Novel Phenolic Glycoside Dimer and Trimer from the Whole Herb of *Pyrola rotundifolia*

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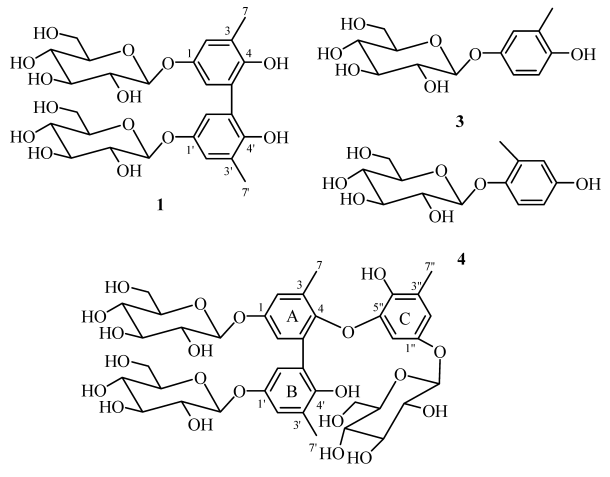
From the water-soluble constituents of the whole herb of *Pyrola rotundifolia* (Pyrolaceae), one novel phenolic glycoside dimer, pyrolaside A (1), and one novel phenolic glycoside trimer, pyrolaside B (2), together with two known phenolic glycosides homoarbutin (3) and isohomoarbutin (4), were isolated. The structures were elucidated by spectroscopic analysis and confirmed with chemical degradation. *In vitro* tests for antimicrobial activity showed pyrolaside B (2) to possess significant activity against two Gram-positive organisms, *Staphylococcus aureus* and *Micrococcus luteus*.

Key words *Pyrola rotundifolia*; Pyrolaceae; phenolic glycoside dimer; phenolic glycoside trimer; antimicrobial activity

Pyrola rotundifolia (Pyrolaceae) has been used for the treatment for neuralgia, gastric hemorrhage, pulmonary hemorrhage, and arthritic diseases as a traditional Chinese medicine. The water extract of the plant was reported to inhibit the growth of many kinds of human pathogenic bacilli *in vitro*.[1] Thus far only nine compounds, *i.e.* arbutin, homoarbutin, isohomoarbutin, *N*-benzyl-2-naphthylamine, chimaphilin, quercetin, gallic acid, protocatechuic acid, and ursolic acid have been isolated from *P. rotundifolia*.[2—4] Wang *et al.* reported that chimaphilin, *N*-benzyl-2-naphthylamine, and gallic acid inhibited the growth of the leukemia cell line P388.[3] Kosuge *et al.* reported that anti-inflammatory and analgesic principles of *P. rotundifolia* were identified as ursolic acid and chimaphilin by *in vivo* models.[4] The focus was set on poplar constituents of *P. rotundifolia* in this report, as a part of the studies on the hydrophilic bioactive constituents from Chinese medicines.[5,6] The 60% aqueous acetone extract of the whole herb of *P. rotundifolia* was subjected to column chromatography on MCI gel CHP 20P, Cosmosil 75 C_{18}-OPN and TSK gel Toyopearl HW-40F to yield one novel phenolic glycoside dimer, pyrolaside A (**1**), and one novel phenolic glycoside trimer, pyrolaside B (**2**), together with two known phenolic glycosides homoarbutin (**3**) and isohomoarbutin (**4**).



