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PESTARHAMNOSES A–C, RHAMNOSYLATED PHENOL DERIVATIVES FROM THE SOFT CORAL-DERIVED FUNGUS *Pestalotiopsis* sp.

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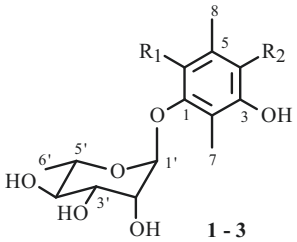
Reinvestigation of the secondary metabolites of the soft coral-derived fungus Pestalotiopsis *sp. (ZJ-2009-7-6), cultured in a bromine-modified medium, led to the isolation of three new rhamnosylated phenol derivatives, pestarhamnoses A–C (**1**–**3**), together with the former metabolites, (±)-pestalachlorides C and D (**4** and **5**). Their structures were elucidated through extensive 1D and 2D NMR spectroscopy and mass spectrometry. The relative configurations of the rhamnosyl moieties were determined as α-rhamnoses by their anomeric $^1J_{CH}$ coupling constants. The absolute configurations of **1**–**3** were determined as L-rhamnose residues by HPLC analysis of their thiocarbamoyl-thiazolidine derivatives and that of authentic L-rhamnose. This is the first report of phenol glycosides with α-L-rhamnose in nature.*

Keywords: rhamnosylated phenol derivative, bromine-modified medium, *Pestalotiopsis* sp.

Pestalone, a chlorinated benzophenone antibiotic, was isolated from the co-culture of a *Pestalotia* fungus and a unicellular marine bacterium (strain CNJ-328) by Fenical and co-workers in 2001 [1]. Recently, pestalone was re-evaluated to display significant antimicrobial activity against different MRSA strains and several plant pathogens [2]. In 2008, Che and co-workers reported the discovery of the antifungal pestalachlorides A–C from the plant endophytic fungus *Pestalotiopsis adusta* [3]. As part of our ongoing investigation on new natural antibacterial and cytotoxic products from marine fungi in the South China Sea [4–7], our group discovered another similar new antibiotic, (±)-pestalachloride D, from the soft coral-derived fungus *Pestalotiopsis* sp. collected from the Yongxing Island in the South China Sea [8]. Since all the above natural compounds possess chlorine atoms, these chlorine atoms should have originated from the cultivation medium and natural seawater. Expecting that bromine-containing compounds might be obtained by the use of a medium in which a bromide solution replaces seawater, we cultivated the same strain in a bromine-modified medium. Although we were unable to isolate the brominated compounds, we succeeded in isolating three new rhamnosylated phenol derivatives, pestarhamnoses A–C (**1**–**3**), which share the same biogenetic phenol moiety of chlorinated diphenylmet, together with (±)-pestalachlorides C and D (**4** and **5**) [3, 8]. Herein, we report the cultivation with bromine-modified medium and isolation, structure determination, and biological evaluation of pestarhamnoses A–C (**1**–**3**).



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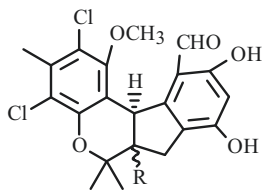

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1: $R_1 = CH_3$, $R_2 = H$; **2:** $R_1 = H$, $R_2 = CH_3$; **3:** $R_1 = R_2 = H$; **4:** R = *β*H; **5:** R = *α*H

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TABLE 1. ^{1}H and ^{13}C NMR Data for **1–3** (δ, ppm, J/Hz)

