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Pseudoferic acids A–C, three novel triterpenoids from the root bark of *Pseudolarix kaempferi*

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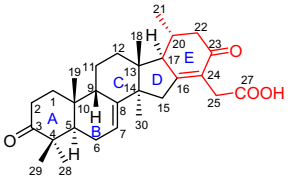
ABSTRACT

Three novel triterpenoids, pseudoferic acids A and B (**1** and **2**) possessed a unique 16,24-cyclo-26-norlanostane skeleton, and pseudoferic acid C (**3**) featured a *cis*-fused D/E-ring system, were isolated from the root bark of *Pseudolarix kaempferi*. Their structures were elucidated on the basis of extensive spectroscopic analysis. Compounds **2** and **3** exhibited weak inhibitory activities against 11β-HSD1 (11β-hydroxysteroid dehydrogenase 1) with IC_{50} values of 0.44 (mouse 11β-HSD1) and 0.75 μM (human 11β-HSD1), respectively.

Pseudolarix kaempferi Gordon (Pinaceae), mainly distributed in southern China, is a monotypic genus plant. Its root bark, known as 'Tu Jin Pi' in Chinese folk medicine, has been used to treat skin disease caused by fungi.[1] Previous phytochemical investigations on this plant have reported a series of compounds including pseudolaric acids,[2] triterpenoids,[3] flavonoids,[4] lignans,[5] and benzoic acid derivatives.[6] Some of these compounds, such as pseudolaric acids A and B, and pseudolarolide B, were found to possess potent cytotoxic,[3c,7] anti-angiogenic,[8] anti-microbial,[9] and anti-fertility[10] activities. In this study, three novel triterpenoids, pseudoferic acids A–C (**1**–**3**), were isolated and characterized from the root bark of *P. kaempferi*, together with the known compound, pseudolarifuroic acid (**4**). Structurally, pseudoferic acids A (**1**) and B (**2**) possessed a unique 16,24-cyclo-26-norlanostane skeleton, and pseudoferic acid C (**3**) featured a *cis*-fused D/E-ring system. Herein, we described the isolation, structural elucidation, and their biological evaluation, as well as the plausible biogenetic pathway of **1** and **2**.

The air-dried and powdered root bark of *P. kaempferi* (50 Kg) was extracted thrice at room temperature with 95% EtOH, and the crude extract (3.5 Kg) was partitioned between H_2O and EtOAc. The EtOAc fraction (1.1 Kg) was subjected to silica gel column chromatography with a gradient elution of petroleum ether–acetone (from1:0 to 0:1) to afford fractions A–H. Fraction B (35 g) was separated into five fractions, B1–B5, by column chromatography over silica gel with petroleum ether–EtOAc (from 9:1 to 6:4) as the eluent. Fraction B2 (1.5 g) was chromatographed repeatedly on silica gel ($CHCl_3$–Me_2CO, 20:1) and Sephadex LH-20 ($CHCl_3$–MeOH, 1:1) columns to yield **1** (10.0 mg) and **4** (23.1 mg). Fraction C (45 g) was fractionated by MPLC (MCI) eluting with MeOH–H_2O (from 60:40 to 100:0) to provide five fractions, C1–C5. Fraction C4 was further chromatographed over a silica gel column (petroleum ether–acetone, 7:3) and then purified by semipreparative HPLC (MeOH–H_2O, 73:27) to afford **2** (8.5 mg) and **3** (30.5 mg).



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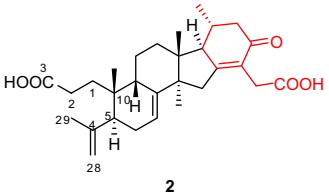

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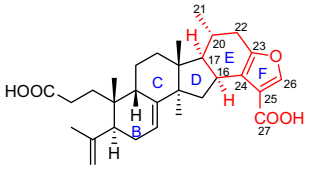

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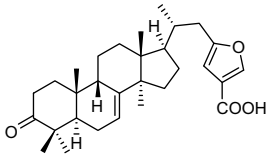

