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Benzoic acid allopyranosides from the bark of *Pseudolarix kaempferi*

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Abstract

Two benzoic acid allopyranosides, pseudolaroside A and pseudolaroside B, along with seven known compounds were isolated from the bark of *Pseudolarix kaempferi*. Their structures were determined by analysis of HR-ESI-MS, 1D and 2D NMR spectroscopic data, chemical analysis and comparison to the literature data.

Keywords: *Pseudolarix kaempferi*; Pinaceae; Benzoic acid; Allopyranoside; Pseudolaroside A; Pseudolaroside B

1. Introduction

The root and trunk bark of *Pseudolarix kaempferi* Gord. (Pinaceae), known as "Tu-Jin-Pi" in traditional Chinese medicine, has been widely used for treatment of skin diseases caused by fungal infections in China. A series of characteristic diterpenoids isolated from the bark of this plant have been reported to have antifungal (Li et al., 1982; Li et al., 1995), antitumor (Pan et al., 1990) and antifertility (Zhang et al., 1990) activities, while triterpene lactones from the seeds have cytotoxic properties (Chen et al., 1993). In the course of our investigations concerning on the antifungal natural products derived from traditional Chinese medicines, a systematic phytochemical investigation on the bark of *P. kaempferi* led to isolation of nine compounds (**1**–**9**) including two benzoic acid allopyranosides (**1**, **2**). All these nine compounds were isolated from this plant for the first time.

2. Results and discussion

The ethanol extract of the bark of *P. kaempferi* was suspended in water and partitioned with petrol, EtOAc and *n*-BuOH, successively. The EtOAc fraction was subjected to silica gel, ODS open column chromatography (CC) and further purified using preparative HPLC to obtain pseudolaroside A (**1**), benzoic acid 4-*O*-β-D-allopyranoside, and pseudolaroside B (**2**), 3-methoxy-benzoic acid 4-*O*-β-D-allopyranoside (Fig. 1), together with seven known compounds, vanillic acid (**3**) (Miyase et al., 1984), methyl caffeate (**4**) (Fujita et al., 1984), 3-hydroxy-1-(4-hydroxyphenyl)propan-1-one (**5**) (Achenbach et al., 1983; Fang et al., 1991), senkyunolide I (**6**) (Naito et al., 1996), protocatechuic acid (**7**) (Teng et al., 2005), gallic acid (**8**) (Nawwar et al., 1982), (−)-gallocatechin (2*S*, 3*R*) (**9**) (Lee et al., 1995).

Pseudolaroside A (**1**) was obtained as a colorless amorphous solid and its molecular formula, $C_{13}H_{16}O_8$, was determined by high-resolution electrospray ionization mass spectrum (HR-ESI-MS) ($[M - H]^-$, m/z 299.0766). The ^1H and ^{13}C NMR spectra of **1** displayed signals that could be attributed to a *para*-disubstituted benzene ring [δ_H 7.89 (2H, dd, $J = 8.2$, 1.7 Hz), 7.08 (2H, dd, $J = 8.2$, 1.7 Hz); δ_C 161.0, 131.2 (2×), 124.0, 115.7 (2×)], a carboxy group [δ_H 12.65 (1H, *brs*); δ_C 166.9], and an anomeric proton of a glycosyl moiety [δ_H 5.21 (1H, d, $J = 7.8$ Hz)]. Enzymatic hydrolysis of **1** furnished D-allose which was confirmed by co-HPLC analysis of its 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxy-alditol acetate derivative with the same derivative of the standard sugar. Since the coupling constant of H-1 and H-2 was 7.8 Hz, the anomeric carbon of

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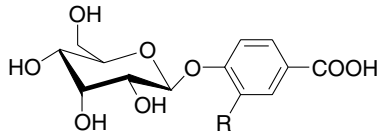