1

3

4

2

Results and Discussion

Compound **1** was obtained as a white amorphous powder. The positive and negative ESI-MS showed quasimolecular ion peaks at *m/z* 593 $[M+Na]^+$ and 569 $[M-H]^+$, respectively, indicating a molecular weight of 570. According to the HR-ESI-MS, ^{1}H- and ^{13}C-NMR spectral data, the molecular formula of **1** was determined to be $C_{26}H_{34}O_{14}$. The IR absorption at 3381 cm^{-1} for hydroxyl groups and 1597 cm^{-1} for an aromatic ring suggested that compound **1** was a phenolic compound, and the UV absorption also indicated the presence of an aromatic moiety (201.1, 292.7 nm). In the ^{1}H-NMR spectrum (Table 1), the *meta*-correlated signals at δ 6.96 (1H, d, J=2.7 Hz, H-2) and δ 6.91 (1H, d, J=2.7 Hz, H-6) defined a 1,3,4,5-tetrasubstituted aromatic ring, which was confirmed in the ^{13}C-NMR spectrum by four quaternary aromatic carbon peaks at δ 153.1 (C-1), 129.0 (C-3), 148.4 (C-4), and 129.2 (C-5) and two methine peaks at δ 120.6 (C-2), and 119.0 (C-6). The high field of ^{1}H-NMR spectrum showed one aromatic methyl at δ 2.25 (3H, s, H-7). The linkage between the methyl group and aromatic ring, established unambiguously by an HMBC experiment, concluded that **1** was the analogue of homoarbutin. The molecular formula showed that **1** was the dehydro homoarbutin dimer. The signals in the NMR spectra indicated the symmetry of the two homoarbutin units in the molecule. The linkage between homoarbutin units must be located on C-5 and C-5′ according to the ^{13}C-NMR spectrum. The glucosyl moiety was identified by acid hydrolysis of **1** and comparison with authentic sample. The β-stereochemistry of anomeric carbon was determined by the coupling constant (J=7.4 Hz) of anomeric proton.[7] With all of these evidence, compound **1** was identified as 5-5′-dehydro-di(3-methyl-4-hydroxy-phenyl-1-*O*-β-D-glucopyranoside), and given the trivial name pyrolaside A.

Compound **2** was obtained as a white amorphous powder. The positive and negative ESI-MS showed quasimolecular ion peaks at *m/z* 877 $[M+Na]^+$ and 853 $[M-H]^+$, respectively, indicating a molecular weight of 854. The molecular formula of **2** was determined to be $C_{39}H_{50}O_{21}$, with the HR-ESI-MS, ^{1}H- and ^{13}C-NMR spectral data. The IR absorption at 3406 cm^{-1} for hydroxyl groups and 1624 and 1603 cm^{-1} for an aromatic ring suggested that compound **2** was also a phenolic compound, and the UV absorption also indicated

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Table 1. NMR Data for Compound **1**

Position	^{1}H[a)]	^{13}C[b)]	HMBC	Position	^{1}H[a)]	^{13}C[b)]
1 (1′)		153.1 s	H-2, H-6, Glc-1	Glc-1 (1′)	4.81 d (7.4)	103.8 d
2 (2′)	6.96 d (2.7)	120.6 d	H-6, H-7	Glc-2 (2′)	3.30 dd (9.3, 7.4)	75.4 d
3 (3′)		129.0 s	H-7	Glc-3′ (3′)	3.40 dd (9.3, 9.2)	78.5 d
4 (4′)		148.4 s	H-2, H-6, H-7	Glc-4′ (4′)	3.32 dd (9.3, 9.2)	71.9 d
5 (5′)		129.2 s	H-6	Glc-5′ (5′)	3.35 m	78.3 d
6 (6′)	6.91 d (2.7)	119.0 d	H-2	Glc-6a (6′a)	3.88 dd (12.0, 2.0)	62.9 t
7 (7′)	2.25 s	17.4 q	H-2	Glc-6b (6′b)	3.67 dd (12.0, 5.7)	

a) 400 MHz, CD_3OD; chemical shifts in ppm relative to TMS; coupling constant (*J*) in Hz. *b*) 100 MHz, CD_3OD; multiplicity was established from DEPT data.