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Pseudoferic acid A (**1**)[11] was obtained as a white amorphous powder and had a molecular formula of $C_{29}H_{40}O_4$, as deduced from HRESIMS at *m/z* 453.3004 $[M+H]^+$ (calcd 453.3004), requiring 10 degrees of unsaturation. The IR spectrum suggested the presence of conjugated carbonyl (1705 cm^{-1}) and olefinic (1639 cm^{-1}) functionalities. The ^1H NMR data of **1** (Table 1) indicated clear signals for six methyls [δ_H 0.76 (s, H_3-18), 0.96 (s, H_3-19), 1.00 (d, J = 6.0 Hz, H_3-21), 1.08 (s, H_3-28 and H_3-29), 1.10 (s, H_3-30)] and one olefinic methine [δ_H 5.73 (m, H-7)]. The ^{13}C NMR (Table 1) and DEPT spectra of **1** displayed 29 carbon signals, consisting of ten quaternary carbons (including one carboxyl, two ketone carbonyls, and three olefinic ones), five methines (including one olefinic carbon), eight methylenes, and six methyls. The aforementioned spectroscopic analysis suggested that **1** was a C_{29} nortriterpenoid with a pentacyclic ring system. Detailed analysis of the ^1H and ^{13}C NMR data of **1** with those of pseudolarifuroic acid (**4**)[7b] suggested that they shared similar substructures for rings A–D, which were supported by the 2D NMR studies (Fig. 1). However, the data for other parts of **1** were quite different from those of **4**. The presence of HMBC correlations from H_2-15 and H-17 to C-16 and C-24, and from H-22b to C-23 and C-24, coupled with the ^1H–^1H COSY correlations of H-17/H-20/H_3-21 and H-20/H_2-22, indicated the connection between C-16 and C-24 to form a six-membered α,β-unsaturated ketone ring E with CH_3-21 attached to C-20. In addition, the key HMBC correlations from H_2-25 to C-16, C-23, C-24, and C-27 suggested the linkage of C-24/C-25/C-27. Therefore, the planar structure of **1** was established as shown in Figure 1.

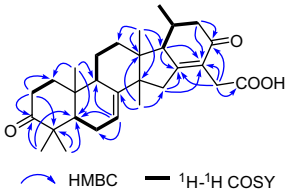


Figure 1. Selected HMBC and ^1H–^1H COSY correlations of **1**.

The relative configuration of **1** was deduced from the ROESY spectrum (Fig. 2). Biogenetically, H-5 and H-17 were tentatively assigned to be α-oriented and H_3-19 to be β-oriented. The observation of ROESY correlations of H_3-28/H-5, H_3-30/H-17, H_3-29/H_3-19, H_3-19/H-9, and H-9/H_3-18 indicated that H-5, H-17, H_3-28, and H_3-30 were in the same orientation (α), while H-9, H_3-18, H_3-19, and H_3-29 were in the opposite orientation (β). Meanwhile, H_3-21 was established as α-oriented in the light of the ROESY correlation between H_3-18 and H-20. Accordingly, compound **1** was identified as a 16,24-cyclo-26-norlanostane triterpenoid with a unique six-membered E-ring and named as pseudoferic acid A.

Table 1
^1H (400 MHz) and ^{13}C (100 MHz) NMR spectroscopic data of **1**–**3**[a] (δ in ppm)