C atom	**1**[a]		**2**[a]		**3**[b]	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	157.0	–	152.8	–	156.5	–
2	115.2	–	110.9	–	111.5	–
3	154.1	–	153.5	–	156.9	–
4	113.0	6.51 s	116.4	–	110.7	6.29 s
5	135.3	–	134.3	–	137.1	–
6	120.2	–	108.4	6.57 s	107.7	6.45 s
7	10.0	2.12 s	11.1	2.10 s	8.4	1.99 s
8	19.9	2.13 s	8.4	2.09 s	21.5	2.18 s
9	12.7	2.09 s	19.7	2.20 s	–	–
1′	105.1	4.90 (d, J = 1.5)	98.7	5.39 (d, J = 1.5)	99.6	5.33 (d, J = 1.8)
2′	71.8	4.26 (dd, J = 3.3, 1.5)	71.0	4.05 (dd, J = 3.4, 1.5)	72.2	3.99 (dd, J = 3.5, 1.8)
3′	72.2	3.90 (dd, J = 9.3, 3.3)	71.6	3.87 (dd, J = 9.4, 3.4)	72.4	3.86 (dd, J = 9.5, 3.5)
4′	72.9	3.53 (t, J = 9.3)	72.8	3.49 (t, J = 9.4)	73.8	3.43 (t, J = 9.5)
5′	71.0	4.07 m	69.3	3.67 m	70.5	3.63 m
6′	17.9	1.26 (d, J = 6.2)	17.4	1.20 (d, J = 6.3)	17.9	1.22 (d, J = 6.1)

[a]Measured at 600 MHz (^{1}H) and 150 MHz (^{13}C), acetone-d_6; [b]measured at 600 MHz (^{1}H) and 150 MHz (^{13}C), methanol-d_4.

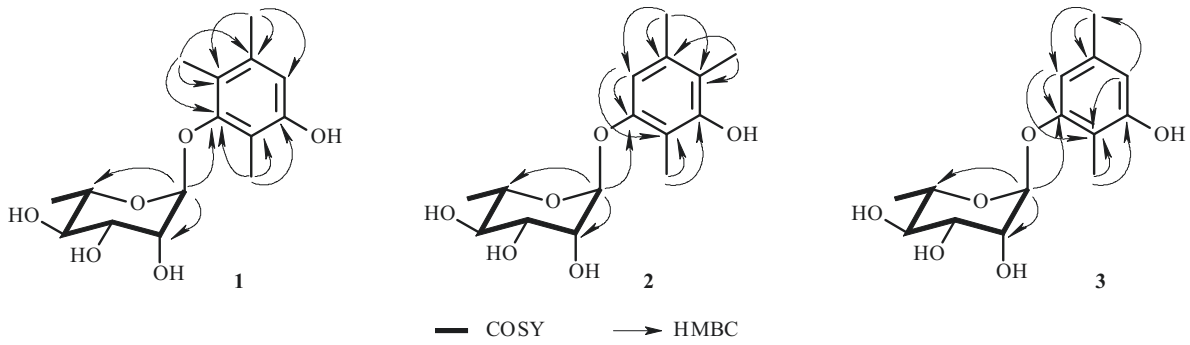

Fig. 1. Key ^{1}H–^{1}H COSY and HMBC correlations of **1**–**3**.

Compound **1** was isolated as a colorless solid with a molecular formula of $C_{15}H_{22}O_6$ as established by HR-EI-MS with five degrees of unsaturation. The glycosidic moiety in **1** was evident from the ^{13}C NMR chemical shifts (Table 1) of methine carbons C-2′ to C-5′ resonating between δ 71.0 and 72.9, which are typical of carbons attached to oxygen. The ^{13}C NMR resonance of C-1′ was shifted further downfield to δ 105.1, as expected for an acetal carbon, whereas C-6′, resonating at δ 17.9, showed a highfield shift, indicating the existence of a deoxy sugar. The presence of a rhamnose residue was confirmed by the contiguous spin system formed by correlations of five oxygen-bearing methines and one doublet methyl signal at δ 1.26 in the COSY spectrum (Fig. 1) together with the vicinal coupling $^{3}J_{HH}$ values ($^{3}J_{1',2'}$ = 1.5 Hz, $^{3}J_{2',3'}$ = 3.3 Hz, $^{3}J_{3',4'}$ = 9.3 Hz, $^{3}J_{4',5'}$ = 9.3 Hz, and $^{3}J_{5',6'}$ = 6.2 Hz) in the ^{1}H NMR spectrum (Table 1). Six sp^2 carbon signals (δ 157.0, 154.1, 135.3, 120.2, 115.2, 113.0) together with characteristic UV absorption spectrum (λ_{max} 199, 224, 280 nm) indicated the presence of one phenyl ring. Further comprehensive analysis of 2D NMR data, especially HMBC data (Fig. 1), enabled the determination of the complete planar structure of **1**. Importantly, the connection of the rhamnosyl moiety to the benzene core unit via an ether bond was established at the C-1 position by the HMBC correlation from H-1′ (δ 4.90) to the aromatic carbon (C-1) at δ 157.0.

