4° (*c* 0.93, CH_3OH); IR (KBr) ν_{max} 3437, 2955, 1735, 1709 and 1647 cm^{-1}; ^1H NMR see Table 2; ^{13}C NMR see Table 1; EIMS *m/z* (rel. int.): 394 $[M]^+$ (2), 334 $[M-AcOH]^+$ (8.1), 302 (20), 290 (16), 258 (13), 257 (10), 222 (18), 204 (14), 192 (11), 191 (52), 159 (10), 131

(42), 99 (100); HREIMS m/z 394.1682 $[M]^+$ ($C_{18}H_{24}O_7$, calcd 394.1628).

Preparation of compound 12–14

An ethanolic solution of pseudolaric acid B was treated with one equivalent of *t*-BuOK (ca. pH = 10–12) at room temperature for 24 h and the mixture was then acidified with AcOH to pH = 7. After removal of solvent under reduced pressure, the dried residue was mixed with a proper amount of acetyl chlororide and stirred for 24 h at rt. The mixture was concentrated under reduced pressure to give a yellow solid, which was dissolved in water and stirred to destroy acyl chlororide of compound **12**. The water solution was then extracted with EtOAc, followed by the purification of silica gel column eluted with petroleum–EtOAc–formic acid (3:1:0.1) to yield **12** (80%). The compounds **13** (76%) and **14** (78%) were prepared by the same procedure in the *n*-propanolic and *n*-butanolic solutions of pseudolaric acid B, respectively.

Compound 12. White amorphous powder; IR (KBr) ν_{max} 3435, 1741, 1707 and 1643 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 1.27 (t, 3H, 7.2), 1.59 (s, 3H), 1.77–1.85 (complex m, 5H), 1.95 (s, 3H), 2.11 (s, 3H), 2.12 (m), 2.59 (br.d, 15.4), 2.74 (dd, 15.0, 8.7), 2.89 (dd, 15.4, 6.2), 3.06 (dd, 13.6, 6.0), 3.29 (d, 3.5), 4.15 (m, 2H), 5.91 (d, 15.0), 6.54 (dd, 15.0, 11.4), 7.18 (m) and 7.25 (d, 11.4); ESIMS m/z 469 $[M+Na]^+$; EIMS m/z (rel. int.): 428 $[M-H_2O]^+$(5), 386(10), 368(8), 260(100), 242(20) and 224(22); HREIMS m/z 428.1829 $[M-H_2O]^+$ ($C_{24}H_{28}O_7$, calcd 428.1835).

Compound 13. White amorphous powder; IR (KBr) ν_{max} 3434, 1741, 1707 and 1645 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 0.94 (t, 3H, 7.4), 1.58 (s, 3H), 1.62–1.89 (complex m, 7H), 1.94 (s, 3H), 2.08 (s, 3H), 2.12 (m), 2.59 (br.d, 14.0), 2.74 (dd, 15.0, 8.7), 2.88 (dd, 15.4, 5.9), 3.06 (dd, 14.0, 5.9), 3.29 (d, 3.8), 4.05 (m, 2H), 5.90 (d, 15.0), 6.54 (dd, 15.0, 11.4), 7.18 (m), 7.25 (d, 11.4); ESIMS m/z 483 $[M+Na]^+$; EIMS m/z (rel. int.): 442 $[M-H_2O]^+$(5), 400(11), 382(6), 260(100), 242(21) and 224 (20); HREIMS m/z 442.1987 $[M-H_2O]^+$($C_{25}H_{30}O_7$, calcd 442.1992).

Compound 14. White amorphous powder; IR (KBr) ν_{max} 3447, 1741, 1705 and 1745 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 0.90 (t, 7.4), 1.33–2.11 (complex m, 10H), 1.58 (s, 3H), 1.94 (s, 3H), 2.11 (s, 3H), 2.59 (br.d, 14.0), 2.74 (dd, 15.0, 8.8), 2.88 (dd, 15.4, 5.9), 3.06 (dd, 14.0, 5.9), 3.29 (d, 3.8), 4.10 (m, 2H), 5.91 (d, 15.0), 6.54 (dd, 15.0, 11.4), 7.16 (m) and 7.25 (d, 11.4); ESI MS m/z 497 $[M+Na]^+$; EIMS m/z (rel. int.): 456 $[M-H_2O]^+$(3), 414(11), 396(5), 260(100), 242(18) and 224(16); HREIMS m/z 456.2173 $[M-H_2O]^+$($C_{26}H_{32}O_7$, calcd 456.2148).

Preparation of compounds 16–19

To a stirred 30% aqueous ammonia solution, a proper amount of pseudolaric acid B was added, and acetic acid was used to adjust the pH of the mixture around 6. After stirred for 6 h at rt, the reaction mixture was diluted with water and extracted with ethyl acetate. The crude was purified with silica gel column (chloroform/methanol; 5:1) to give two compounds **16** and **17** (34%, 58%). Compounds **18** and **19** (6%, 92.8%) were prepared via the same method just by replacement of ammonia with methylamine.