Table 2. NMR Data for Compound **2**

Position	^{1}H[a)]	^{13}C[b)]	HMBC	Position	^{1}H[a)]	^{13}C[b)]
1		155.9 s	H-2, H-6, Glc-1	Glc-1	4.85 d (7.4)	102.5 d
2	6.94 d (2.8)	120.0 d	H-6, H-7	Glc-2	3.20 m	74.9 d
3		134.1 s	H-7	Glc-3	3.35 m	77.8 d
4		146.7 s	H-2, H-6, H-7	Glc-4	3.27 m	71.1 d
5		127.6 s	H-6	Glc-5	3.30 m	77.6 d
6	6.86 d (2.8)	118.6 d	H-2, H-6′	Glc-6a	3.72 d (12.0)	62.3 t
7	2.04 s	16.8 q	H-2	Glc-6b	3.54 m	
1′		152.1 s	H-2′, H-6′, Glc-1′	Glc-1′	4.40 d (7.8)	104.0 d
2′	6.66 d (2.6)	120.8 d	H-6′, H-7′	Glc-2′	3.22 m	75.0 d
3′		128.3 s	H-7′	Glc-3′	3.37 m	78.2 d
4′		148.1 s	H-2′, H-7′	Glc-4′	3.28 m	71.5 d
5′		134.7 s		Glc-5′	3.31 m	77.8 d
6′	6.63 br s	118.0 d		Glc-6′a	3.79 dd (12.0, 2.0)	62.5 t
7′	2.07 s	17.0 q	H-2′	Glc-6′b	3.55 m	
1″		151.5 s	H-2″, H-6″, Glc-1″	Glc-1″	4.40 d (7.8)	104.0 d
2″	6.31 d (2.7)	113.4 d	H-6″, H-7″	Glc-2″	3.22 m	75.0 d
3″		126.4 s	H-7″	Glc-3″	3.37 m	78.2 d
4″		140.6 s	H-2″, H-6″, H-7″	Glc-4″	3.28 m	71.5 d
5″		147.0 s	H-6″	Glc-5″	3.31 m	77.8 d
6″	5.93 d (2.7)	102.8 d	H-2″	Glc-6″a	3.79 dd (12.0, 2.0)	62.5 t
7″	1.99 s	16.2 q	H-2″	Glc-6″b	3.55 m	

a) 400 MHz, D_2O; chemical shifts in ppm relative to TMS; coupling constant (*J*) in Hz. *b*) 100 MHz, D_2O; multiplicity was established from DEPT data.

the presence of an aromatic moiety (202.4, 288.0 nm). The ^{1}H-NMR spectrum (Table 2), showed three pairs of the *meta*-correlated signals at δ 6.94 (1H, d, J=2.8 Hz, H-2) and δ 6.86 (1H, d, J=2.8 Hz, H-6), δ 6.66 (1H, d, J=2.6 Hz, H-2′) and δ 6.63 (1H, br s, H-6′), and δ 6.31 (1H, d, J=2.7 Hz, H-2″) and δ 5.93 (1H, d, J=2.7 Hz, H-6″). The high field of ^{1}H-NMR spectrum also showed three aromatic methyls at δ 2.04 (3H, s, H-7), δ 2.07 (3H, s, H-7′), and δ 1.99 (3H, s, H-7″). The location of the methyl groups on the aromatic rings were established unambiguously by an HMBC experiment. The molecular formula showed that **2** was the homoarbutin trimer. The linkage between ring A and B must be located between C-5 and C-5′ according to the ^{13}C-NMR spectrum, in which there are two quaternary aromatic carbons at δ 127.6 (C-5) and 134.7 (C-5′). The two deshielded signals at δ 147.0 (C-5″) and δ 155.9 (C-1) and one shielded signal at δ 146.7 (C-4) indicated that C-5″ was an oxygen-bearing quaternary aromatic carbon, and ring C was connected with ring A by ether bond at C-4 and C-5″. The glucosyl moiety was identified by acid hydrolysis of **2** and comparison with authentic sample, and the β-stereochemistry of anomeric carbon was determined by the coupling constants (J=7.4, 7.8, 7.8 Hz) of anomeric protons.[7] Consequently, compound **2** was identified as 5-5′-dehydro-[(4-*O*-5″)-dehydro-di(3-methyl-4-hydroxy-phenyl-1-*O*-β-D-glucopyranoside)]-(3-methyl-4-hydroxy-phenyl-1-*O*-β-D-glucopyranoside), and given the trivial name pyrolaside B.