Generally, the relative configuration of the glycosidic residue could be determined by the chemical shift of the anomeric carbon and the vicinal coupling $^{3}J_{HH}$ values. Because of the unusual feature of rhamnose, the large range of chemical shifts of the anomeric carbons of rhamnosides in natural compounds, in the case of α-rhamnosyl residues from δ_C 98 to 105 [9–12], is not suitable for identifying the relative configuration of rhamnosyl residues. It was reported that the α- and β-rhamnosides could be determined by their anomeric $^{1}J_{CH}$ coupling constants (172 Hz for the α- and 156 Hz for the β-rhamnoside) [13]. In present study, the anomeric $^{1}J_{CH}$ coupling constant of **1** was determined to be 168.5 Hz, which differs noticeably from that of a β-rhamnoside (below 160 Hz). Therefore, the rhamnosyl residue of **1** was deduced to be in the α-configuration.

To determine the absolute configuration of the rhamnosyl residue, **1** was hydrolyzed with CF_3COOH to obtain the monosaccharide residue, which was subsequently derived with L-cysteine methyl ester hydrochloride and phenyl isothiocyanate to yield the thiocarbamoyl-thiazolidine derivative. The absolute configuration of monosaccharide in **1** was identified as a L-rhamnose by directly comparing the specific optical rotation of the monosaccharide from the acid hydrolysis of **1** ($[\alpha]_D^{26}$–59.1° (*c* 0.1 H_2O)) with that of authentic L-rhamnose ($[\alpha]_D^{26}$–63.3° (*c* 0.1 H_2O)). The L-rhamnosyl moiety was also verified by the retention time in HPLC of the thiocarbamoyl-thiazolidine derivative of the monosaccharide from **1** (21.3 min) compared with that of the standard sample of L-rhamnose (21.3 min). Since there are no commercial standard samples of D-rhamnose available, **1** was tentatively determined as 1-*O*-[α-L-rhamnopyranosyl]-2,5,6-trimethyl-3-phenol and named pestarhamnose A.

Compound **2** was also obtained as a colorless solid with the same molecular formula as **1** as established by HR-ESI-MS. The ^1H and ^{13}C NMR data of **2** were similar to those of **1** (Table 1). The significant difference in the ^{13}C NMR spectrum of **2** was the anomeric carbon shifted upfield from δ 105.1 to 98.7. Furthermore, the position of the only aromatic proton was at C-6 (δ 108.4), which was confirmed on the basis of the HMBC correlations from H-6 (δ 6.57) to C-1 (δ 152.8), C-2 (δ 110.9), C-4 (δ 116.4), and C-9 (δ 19.7). The glycosidic unit, with an anomeric proton at δ_H 5.39 (d, J = 1.5 Hz) and a methyl doublet at δ 1.20 (d, J = 6.3 Hz), was identified as L-rhamnose [14], which was further confirmed by comparison of the retention time of the monosaccharide derivatives in the HPLC spectra. The small coupling constant of the anomeric proton (1.5 Hz) and the chemical shift of C-5′ (δ 69.3) indicated the usual α-configuration for this sugar [14], which was further confirmed by the anomeric $^1J_{CH}$ coupling constant (169.4 Hz). The ^1H–^{13}C long-range couplings from H-1′ (δ 5.39) to C-1 (δ 152.8) established the connectivity between the sugar and the benzene moiety (Fig. 1). Compound **2** was therefore established as 1-*O*-[α-L-rhamnopyranosyl]-2,4,5-trimethyl-3-phenol and named pestarhamnose B.

