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Two Novel Cytotoxic and Antimicrobial Triterpenoids from *Pseudolarix kaempferi*

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Abstract—Two novel antimicrobial and cytotoxic triterpenoids, isopseudolarifuroic acids A (**1**) and B (**2**), were isolated from the bark of *Pseudolarix kaempferi*. The structural elucidation of two novel compounds was carried out mainly by spectroscopic methods, and also by computer modeling. Compounds **1** and **2** exhibited significant cytotoxic activities against several tumor cell lines. Compound **1** also showed most potent antimicrobial activities against both Gram-positive and Gram-negative bacteria. © 2001 Published by Elsevier Science Ltd.

Introduction

Pseudolarix kaempferi Gord. (Pinaceae) is a plant indigenous to the east of China. It's root bark known as "Tu Jin Pi" was used in traditional Chinese medicine for the treatment of skin diseases caused by microbial infection. A series of novel diterpenoids have been isolated from its root bark in the past decades. Some of them have been demonstrated to have a variety of biological activities, for example, pseudolaric acids A and B were reported to possess significant antifungal, antifertility[1] and cytotoxic[2] activities. A triterpenoid lactone, pseudolarolide B isolated from the seeds of *P. kaempferi*, also showed potent cytotoxic activity.[3] Two novel triterpenoids, isopseudolarifuroic acids A (**1**) and B (**2**), were isolated from the bark of this plant by bioassay-guided fractionation and their structures including stereochemistry were elucidated by spectroscopic method, especially 2D NMR techniques, and also by computer modeling. This communication covers the isolation, structure elucidation, and biological evaluation of compounds **1** and **2**.

The bark of *P. kaempferi* collected in Jiangxi province of China was identified by its morphology.[4] The dried bark (10 kg) of *P. kaempferi* was ground and extracted with 95% EtOH by maceration. After filtration and removal of the solvent, the ethanol extract was dissolved in 2 L 5% $NaHCO_3$ solution to make a suspension and immediately partitioned with EtOAc to give a neutral EtOAc-soluble fraction (40 g). The aqueous solution was then acidified with 5% HCl solution to about pH = 6 and extracted with EtOAc again to afford acidic EtOAc-soluble fraction (78 g). The neutral EtOAc-soluble fraction showed significant antibacterial and cytotoxic activities, and the acidic EtOAc-soluble fraction showed strong antifungal and cytotoxic activities, which has been demonstrated to contain mainly the components, pseudolaric acids A and B. Bioassay-guided fractionation of the antibacterial neutral EtOAc-soluble fraction using these in vitro assays,[5] applying successive silica gel and reversed-phase (C18) silica gel column chromatography, has led to isolation of two new novel triterpenoids, isopseudolarifuroic acid A (**1**) (100 mg) and isopseudolarifuroic acid B (**2**) (10 mg).

Results and Discussion

Structural elucidation

Isopseudolarifuroic acid A (**1**), obtained as white powder, has the molecular formula $C_{30}H_{42}O_4$ as found from its HREIMS (*m*/*z* 466.3082). Its IR absorption bands at 1705 and 3000–2500 cm^{-1} showed the presence of a carboxyl group, and supported by carbon signal at 168.10 ppm in the ^{13}C NMR. A strong IR absorption at 3437 cm^{-1} and carbon signal at 76.96 ppm indicated the

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presence of a hydroxyl group. ^{1}H NMR and ^{13}C NMR (Table 1) showed the presence of six methyls, seven methenes, eight methines and nine quaternary carbons. The ^{1}H NMR, ^{13}C NMR, DEPT, HMQC and HMBC spectra characterized the occurrence of four trisubstituted double bonds. The ^{1}H NMR indicated six methyls, including one secondary (δ 0.74), and five tertiary (δ 0.85, 0.87, 0.90, 0.90 and 0.93) methyls. These data were consistent with the HREIMS empirical formula and suggested that **1** was a triterpenoid. The significant fragment ion at *m*/*z* 313 produced by loss of the side chain in the EIMS spectrum inferred that **1** was a tetracyclic triterpenoid.[6,7]