Homoarbutin (**3**) and isohomoarbutin (**4**) were also identified by comparison of their spectroscopic data with the values in literature.[2] Because of the reported pharmacological activities and the traditional usage of the whole herb of *Pyrola rotundifolia*, compounds **1** and **2** were tested in an antimicrobial assay.[8] Pyrolaside B (**2**) showed significant activity against two Gram-positive organisms, *Staphylococcus aureus* (MIC 35.0 μM) and *Micrococcus luteus* (MIC 20.5 μM), using bakuchiol as a positive control (MIC 20.0, 10.0 μM, respectively).

Experimental

General Experimental Procedures Optical rotation data were obtained on a Perkin-Elmer 241 automatic digital polarimeter. UV spectral data were measured on a Shimadzu UV-260 instrument. IR spectral data were measured on a Perkin-Elmer 599B instrument with KBr disks. ^{1}H-, ^{13}C-NMR, COSY, HMQC, and HMBC spectra were recorded on a Bruker DRX-400 spectrometer (^{1}H 400 MHz and ^{13}C 100 MHz). The carbon multiplicities were obtained by DEPT experiment. ESI-MS data were measured on a Quattro instrument. Gas chromatography (GC) was run on a HP 1890 gas chromatography. Reversed-phase chromatography utilized TSK gel Toyopearl HW-40F (30—60 μm, Toso Co., Ltd.), MCI gel CHP 20P (75—150 μm, Mitsubishi Chemical Industries Co., Ltd.) and Cosmosil 75 C_{18}-OPN (42—

105 μm, Nacalai Tesque Inc.) columns. TLC was performed using precoated silica gel 60 F_{254} plates (0.2 mm, Merck).

Plant Material The whole herb of *Pyrola rotundifolia* (Pyrolaceae) were collected in Jiangxi Province, People's Republic of China in 2004, and authenticated by Dr. Chang J. A voucher specimen has been deposited in the Herbarium of our lab (DMP 2004040).

The whole herb of *Pyrola rotundifolia* (Pyrolaceae) (2 kg) was extracted three times with 60% aqueous acetone at room temperature (3×10 l). The solvent was evaporated under reduced pressure to 1 l and filtered with celite. The filtrate was concentrated *in vacuo* to yield 75 g of a gummy residue. The extract was dissolved in 400 ml water, and subjected to MCI gel CHP 20P (8×60 cm) eluting with MeOH/H_2O gradient with a flow rate of 15 ml/min to obtain fraction 1 [1.0 l, H_2O], fraction 2 [0.6 l, MeOH/H_2O (10 : 90)], fraction 3 [0.6 l, MeOH/H_2O (30 : 70)], fraction 4 [0.6 l, MeOH/H_2O (50 : 50)], fraction 5 [1.0 l, MeOH/H_2O (70 : 30)], and fraction 6 [2.0 l, MeOH]. Fraction 2 (5.4 g) was chromatographed on Toyopearl HW-40F (6×60 cm) using water as eluent to obtained six fractions 2A—F (eluent volume: 200 ml/fraction). Fraction 2C (0.31 g) was further purified by MCI gel CHP 20P (5×40 cm, eluted with H_2O→10% MeOH) and Cosmosil 75 C_{18}-OPN (4×30 cm, eluted with H_2O→10% MeOH) to give **3** (15 mg) and **4** (21 mg). Fraction 3 (6.2 g) was chromatographed on Toyopearl HW-40F (6×60 cm) using water as eluent to obtained five fractions 3A—E (eluent volume: 200 ml/fraction). Fraction 3C (0.22 g) was further purified by MCI gel CHP 20P (5×40 cm, eluted with 10—30% MeOH) and Cosmosil 75 C_{18}-OPN (4×30 cm, eluted with H_2O→30% MeOH) to give **1** (16 mg). Fraction 4 (6.0 g) was chromatographed on Toyopearl HW-40F (6×60 cm) using water as eluent to obtained six fractions 4A—F (eluent volume: 200 ml/fraction). Fraction 4C (0.25 g) was further purified by MCI gel CHP 20P (5×40 cm, eluted with 30—50% MeOH) and Cosmosil 75 C_{18}-OPN (4×30 cm, eluted with H_2O→50% MeOH) to give **2** (15 mg). Experiments were monitored using TLC developed in benzene/formic acid/ethyl acetate (3 : 2 : 5), and detected by spraying with sulfuric acid/ethanol (1 : 4) reagent followed by heating.