1 R = H

2 R = OCH_3

Fig. 1. Structure of **1** and **2**

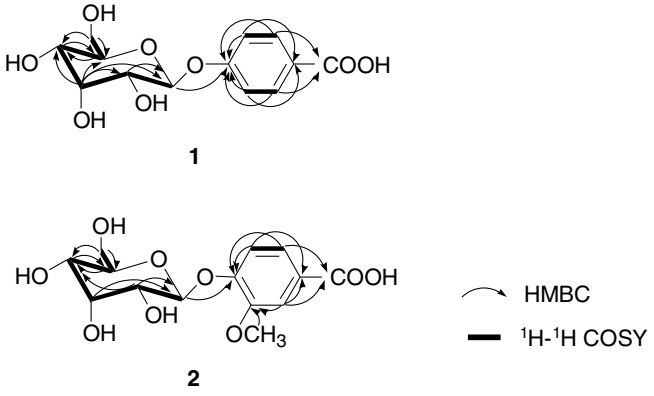

Fig. 2. Key HMBC and ^{1}H-^{1}H COSY correlations in **1** and **2**

1 was determined to be in the β-configuration. In the HMBC spectrum, a correlation was observed between δ_H 5.21 and δ_C 161.0, which suggested that the sugar linkage was at C-4 (Fig. 2). The complete spectroscopic assignment was performed on the basis of analysis of HMQC, HMBC and ^{1}H-^{1}H COSY data (Fig. 2 and Table 1). Consequently, the structure of **1** was established to be benzoic acid 4-*O*-β-D-allopyranoside, named pseudolaroside A.

Pseudolaroside B (**2**) was obtained as a colorless amorphous solid and its molecular formula, $C_{14}H_{18}O_9$, was also determined by HR-ESI-MS ($[M - H]^-$, *m/z* 329.0867). The ^{1}H and ^{13}C NMR spectra of **2** showed that the signals belonged to an ABX-pattern substituted benzene ring [δ_H 7.52 (1H, dd, $J = 8.4$, 1.9 Hz), 7.47 (1H, d, $J = 1.9$ Hz), 7.11 (1H, d, $J = 8.4$ Hz)], a carboxy group [δ_H 12.67 (1H, *brs*); δ_C 167.0], a methoxy group [δ_H 3.81 (3H, s); δ_C 55.6], and an anomeric proton of a glycosyl moiety [δ_H 5.24 (1H, d, $J = 7.8$ Hz)]. The glycosyl moiety of **2** had almost the same ^{1}H and ^{13}C NMR spectroscopic data as that of **1**. The corresponding derivative of **2** showed the same retention time as those of **1** and D-allose, hence **2** was confirmed to have the same glycosyl group as **1**. The anomeric proton appeared at δ_H 5.24, which showed a HMBC correlation with δ_C 150.5, suggesting glycosylation at C-4. Another obvious HMBC correlation was observed between δ_H 3.81 and δ_C 148.5, indicating methoxylation at C-3 (Fig. 2). Thus, structure of **2** was elucidated as 3-methoxy-benzoic acid 4-*O*-β-D-allopyranoside, named pseudolaroside B.

To our knowledge, this is the first example of the presence of benzoic acid alloside derivatives in natural products. It is noteworthy that we have initially assigned the glycosyl moieties of **1** and **2** as α-D-altrose, because the ^{13}C NMR spectroscopic data of D-altrose and D-allose were similar and the R_f values of the enzymatic hydrolysis products of **1** and **2** were more like that of D-altrose in TLC. However, the results of more detailed analysis by NMR (i.e., raising the testing temperature and diluting the sample

Table 1
^{13}C and ^{1}H NMR spectroscopic data for **1** and **2**

Position	**1**[a]		**2**[a]		**1**[b]		**2**[b]	
	^{13}C	^{1}H [m, *J* (Hz)]	^{13}C	^{1}H [m, *J* (Hz)]	^{13}C	^{1}H [m, *J* (Hz)]	^{13}C	^{1}H [m, *J* (Hz)]
1	124.0	–	124.0	–	126.1	–	127.0	–
2	131.2	7.89 (dd, 8.2; 1.7)	112.7	7.47 (d, 1.9)	133.1	7.96 (d, 9.0)	114.9	7.60 s
3	115.7	7.08 (dd, 8.2; 1.7)	148.5	–	117.6	7.13 (d, 9.0)	150.8	–
4	161.0	–	150.5	–	163.5	–	152.6	–
5	115.7	7.08 (dd, 8.2; 1.7)	114.1	7.11 (d, 8.4)	117.6	7.13 (d, 9.0)	116.8	7.20 (d, 8.5)
6	131.2	7.89 (dd, 8.2; 1.7)	122.9	7.52 (dd, 8.4; 1.9)	133.1	7.96 (d, 9.0)	125.2	7.63 (d, 8.5)
–*C*=O	166.9	–	167.0	–	170.2	–	170.6	–
OCH_3			55.6	3.81 s			57.2	3.89 s
1′	98.0	5.21 (d, 7.8)	97.9	5.24 (d, 7.8)	100.1	5.34 (d, 7.5)	100.5	5.35 (d, 8.0)
2′	70.2	3.40–3.48*	70.1	3.41–3.49*	72.5	3.59–3.62*	72.5	3.65–3.69*
3′	71.4	3.94 (t, 2.7)	71.6	3.92 (t, 2.9)	73.5	4.14 (t, 3.0)	73.4	4.15 (t, 3.0)
4′	66.9	3.40–3.48*	66.9	3.41–3.49*	69.1	3.59–3.62*	69.1	3.60 (dd, 3.0; 9.5)
5′	74.7	3.66–3.74*	74.7	3.63–3.71*	76.3	3.86–3.90*	76.4	3.85–3.87*
6′	60.8	3.40–3.48*	60.9	3.41–3.49*	63.3	3.68 (*q*, 6.0)	63.3	3.65–3.69*
		3.66–3.74*		3.63–3.71*		3.86–3.90*		3.85–3.87*
OH-2′	–	5.14 (d, 6.8)	–	5.02 (d, 7.3)	–		–	
OH-3′	–	5.00 (d, 3.5)	–	4.99 (d, 3.6)	–		–	
OH-4′	–	4.71 (d, 7.7)	–	4.69 (d, 7.3)	–		–	
OH-6′	–	4.52 (t, 5.5)	–	4.48 (t, 5.6)	–		–	