A direct comparison of the ^{1}H NMR and ^{13}C NMR data of **1** with those of pseudolarifuroic acid A, which was isolated from the seeds of *P. kaempferi*,[8] suggested that two compounds have the same side chain. The olefinic protons at δ 6.29 and 7.89 were, respectively, assigned to the H-24 and H-27 at the furan ring in the side chain. The above functionalities account for eight (four for tetracyclic ring, three for furan ring and one for carboxyl group) of 10 degrees of unsaturation present in the compound **1**. The remaining two degrees of unsaturation are attributable to another two trisubstituted double bonds. For tetracyclic triterpenoids, the quaternary carbon signals of C-4 (δ 37.21) and C-10 (δ 34.83) were more easily distinguishable in the ^{13}C NMR, the H-5 and H-9 were thus assigned by the correlations with C-4 and C-10 in the HMBC, respectively. The C-5 and C-9 were then easily identified respectively by the correlations with the H-5 and H-9 in the HMQC. In the HMBC spectrum, one olefinic proton signal at δ 5.51 (1H, br.d, $J = 5.0$ Hz) correlated with the carbon signals at δ 38.09 (C-5), 23.18 (C-6) and 53.12 (C-9), indicated a δ^7 double bond. The remaining one olefinic proton signal (δ 5.12, br.s, H-15) correlated with four quaternary carbon signals at δ 50.84 (C-17), 51.84 (C-13), 136.85 (C-8) and 153.05 (C-14) revealed a $\delta^{14(15)}$ double bond, suggesting that **1** was a methyl-rearranged lanostane type triterpenoid, like mariesiic acid A, 23-oxo-mariesiic acid A, methyl mariesiate A and methyl 23-oxo-mariesiate A, which were isolated from the seeds of *Abies mariesii*.[9–11] One broad singlet proton signal at δ 3.43 ($W/2 = 7$ Hz) in the ^{1}H NMR and a tertiary carbon signal at δ 76.96 were respectively assigned to H-3β and C-3 bearing an α-hydroxyl group.[7] The small coupling constants between H-3β and two H-2 protons indicated that H-3β took a preferred equatorial orientation. The γ-gauche effects of 3α-hydroxyl caused high-field chemical shift of C-1 (δ 28.84) and C-5 (δ 38.09) compared with 3β-hydroxyl lanostane type triterpenoids (Fig. 1). The C-29 (δ 23.12) was down-field shifted about δ +2 ppm compared with that of its 3β-hydroxyl analogues since the 3α-hydroxyl only has γ-gauche effect with C-28, while the 3β-hydroxyl has γ-gauche effects with both C-28 and C-29 (Fig. 1).[10,12] The severe higher-field shifted H-9β proton signal at δ 1.36 was possibly caused by the shield effect of conjugated δ^7 and δ^{14} double bonds. The H-12α proton signal at δ 1.38 in