Compound **3** was isolated as a colorless solid with a molecular formula of $C_{14}H_{20}O_6$ (five degrees of unsaturation) through HR-ESI-MS. The ^1H and ^{13}C NMR data of **3** were similar to those of **2** (Table 1). The main difference was that **3** showed two aromatic protons revealed by the ^1H NMR spectrum,which was also confirmed by the presence of a methine carbon at C-4 (δ 110.7) in **3** instead of a quaternary carbon at C-4 (δ 116.4) in **2** in the ^{13}C NMR spectra. The sugar moiety, the same as that of **2**, was also identified as α-L-rhamnose through ^1H NMR, ^{13}C NMR, and HPLC analysis of the acid hydrolysate derivative. Compound **3** was designated as 1-*O*-[α-L-rhamnopyranosyl]-2,5-dimethyl-3-phenol and named pestarhamnose C.

Pestarhamnoses A–C (**1**–**3**) have possibly similar phenol units compared with (±)-pestalachlorides C and D from the same fungal strain in a different cultivation medium in our previous study [8]. Based on this point, we proposed that these compounds were probably produced by the same biogenetic pathway. Our current research further confirmed the possibility of our proposed biogenetic pathway of (±)-pestalachlorides C and D before [8]. Obviously, the production of pestarhamnoses A–C (**1**–**3**) was partly affected by the addition of KBr in the modified culture medium. Thus, it may be possible to use a bromine-modified medium to discover new natural compounds from the same microorganism resources.

The antimicrobial activities of **1**–**3** against a panel of pathogenic bacteria, including Gram-positive bacteria (*Bacillus subtilis*, *B. cereus*, *Kocuria rhizophila*, *Staphylococcus epidermidis*, *S. aureus*, and *S. albus*) and Gram-negative bacteria (*Pseudomonas putida*, *Acinetobacter calcoaceticus*, and *Klebsiella pneumoniae*), along with seven agricultural pathogenic fungi (*Colletotrichum capsici*, *Thielaviopsis paradoxa*, *Glorosprium musarum*, *Pestalotia mangiferae*, *Pestalotiopsis theae*, *Setosphaeria turcica*, and *Aspergillus niger*), were evaluated using a standard screening protocol [15]. However, pestarhamnoses A–C (**1**–**3**) only showed weak antimicrobial activities in these assays.

Moreover, compounds **1**–**3** were also evaluated for their cytotoxic activities against human cervical carcinoma cell line HeLa, human lung carcinoma cell line A549, human promyelocytic leukemia cell line HL-60, and human erythroleukemia cell line K562 at a concentration of 20 μM [16]. Pestarhamnoses A–C (**1**–**3**) only exhibited weak cytotoxic activities against the tested cell lines.

In conclusion, three new rhamnosylated phenol derivatives, pestarhamnoses A–C (**1**–**3**), were isolated from a soft coral-derived fungus *Pestalotiopsis* sp. cultured in a bromine-modified medium. The relative configurations of the rhamnosyl moieties were determined by their anomeric $^1J_{CH}$ coupling constants, while the absolute structures were determined by HPLC analysis of the thiocarbamoyl-thiazolidine derivatives of the acid hydrolysates. To the best of our knowledge, this is the first report of phenol glycosides with α-L-rhamnose in nature. Pestarhamnoses A–C (**1**–**3**) showed weak antimicrobial and cytotoxic activities.