No.	**1**[b]		**2**[b]		**3**[c]	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1a	1.59 m	33.7 t	1.63 m	28.8 t	1.92 overlap	29.5 t
1b	1.70 overlap		1.71 m		1.99 m	
2a	2.43 overlap	34.1 t	2.30 overlap	29.1 t	2.53 overlap	29.9 t
2b	2.53 m		2.30 overlap		2.53 overlap	
3		219.4 s		180.6 s		176.9 s
4		46.9 s		149.7 s		150.3 s
5	1.42 m	52.8 d	2.12 m	45.2 d	2.21 m	45.7 d
6a	1.77 m	22.7 t	2.04 m	29.7 t	1.92 overlap	30.1 t
6b	1.93 m		2.32 m		2.30 m	
7	5.73 m	122.7 d	5.41 br s	118.9 d	5.23 br s	118.3 d
8		146.2 s		144.2 s		146.2 s
9	2.25 m	44.9 d	2.69 m	38.4 d	2.67 m	38.8 d
10		35.9 s		36.4 s		36.9 s
11a	1.70 overlap	19.9 t	1.65 m	18.1 t	1.57 m	18.0 t
11b	1.70 overlap		1.73 m		1.69 overlap	
12a	1.70 overlap	32.4 t	1.77 m	32.3 t	1.59 m	31.7 t
12b	1.98 m		1.99 m		1.69 overlap	
13		44.3 s		43.8 s		45.3 s
14		49.1 s		49.1 s		51.2 s
15a	2.37 d (12.8)	41.3 t	2.48 overlap	42.2 t	1.68 m	43.4 t
15b	2.48 d (12.8)		2.48 overlap		2.88 m	
16		170.8 s		171.4 s	3.85 m	31.7 d
17	2.40, overlap	58.3 d	2.41 d (10.2)	58.6 d	2.03 m	52.4 d
18	0.76 s	22.8 q	0.77 s	22.1 q	0.81 s	23.5 q
19	0.96 s	23.0 q	0.86 s	23.8 q	0.87 s	24.4 q
20	2.06 m	32.4 d	2.09 m	32.9 d	2.18 m	27.5 d
21	1.00 d (6.0)	19.7 q	1.02 d (6.1)	19.8 q	1.00 d (6.8)	25.3 q
22a	2.14 t (13.6)	46.3 t	2.17 t (13.4)	46.5 t	2.35 m	29.3 t
22b	2.45 overlap		2.49 overlap		2.85 m	
23		198.9 s		198.8 s		150.5 s
24		128.5 s		128.1 s		121.1 s
25a	3.21 d (16.2)	31.7 t	3.13 d (16.1)	31.7 t		120.0 s
25b	3.28 d (16.2)		3.45 d (16.1)			
26					8.44 br s	147.7 d
27		174.3 s		176.2 s		166.2 s
28a	1.08 s	28.3 q	4.78 br s	112.1 t	4.90 br s	122.2 t
28b			4.90 br s		4.98 br s	
29	1.08 s	20.9 q	1.80 s	26.1 q	1.80 s	26.1 q
30	1.10 s	27.2 q	1.13 s	27.6 q	1.18 s	27.6 q

[a] Assignments are based on 1D and 2D NMR experiments.
[b] Data measured in $CDCl_3$.
[c] Data measured in pyridine-d_5.

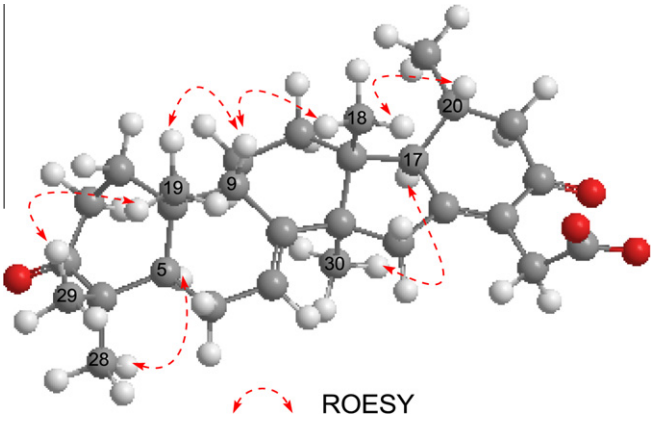
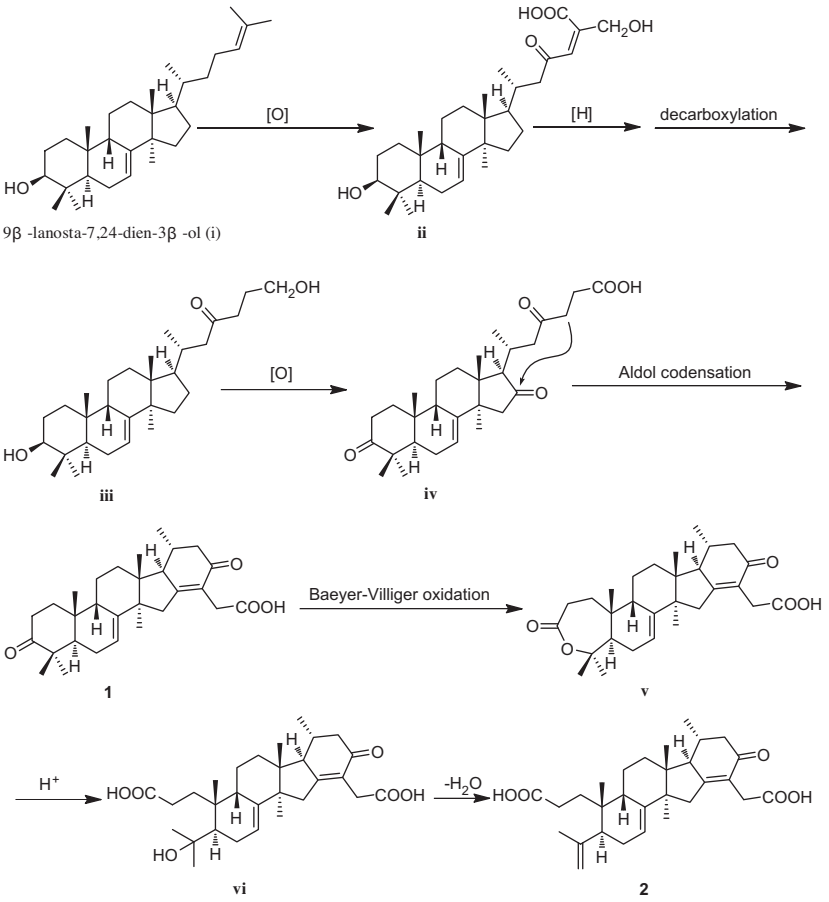