Table 1. ^{1}H NMR and ^{13}C NMR of compounds **1** and **2**

	Compd **1**[a]		Compd **2**[b]	
No.	^{13}C NMR δ_C (DEPT)	^{1}H NMR δ_H (J/Hz)	^{13}C NMR δ_C (DEPT)	^{1}H NMR δ_H (J/Hz)
1	28.84	1.95 (m) 0.82 (m)	29.48	2.17 (m) 0.87 (m)
2	25.39	1.75 (m) 1.56 (m)	26.16	1.54 (m) 1.41 (m)
3	76.96	3.43 (br.s)	75.93	3.37 (br.d, 2.0)
4	37.21		37.69	
5	38.09	1.50 (dd, 11.8, 5.0)	38.59	1.64 (dd, 11.8, 4.9)
6	23.18	1.90 (2H, m)	23.68	1.93 (2H, m)
7	120.79	5.51 (d, 5.0)	121.57	5.56 (d, 5.6)
8	136.85		137.72	
9	53.12	1.36 (m)	54.02	1.40 (m)
10	34.83		35.41	
11	33.66	1.78 (m) 1.44 (m)	34.01	1.85 (m) 1.51 (m)
12	25.32	1.91 (m) 1.38 (m)	25.99	1.92 (m) 1.84 (m)
13	51.84		52.39	
14	153.05		154.00	
15	115.09	5.12 (br.s)	115.36	5.26 (br.s)
16	45.31	2.11 (m) 1.85 (dd, 15.6, 3.2)	45.62	2.19 (dd, 15.3,3.3) 1.89 (m)
17	50.84		51.16	
18	16.60	0.85 (s)	17.12	0.86 (s)
19	22.41	0.90 (s)	22.69	0.94 (s)
20	37.54	2.15 (m)	35.88	2.34 (m)
21	15.81	0.74 (d, 6.2)	16.58	0.88 (d, 6.0)
22	31.65	2.66 (d, 13.2) 2.17 (m)	38.49	2.36 (m) 1.94 (m)
23	158.08		174.96	
24	105.71	6.29 (s)		
25	119.18			
26	168.10			
27	147.53	7.89 (s)		
28	28.33	0.93 (s)	28.83	0.94 (s)
29	23.12	0.87 (s)	23.35	0.89 (s)
30	19.24	0.90 (s)	19.45	0.90 (s)

[a]^{1}H NMR and ^{13}C NMR of compound **1** was measured in $CDCl_3$.
[b]^{1}H NMR and ^{13}C NMR of compound **2** was measured in CD_3COCD_3.

compound **1** was high-field shifted about δ −0.5 ppm compared with that of compound **2**, suggesting that the H-12α of **1** was likely shielded by the furan ring in the side chain.

The NOESY spectrum of **1** exhibited strong correlations between following protons, $H_{1\alpha}$–$H_{12\alpha}$, $H_{5\alpha}$–H_{30}, $H_{9\beta}$–$H_{11\beta}$, H_{18}–$H_{22\alpha}$, $H_{9\beta}$–H_{19}, $H_{6\beta}$–H_{19}, $H_{12\beta}$–H_{18} and $H_{5\alpha}$–$H_{1\alpha}$, indicating that the six-membered A-ring, B-ring and C-ring took chair-conformation, boat-conformation and twist boat-conformation, respectively, and the five-membered D-ring took envelope-conformation.

A computer modeled 3D structure (Fig. 2) of **1** was generated by using the molecular modeling program CS Chem3D Pro Version 5.0, using MM2 force field calculations for energy minimization. The computer modeling offered a favorable conformation of **1** and close contacts of the atoms in space, especially the close contacts of some key hydrogen atoms,[13] which were consistent with the stereochemistry of **1** assigned by the NOESY spectrum.

The assignments for C-21 and C-30 were reversed in our research compared with the literature.[9–11] The doublet proton signal for H-21 at δ 0.74 (3H, d, $J=6.2$) was easily distinguishable from the other angular methyl groups, and the carbon signal at δ 15.81 was then definitely assigned to the C-21 by the correlation with H-21 in the HMQC. The proton signal of one angular methyl group at δ 0.90 (3H, s) correlated with the C-13 at δ 51.84 in the HMBC

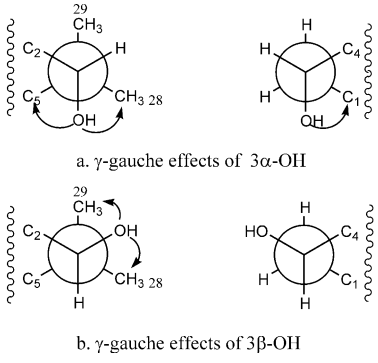


Figure 1. The major γ-gauche effects of 3α-OH and 3β-OH.