[a] ^{13}C (100 MHz) and ^{1}H (400 MHz) NMR in DMSO-d_6.
[b] ^{13}C (125 MHz) and ^{1}H (500 MHz) NMR in CD_3OD.
* Overlapped signals.

with CCl_4) indicated that the sugar was not α-D-altrose (Angyal and Pickles, 1972; Fujita et al., 1999). So the 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxy-alditol acetate derivatives of the hydrolytic products of **1** and **2** were produced and then analyzed by HPLC. This established that they had the same retention times with the corresponding derivative of D-allose, and proved that the glycosyl moieties of **1** and **2** were D-allose. This experience suggests that more patience and efforts should be paid in the identification of allose or altrose. On the other hand, it was also demonstrated that TLC was not a sufficient method for the identification of sugars and the method of preparing chiral derivatives and comparison with standards using HPLC should instead give unambiguous results. Furthermore, a series of ^{1}H NMR spectroscopic experiments of **1** and **2** were also conducted in CD_3OD under different magnetic fields (300, 500, 600 MHz) to obtain better resolution of the ^{1}H signals. The results showed that one more proton could be distinguished from the overlapped signals in CD_3OD, rather than DMSO-d_6, however, there were still two overlapping resonances involving four protons, and the overlapping patterns were almost same under the different magnetic fields in CD_3OD. So the overlap of signals should be attributed to the close chemical shifts of hydrogen atoms, and that was not improved by increasing the intensity of magnetic field. It was also observed that the glycosyl proton signals resolved better in CD_3OD than in DMSO-d_6. In addition, seven known compounds were isolated and identified by comparison with the literature data and authentic substances.

3. Experimental

3.1. General experimental procedures

IR and UV were recorded on Nicolet Avatar FT-IR and TU-1901 UV–Vis spectrophotometers, respectively. Optical rotations were performed with an AA10R digital polarimeter in MeOH at 25 °C. HR-ESI-MS was conducted using an ABI Qstar mass spectrometer. The NMR spectra were recorded on Bruker ARX-400 spectrometer in DMSO-d_6 and Bruker ARX-300, DRX-500 and DRX-600 spectrometers in CD_3OD. The chemical shifts are given in δ (ppm) values and the coupling constants (J values) are reported in Hertz (Hz). Analytical HPLC was performed on an Agilent 1100 liquid chromatograph system consisting of a quaternary pump, diode array detector, using Senshu Pak PEGASIL ODS-II (4.6 mm i.d. × 250 mm) and Inertsil ODS-3 (4.6 mm i.d × 250 mm) column. Preparative HPLC was performed using a TSP P100 pump connected with a TSP UV100 detector, using a Senshu Pak PEGASIL ODS-II column (20 mm i.d. × 150 mm) with flow rate set at 4 ml/min and wavelength detection at 254 nm. Silica gel (200–300 mesh, Qingdao Marine Chemical Co. Ltd.), ODS (Fuji silysia chemical Ltd.) were used in open CC fractionations. All chemical solvents used for product isolation were of analytical grade. Standard sugars and reagents for derivatization were purchased from Sigma and Fluka.