Compound 16. White amorphous powder; ^1H NMR (DMSO-d_6) δ: 1.47 (s, 3H), 1.50–1.72 (complex m, 5H), 1.82 (s, 3H), 2.02 (m, 3H), 2.23 (br.d, 5.6), 2.33 (dd, 13.9, 8.6), 2.50 (overlap), 6.21 (d, 15.1), 6.32 (dd, 14.9, 11.2), 6.55 (br.s), 6.74 (br.s), 7.12 (d, 10.9) and 7.28 (br.s); ESI MS m/z 398 $[M+Na]^+$, 773 $[2M+H]^+$; EIMS m/z (rel. int.): 375 $[M]^+$(3), 357$[M-H_2O]^+$(18), 313(30), 296(10), 178(100), 160(76) and 133(82); HREIMS m/z 375.1658 $[M]^+$ ($C_{20}H_{25}NO_6$, calcd 375.1682).

Compound 17. White amorphous powder; IR (KBr) ν_{max} 3431, 3203, 2952, 1743, 1689, 1443, 1371, 1252, 1167, 1072 and 957 cm^{-1}; ^1H NMR ((DMSO-d_6) δ: 1.52 (s, 3H), 1.56–1.77 (complex m, 5H), 1.83 (s, 3H), 2.04 (m), 2.12 (s, 3H), 2.53 (br.d, 14.2), 2.64 (dd, 14.9, 5.6), 2.94 (dd, 13.8, 5.9), 3.16 (br.s), 3.37 (t, 6.3), 6.24 (d, 15.1), 6.36 (dd, 14.9, 11.3), 6.60 (br.s), 6.82 (br.s), 7.11 (d, 10.9) and 7.34 (br.s); ESI MS m/z 416 $[M-H]^-$, 833 $[2M-H]^-$; EIMS m/z (rel. int.): 399$[M-H_2O]^+$(16), 357(20), 339(20), 311(24), 296(10), 260(100), 242(40) and 229(45); HREIMS m/z 399.1683 $[M-H_2O]^+$ ($C_{22}H_{27}NO_6$, calcd 399.1682).

Compound 18. White amorphous powder; ^1H NMR (DMSO-d_6) δ 1.47 (s, 3H), 1.47–2.36 (complex m, 11H), 1.82 (s, 3H), 6.21 (d, 15.1), 6.32 (br.t, 13.1), 6.44 (br.s), 7.13 (d, 10.9) and 7.71 (br.s); ESI MS m/z 390 $[M+H]^+$, 779 $[2M+H]^+$; EIMS m/z (rel. int.): 389 $[M]^+$(5), 371$[M-H_2O]^+$(15), 327(34), 296(8), 178 (100), 160(70) and 133(78); HREIMS m/z 389.1844 $[M]^+$ ($C_{21}H_{27}NO_6$, calcd 389.1838).

Compound 19. White amorphous powder; IR (KBr) ν_{max} 3390, 1712, 1655 and 1612 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.57 (s, 3H), 1.67–1.84 (complex m, 5H), 1.94 (s, 3H), 2.10 (s, 3H), 2.23 (br.t, 13.8), 2.59 (br.d, 2H, 7.1), 2.69 (dd, 15.1, 6.0), 3.04 (dd, 14.0, 6.3), 3.29 (br.d, 3.3), 2.81 (d, 3H, 4.1), 5.89 (d, 15.1), 5.91 (br.s), 6.42 (m), 6.52 (dd, 15.1, 11.6) and 7.23 (d, 11.6); ESI MS m/z 454 $[M+Na]^+$, 863 $[2M+H]^+$; EIMS m/z (rel. int.): 431 $[M]^+$(2), 413 $[M-H_2O]^+$(10), 371(25), 353(20), 325(40), 296(22), 260(100), 242(60), 229(40) and 190(56); HREIMS m/z 431.1958 $[M]^+$ ($C_{23}H_{29}NO_6$, calcd 431.1944).

Purity of compounds 1–19

Purities of all the tested compounds were checked by HPLC analysis (the basic HPLC conditions see General in the Experimental) are higher than 95%, some of the active antifungals, that is compounds **7**, **8** and **12–14**, the purity of them are more than 98.5%.

Microdilution assay

The twofold serial broth dilution assay was applied to measure the MIC values and all the compounds were tested at the concentration of 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39 and 0.20 μg/mL. The fungus, *C. albicans* (10^4–10^5 CFUs/mL), was incubated in Sabouraud dextrose broth at 37 °C for 48 h with the respective compounds and the positive control dissolved in DMSO. The black controls of fungal culture were incubated with limited DMSO under the same condition. DMSO was determined not to be toxic at a limited amount under these experimental conditions. MIC was defined as the lowest concentration that inhibited visible growth and the MIC > 100 μg/mL was considered to be inactive. Amphotericin B was used as positive control.

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