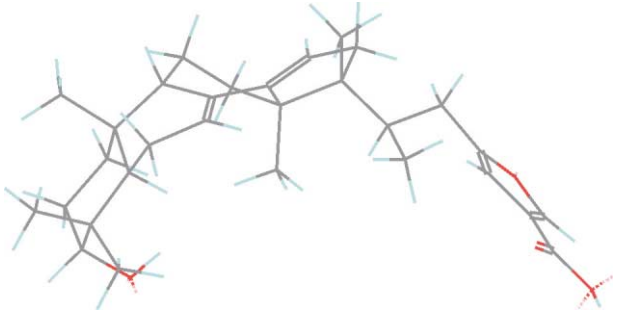
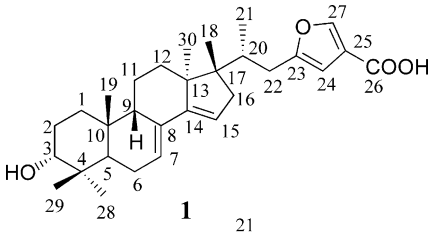
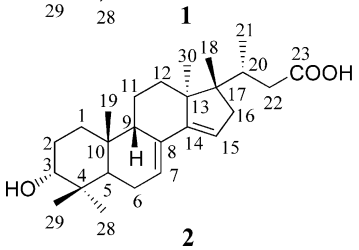


Figure 2. Stereoview of isopseudolarifuroic acid A (**1**) generated from computer modeling.

was assigned to H-30, the C-30 at δ 19.24 was then identified by the correlation with H-30 in the HMQC. The assignments for C-23 and C-25 were also reversed with the literature based on the HMBC spectrum.[8]



1



2

The structure of compound **1** was thus unambiguously elucidated.[14]

Isopseudolarifuroic acid B (**2**), obtained as white powder, possessed the molecular formula $C_{26}H_{40}O_3$ as found from its HREIMS (m/z 400.2967). Its IR absorptions at 3000–2500 and 1709 cm^{-1} showed the presence of one carboxyl group. This was supported by the carbon signal at δ 174.96 in the ^{13}C NMR. IR absorption at 3435 cm^{-1} and ^{13}C NMR signal at δ 75.93 suggested the presence of one hydroxyl. The ^{1}H NMR, ^{13}C NMR and 2D NMR techniques (Table 1) indicated that compound **2** composed of two trisubstituted double bonds, six methyls, seven methenes, three methines and four quaternary carbons. The ^{1}H NMR spectrum of **2** indicated six methyls including one secondary and five angular methyls. Furthermore, comparison of the ^{1}H NMR and ^{13}C NMR of **2** with those of **1** inferred that the both compounds **1** and **2** possessed the same tetracyclic skeleton, the only difference was in the side chain. The doublet proton signal for H-21 at δ 0.88 (3H, d, $J=6.0$ Hz) was easily distinguishable, and the carbon signal at δ 16.58 was then assigned to the C-21 by the correlation with H-21 in the HMQC. The C-22 was assigned to the carbon signal at δ 38.49 by the correlation with H-20 at δ 2.34 (1H, m) in the HMBC. The only remaining carbon signal for the carboxyl at δ 174.96 was attributable to the C-23 by the correlations with H-22a, 22b (δ 2.36, 1.94, each 1H, m). The structure of isopseudolarifuroic acid B was therefore elucidated to be **2**.[14] The complete assignments of the proton and carbon signals of compounds **1** and **2** (Table 1) were made by utilization of ^{1}H NMR, ^{13}C NMR, DEPT, HMQC, HMBC and NOESY spectra.

Biological activity evaluation

The microbial strains (Table 2) were from the American Type Culture Collection, and the antimicrobial susceptibility tests were carried out by microdilution assay.[5,15] The antibacterial activity of compound **1** is showed in Table 2. Compound **1** was found to be very active against two Gram positives, *Staphylococcus aureus* and

Table 2. Antimicrobial activities of compound **1**

Microbes	MIC (mM) [a]		
	1	Bakuchiol	Magnolol
Staphylococcus aureus ATCC 25923	0.027	0.019	0.048
Micrococcus luteus ATCC 9341	0.013	0.010	0.048
Escherichia coli ATCC 259277	0.107	>0.78	>0.75
Pseudomonas aeruginosa ATCC 2785	>0.215	>0.78	>0.75

[a]MIC was defined as the lowest concentration that inhibited visible growth.