3.2. Plant material

The bark of *P. kaempferi* was collected from Zhejiang Province, China and identified by Dr. Hongzhu Guo. A voucher specimen (040309TJP01) was deposited in the Division of Pharmacognostical Biotechnology, School of Pharmaceutical Sciences, Peking University Health Science Center, China.

3.3. Extraction and isolation

The bark of *P. kaempferi* (15 kg) was refluxed with 95% EtOH (2 × 45 l, 2 × 2 h). The resulting EtOH extract was concentrated (2 kg), suspended in H_2O and partitioned successively with petrol, EtOAc and *n*-BuOH. The EtOAc fraction (154.7 g) was then subjected to silica gel CC eluted with $CHCl_3$–MeOH (30:1–2:1) to furnish fractions A–G. Fraction D was applied to silica gel ($CHCl_3$–MeOH 20:1), ODS open CC (40–60% MeOH), and further purified with HPLC (MeOH–H_2O 45:55) to furnish **3** (140 mg), **4** (10 mg), **5** (24 mg) and **6** (11 mg), respectively. Fraction F was isolated using ODS open CC (20–60% MeOH) and HPLC (MeOH–H_2O 1:4) to obtain **7** (42 mg). Fraction G was subjected to silica gel CC eluted with $CHCl_3$–MeOH (10:1–2:1) and further purified using ODS CC (20–60% MeOH) and then HPLC (MeOH–H_2O 12:88) to furnish **8** (5 mg), **1** (30 mg), **9** (4 mg) and **2** (13 mg), respectively.

3.4. Pseudolaroside A (*1*)

Colorless amorphous solid; $[\alpha]_D^{25}$ − 126.3 (MeOH; c 0.095); UV λ_{max}^{MeOH} nm (log ε): 246.60 (5.26); IR ν_{max}^{KBr} cm^{-1}: 3430 (–OH), 1663 (>C=O), 1608 (benzene ring); The ^{1}H and ^{13}C NMR spectroscopic data, see Table 1; HR-ESI-MS $[M - H]^-$ m/z 299.0766 (Calcd. for $C_{13}H_{15}O_8$, 299.0772).

3.5. Pseudolaroside B (*2*)

Colorless amorphous solid; $[\alpha]_D^{25}$ − 145.4 (MeOH; c 0.055); UV λ_{max}^{MeOH} nm (log ε): 249.60 (3.89), 287.60 (3.52); IR ν_{max}^{KBr} cm^{-1}: 3377 (–OH), 1695 (>C=O), 1599 (benzene ring); The ^{1}H and ^{13}C NMR spectroscopic data, see Table 1; HR-ESI-MS $[M - H]^-$ m/z 329.0867 (Calcd. for $C_{14}H_{17}O_9$, 329.0878).

3.6. Enzymatic hydrolysis of *1, 2*

The solution of compound **1** or **2** (3.0 mg) in 0.1 M acetate buffer (pH 4.0, 0.5 ml) was treated with naringinase (Sigma Chemical Co., 6.0 mg) and the reaction mixture was stirred at 40 °C for 24 h. The reaction mixture was then passed through a Sep-Pak C_{18} cartridge (Waters Co.)

eluted with H_2O and MeOH, successively. The H_2O eluate was concentrated and the residue was dissolved in 1 ml H_2O, to which L-(−)-α-methylbenzylamine (5 mg) and $NaBH_3CN$ (8 mg) in EtOH (1 ml) was added. After being stirred at 40 °C for 4 h followed by addition of glacial HOAc (0.2 ml) and evaporated to dryness, the resulting solid was acetylated with Ac_2O (0.3 ml) in pyridine (0.3 ml) for 24 h at room temp. The reaction mixture was evaporated 5 times by adding H_2O to remove pyridine, and then passed through a Sep-Pak C_{18} cartridge with H_2O–CH_3CN (4:1) and H_2O–CH_3CN (1:1) (each 10 ml), successively. The H_2O–CH_3CN (1:1) eluate was analyzed and the 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivative was identified by co-HPLC analysis with the derivative of standard sugar prepared under the same conditions (Oshima and Kumanotani, 1981; Oshima et al., 1982). HPLC conditions: Inertsil ODS-3 column (4.6 mm i.d. × 250 mm); solvent, CH_3CN–H_2O (2:3); flow rate, 0.8 ml/min; column temperature, 40 °C ; detection, UV 230 nm. The derivative of D-allose was detected with t_R of 29.77 min.

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