Figure 2. Key ROESY correlations of **1**.

Pseudoferic acid B (**2**)[12] was obtained as a white amorphous powder. The molecular formula of **2** was established as $C_{29}H_{40}O_5$ by HRESIMS at *m/z* 469.2957 $[M+H]^+$ (calcd 469.2953). The NMR data for **2** (Table 1) were similar to those of pseudoferic acid A (**1**) except for the signals ascribed to ring A. The presence of a terminal double bond was revealed by two proton signals at δ_H 4.78 (br s, H-28a) and δ_H 4.90 (br s, H-28b), along with the carbon signals at δ_C 149.7 (C-4) and δ_C 112.1 (C-28).[13] The HMBC correlations of H_3-29 with C-4, C-5, and C-28, suggested that the terminal double bond was located between C-4 and C-28. In addition, the ^{13}C NMR signal at δ_C 180.6 indicated the presence of carboxylic acid. This group was positioned at C-3 by the HMBC correlations of H-1a and H_2-2 with C-3. According to the evidence mentioned above, compound **2** was deduced to be a 3,4-seco-16,24-cyclo-26-norlanostane triterpenoid and named as pseudoferic acid B.

Pseudoferic acid C (**3**)[14] was isolated as a white amorphous powder. Its molecular formula was established as $C_{30}H_{40}O_5$ on the basis of HRESIMS at *m/z* 481.2953 $[M+H]^+$ (calcd 481.2953) with 11 degrees of unsaturation. The IR spectrum showed absorption bands for double bond (1637 cm^{-1}) and carboxyl groups (1698 cm^{-1}). The NMR data of **3** (Table 1) displayed the presence of the same 3,4-seco-ring A unit as in **2**, including the characteristic data of C-3 (δ_C 176.9), C-4 (δ_C 150.3), and C-28 (δ_C 122.2). Detailed 1D and 2D NMR data analyses indicated that other parts of **3** were similar to those of pseudolarifuroic acid (**4**),[7b] except for the C-16 and C-24 moieties. In the HMBC spectrum, the key correlations of H-16 with C-23, C-24, and C-25, suggested the linkage between C-16 and C-24 to construct an unusual six-membered ring. The relative configuration of **3** was deduced from the ROESY spectrum, together with 1D NMR data compared with those of **1** (Table 1). The ROESY correlation of H-16 with H_3-30 revealed α-orientation of H-16. Therefore, the structure of **3** was assigned and named as pseudoferic acid C.