Table 3. Cytotoxic activities of compounds **1** and **2**

Cell lines	IC_{50} ($\times 10^{-5}$ M)	
	1	**2**
P 388	5.0	5.5
A 549	5.4	6.0

Micrococcus luteus at the MICs of 0.027 and 0.013 mM, respectively. Compound **1** also showed strong activity against one of the Gram negative, *Escherichia coli* at the MIC of 0.107 mM. In our tests, two of the most potent natural antimicrobial agents, bakuchiol and magnolol, isolated from plant resources were used as positive controls (Table 2).[16,17] Against the Gram positives, compound **1** is comparable with bakuchiol, and stronger than magnolol. It is more important that compound **1** showed potent activity against Gram negative, *E. coli*, while bakuchiol and magnolol were inactive at the comparable dosage.

Compounds **1** and **2** were evaluated for their cytotoxic activities according to established protocols.[18] Both of them showed moderate cytotoxic activities against two tested cell lines (Table 3). The test result indicated that the cytotoxic activities of compounds **1** and **2** were not related with the changes of the side chain.

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4. *Pseudolarix kaempferi* Gord. (Pinaceae), which collected in Jiangxi province of China, was identified by Professor Zeng-Tao Wang of Shanghai Chinese Traditional Medical University, where a voucher specimen was deposited in the Herbarium (accession number TJP-1999-1Y).
5. The microbial cells were suspended in Mueller Hinton broth to form a final density of 5×10^{-5}–10^{-6} CFU/ml and incubated at 37 °C for 18 h under aerobic conditions with the respective compounds which have been dissolved in DMSO. The blank controls of microbial 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.
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13. The close contacts of atoms in space calculated for compound **1** (Å): C_5–C_8, 2.713, C_6–C_9, 2.843, C_{11}–C_{14}, 2.690, C_7–$H_{5\alpha}$, 2.409, C_8–$H_{5\alpha}$, 2.557, C_{18}–$H_{12\beta}$, 2.427, C_{18}–$H_{16\beta}$, 2.461, C_{18}–$H_{22\alpha}$, 2.555, C_{19}–$H_{9\beta}$, 2.362, C_{21}–$H_{16\alpha}$, 2.523, C_{30}–$H_{12\alpha}$, 2.395, $H_{1\alpha}$–$H_{12\alpha}$, 2.225, $H_{5\alpha}$–H_{30}, 2.227, $H_{9\beta}$–$H_{11\beta}$, 2.239, H_{18}–$H_{22\alpha}$, 2.192.
14. Physical and spectroscopic data: **Isopseudolarifuroic acid A (1)**: White powder, $[\alpha]_D$ +34.5 (c, 0.85, $CHCl_3$), UV($CHCl_3$) 238 nm, IR (KBr) γ_{max} cm^{-1}: 3437, 2926, 1705, 1551, 1450, 1367, 1317, 1213, 1142, 980, 905, 760, ^1H NMR and ^{13}C NMR data of **1**, see Table 1, HREIMS *m/z* 466.3082 $[M]^+$ ($C_{30}H_{42}O_4$, calcd 466.3083), EIMS *m/z* (%) 466 (48), 451 (9), 434 (30), 433 (52), 400 (29), 382 (8), 367 (8), 315 (26), 314 (64), 313 (40), 301 (10), 300 (46), 299 (100), 295 (13), 282 (20), 281 (48), 280 (14), 265 (11) and 159 (5); **Isopseudolarifuroic acid B (2)**: White powder, $[\alpha]_D$ +67 (c, 0.28, $CHCl_3$), UV($CHCl_3$) 227 nm, IR (KBr) γ_{max} cm^{-1}: 3435, 2964, 1709, 1454, 1385, 1367, 1300, 1205, 1065, 980, 905, 802, ^1H NMR and ^{13}C NMR see Table 2, HREIMS *m/z* 400.2967 $[M]^+$ ($C_{30}H_{42}O_4$, calcd 400.2977), EIMS *m/z* (%) 400 (100), 382 (30), 367 (23), 313 (67), 295 (22), 187 (20), 159 (31), 149 (20), 135 (24), 133 (24), 131 (24) and 105 (21).
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