Pyrolaside A (**1**): White amorphous powder; $[\alpha]_D^{20}$ 0° (c=0.10, H_2O); ESI-MS (positive and negative) m/z 593 $[M+Na]^+$, 569 $[M-H]^+$; HR-ESI-MS $[M+Na]^+$ m/z 593.1848, Calcd 593.1846 for $C_{26}H_{34}O_{14}Na$; UV (MeOH) λ_{max} (log ε) 201.1 (3.80), 292.7 (2.10) nm; IR (KBr) ν_{max} 3381, 2926, 1597, 1481, 1355, 1200, 1080, 1051, 860, 630 cm^{-1}; ^{1}H- and ^{13}C-NMR spectral data were listed in Table 1.

Pyrolaside B (**2**): White amorphous powder; $[\alpha]_D^{20}$ −47.4° (c=0.10, H_2O); ESI-MS (positive and negative) m/z 877 $[M+Na]^+$, 853 $[M-H]^+$; HR-ESI-MS $[M+Na]^+$ m/z 877.2746, Calcd 877.2742 for $C_{39}H_{50}O_{21}Na$; UV (MeOH) λ_{max} (log ε) 202.4 (3.93), 288.0 (2.15) nm; IR (KBr) ν_{max} 3406, 2920, 1624, 1603, 1497, 1196, 1074, 1043, 588 cm^{-1}; ^{1}H- and ^{13}C-NMR spectral data were listed in Table 2.

Acid Hydrolysis of 1 and 2 A solution of **1** and **2** (6 mg each) in 2 M HCl was heated (90 °C) for 2 h. After removing HCl by evaporation in vacuum, the mixture was diluted with H_2O and extracted with EtOAc. The aqueous layer was neutralized with 1 M NaOH and subjected to TLC analysis on Kieselgel 60 F_{254} (Merck) [using $CHCl_3$–MeOH–H_2O (30 : 12 : 9), 9 ml and HOAc, 1 ml] and paper chromatography [using *n*-BuOH–HOAc–H_2O (4 : 1 : 5)] with authentic sugars, in which the presence of glucose was established. The remaining aqueous layer was then passed through an Amberlite IRA-60E column, and the aqueous eluate was concentrated and derivative with thiazolidine as reported previously.[9] Only D-glucose derivative was detected by GC. (GC conditions: column, Supelco SPB^{-1}, 0.25 mm×27 m, column temperature 230 °C; carrier gas, N_2; t_R, D-glucose derivative 17.9 min, L-glucose derivative 17.3 min).

Bioassay Procedures The microbial strains were from the American Type Culture Collection, and the antimicrobial susceptibility tests were carried out by a microdilution assay.[8] 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 dissolved in DMSO. The blank controls of microbial culture were incubated with a limited amount of DMSO under the same conditions. DMSO was determined not to be toxic under these experimental conditions.

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