Pseudoferic acids A–C (**1**–**3**) were three novel triterpenoids and possessed unusual 16,24-cyclolanostane skeletons. The co-occurrence of **1**–**3** with pseudolarifuroic acid (**4**) in the same plant implied that they probably originated from the same lanostane precursor, 9β-lanosta-7,24-dien-3β-ol, which could be generated from oxidosqualene.[15] Herein, a hypothetical biogenetic pathway for pseudoferic acids A (**1**) and B (**2**) was proposed as shown in Scheme 1. The lanostane precursor, 9β-lanosta-7,24-dien-3β-ol (**i**), would be transformed into **1** and **2** through a series of biochemical reactions



Scheme 1. Hypothetical biogenetic pathway of **1** and **2**.

Table 2
Inhibitory activities of compounds **1**–**3** against isozymes 11β-hydroxysteroid dehydrogenases

Compounds	Mouse HSD1 IC_{50} (μM)	Mouse HSD2 IC_{50} (μM)	Human HSD1 IC_{50} (μM)	Human HSD2 IC_{50} (μM)
1	>1	ND[a]	>1	ND
2	0.44	>100	>1	ND
3	>1	ND	0.75	>100
Glycyrrhizinic acid	0.0031	ND	0.0021	0.0008

[a] Not determined.

including oxidation, hydrogenation reduction, decarboxylation, intramolecular aldol condensation, Baeyer–Villiger oxidation, ring-opening, and dehydration.

Compounds **1**–**3** were tested for their cytotoxicity against breast cancer (SK-BR-3), hepatocellular carcinoma (SMMC-7721), human myeloid leukemia (HL-60), pancreatic cancer (PANC-1), and lung cancer (A-549) cell lines using the MTT method as previously reported,[16] with cisplatin (sigma) as the positive control. However, none of them were active against the above cancer cell lines with IC_{50} values of more than 40 μM. In addition, compounds **1**–**3** were tested for inhibitory activities against mouse and human 11β-hydroxysteroid dehydrogenases (11β-HSD1 and 11β-HSD2) by Scintillation proximity assay (SPA), using glycyrrhizinic acid as the positive control. The results showed that compounds **2** and **3** exhibited weak inhibitory activities against 11β-HSD1 with IC_{50} values of 0.44 (mouse 11β-HSD1) and 0.75 μM (human 11β-HSD1), respectively (Table 2).

Acknowledgments

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11. Pseudoferic acid A (**1**), white amorphous powder; $\alpha_D^{19.6}$ + 3.26 (*c* 0.53, MeOH); UV (MeOH) λ_{max} (log ε): 248 (4.06), 202 (3.84) nm; IR (KBr) ν_{max} 3440, 2960, 2935, 2872, 1705, 1656, 1639, 1527, 1460, 1382, 1363, 1172, 1146, 1105, 1014, 902, 807, 754 cm^{-1}; NMR data found in Table 1; positive ESIMS: *m*/*z* 453 $[M+H]^+$; positive HRESIMS $[M+H]^+$ *m*/*z* 453.3004 (calcd for $C_{29}H_{41}O_4$ $[M+H]^+$, 453.3004).
12. Pseudoferic acid B (**2**), white amorphous powder; $\alpha_D^{19.3}$ − 77.79 (*c* 0.23, MeOH); UV (MeOH) λ_{max} (log ε): 247 (4.06), 202 (3.95) nm; IR (KBr) ν_{max} 3432, 2973, 2949, 1736, 1685, 1598, 1569, 1453, 1387, 1372, 1290, 1241, 1203, 1129, 1100, 1045, 987, 823, 752 cm^{-1}; NMR data found in Table 1; positive ESIMS: *m*/*z* 469 $[M+H]^+$; positive HRESIMS $[M+H]^+$ *m*/*z* 469.2957 (calcd for $C_{29}H_{41}O_5$ $[M+H]^+$, 469.2953).
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14. Pseudoferic acid C (**3**), white amorphous powder; $\alpha_D^{19.2}$ − 6.89 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε): 203 (4.20), 251 (3.41) nm; IR (KBr) ν_{max} 3431, 2958, 2924, 1709, 1639, 1546, 1460, 1378, 1365, 1153, 1032, 901, 754 cm^{-1}; NMR data found in Table 1; positive ESIMS: *m*/*z* 481 $[M+H]^+$; positive HRESIMS $[M+H]^+$ *m*/*z* 481.2953 (calcd for $C_{30}H_{41}O_5$ $[M+H]^+$, 481.2953).
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