EXPERIMENTAL

Melting points were determined on an X-6 micromelting point apparatus and are uncorrected. Optical rotations were measured on a JASCO P-1020 digital polarimeter. UV spectra were obtained on a Beckman DU 640 spectrophotometer. IR spectra were recorded on a Bruker EQUINOX 55 spectrometer using KBr pellets. NMR spectra were recorded on a JEOL JEM-ECP NMR spectrometer (600 MHz for ^{1}H and 150 MHz for ^{13}C) and on a Bruker Avance 400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C). Chemical shifts δ are reported in ppm using TMS as internal standard, and coupling constants (J) are in Hz. EI-MS spectra were measured on a Thermo DSQ EI-mass spectrometer, and HR-EI-MS on a Thermo MAT95XP high-resolution mass spectrometer. APCIMS spectra were measured on an amaZon SL BRUKER spectrometer, and HR-ESI-MS on a Thermo LTQ Orbitrap XL spectrometer. HPLC analysis and separation was performed in a Waters 1525 preparative HPLC system coupled with a Waters 2996 photodiode array detector. A Kromasil C_{18} preparative HPLC column (250 × 10 mm, 5 μm) was used. Silica gel (Qing Dao Hai Yang Chemical Group Co.; 200–300 mesh), octadecylsilyl silica gel (Unicorn; 45–60 μm), and Sephadex LH-20 (GE Healthcare) were used for column chromatography (CC). Precoated silica gel plates (Yan Tai Zi Fu Chemical Group Co.; G60, F-254) were used for thin-layer chromatography (TLC).

Isolation of the Fungal Material and Identification of Fungal Cultures. The procedures are the same as in [8].

Fermentation, Extraction, and Isolation. The fungal strain *Pestalotiopsis* sp. (ZJ-2009-7-6) was cultivated on solid rice medium (composition of artificial seawater: sea salt 15 g and KBr 15 g in 1 L distilled water; composition of medium: parboiled rice in bromine-modified artificial seawater, in 1 L Erlenmeyer flask each containing 30 g rice and 30 mL bromine-modified artificial seawater) in 60 Erlenmeyer flasks at 27°C without shaking for 4 weeks. Then the culture was extracted three times with an equal volume of $CHCl_3$–MeOH (1:1, v/v). The organic extracts were combined and concentrated under vacuum to afford a dry crude extract. The resulting extract (9.8 g) was subjected to silica gel column chromatography (CC) (petroleum ether, EtOAc, v/v, gradient elution) to afford four fractions (Fr.1–Fr.4). Fraction 2 was subjected to silica gel CC with petroleum ether–EtOAc (4:1, v/v) and Sephadex LH-20 CC eluting with mixtures of petroleum ether–$CHCl_3$–MeOH (2:1:1, v/v/v). Further purification by semipreparative HPLC using a C18 column eluting with 90% of MeOH–H_2O at a flow rate of 2.0 mL/min yielded compounds **4** (7.7 mg) and **5** (3.5 mg). Fraction 4 was subjected to silica gel CC with $CHCl_3$–MeOH (9:1, v/v) and Sephadex LH-20 CC eluting with mixtures of $CHCl_3$–MeOH (1:1, v/v). Further purification by semipreparative HPLC using an ODS C18 column eluting with 50% of MeOH–H_2O at a flow rate of 2.0 mL/min yielded **1** (8.0 mg), **2** (6.0 mg), and **3** (3.5 mg).

Pestarhamnose A (1). Colorless solid, mp 72–74°C; $[\alpha]_D^{26}$–85.0° (*c* 0.405, MeOH). UV (MeOH, λ_{max}, nm) (log ε): 199 (2.88), 224 (2.76), 280 (2.67). IR (KBr, ν_{max}, cm^{-1}): 3456, 2925, 1643, 1562, 1416, 1375, 1262, 1148. For ^{1}H NMR and ^{13}C NMR, see Table 1. EI-MS *m/z* 298 $[M]^{\cdot+}$; HR-EI-MS *m/z* 298.1406 $[M]^{\cdot+}$ (calcd for $C_{15}H_{22}O_6$, 298.1411).

Pestarhamnose B (2). Colorless solid, mp 169–171°C; $[\alpha]_D^{26}$–82.2° (*c* 0.190, MeOH). UV (MeOH, λ_{max}, nm) (log ε): 200 (2.51), 225 (2.32), 278 (2.18). IR (KBr, ν_{max}, cm^{-1}): 3456, 2927, 1643, 1556, 1458, 1386, 1129, 1057. For ^{1}H NMR and ^{13}C NMR, see Table 1. APCIMS *m/z* 297.1 $[M - H]^-$; HR-ESI-MS *m/z* 299.1485 $[M + H]^+$ (calcd for $C_{15}H_{23}O_6$, 299.1489).

Pestarhamnose C (3). Colorless solid, mp 92–95°C; $[\alpha]_D^{26}$ –90.3° (*c* 0.035, MeOH). UV (MeOH, λ_{max}, nm) (log ε): 202 (3.05), 226 (2.93), 270 (2.86). IR (KBr, ν_{max}, cm^{-1}): 3439, 2918, 1654, 1555, 1450, 1378, 1269, 1133. For ^{1}H NMR and ^{13}C NMR, see Table 1. APCIMS *m/z* 283.0 $[M - H]^-$; HR-ESI-MS *m/z* 285.1329 $[M + H]^+$ (calcd for $C_{14}H_{21}O_6$, 285.1333).

Acid Hydrolysis and Absolute Configuration Determination of Pestarhamnoses A–C (1–3). Pestarhamnoses A–C (**1**–**3**) (each 0.5 mg) were dissolved in 2 M CF_3COOH (aqueous) (1.0 mL) at 120°C for 6 h. The mixture was evaporated to dryness, and the residue was partitioned between CH_2Cl_2 and H_2O. The aqueous phase was concentrated to furnish a monosaccharide residue. After drying under vacuum, the residue was dissolved in 0.5 mL of pyridine containing 2 mg of L-cysteine methyl ester hydrochloride and heated at 60°C for 1 h. Phenyl isothiocyanate (2 μL) was then added, and the mixture was heated at 60°C for 1 h. The reaction mixture was analyzed by reversed-phase HPLC with a Kromasil C_{18} preparative HPLC column (250 × 10 mm, 5 μm) at 30°C with isocratic elution of 25% of MeOH–H_2O at a flow rate of 2.0 mL/min. The injection volume was 10 μL, and peaks were detected at 254 nm. The reaction conditions for authentic L-rhamnose were the same as described above. The absolute configurations of the rhamnosyl moieties were determined by comparison of the retention time of the thiocarbamoyl-thiazolidine derivative of the acid hydrolysate of **1**–**3** with that of standard samples of L-rhamnose.

Antimicrobial Assays. The antimicrobial activities against nine pathogenic bacteria (*B. subtilis*, *B. cereus*, *K. rhizophila*, *S. epidermidis*, *S. aureus*, *S. albus*, *P. putida*, *A. calcoaceticus*, and *K. pneumoniae*) and seven agricultural pathogenic fungi (*C. capsici*, *T. paradoxa*, *G. musarum*, *P. mangiferae*, *P. theae*, *S. turcica*, and *A. niger*) were evaluated by a serial dilution technique using 96-well microtiter plates [15]. Ciprofloxacin and carbendazim were used as positive control in antibacterial and antifungal assays, respectively. The results were observed with a Multiskan Mk3 (Thermo Labsystems).

Cytotoxicity Assays. The cytotoxic activities were evaluated against four human cancer cell lines (human cervical carcinoma HeLa, human lung carcinoma A549, human promyelocytic leukemia HL-60, and human erythroleukemia K562) by the MTT method [16]. Adriamycin was used as a positive control.

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