Two New Galloylated Monoterpene Glycosides, 4-*O*-Galloylalbiflorin and 4′-*O*-Galloylpaeoniflorin, from the Roots of *Paeonia lactiflora* (Paeoniae Radix) Grown and Processed in Nara Prefecture, Japan

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Two new galloylated monoterpene glycosides, 4-*O*-galloylalbiflorin and 4′-*O*-galloylpaeoniflorin, were isolated from the roots of *Paeonia lactiflora* that had been grown and processed in Nara prefecture, Japan. Their structures were elucidated based on spectroscopic analysis. These compounds showed androgen receptor (AR) binding activity.

Key words *Paeonia lactiflora*; androgen receptor; monoterpene glycoside; crude drug; Paeoniaceae

The roots of *Paeonia lactiflora* (Paeoniae radix, *shakuyaku* in Japanese) are one of the most important crude drugs in Japan and China, being used in many traditional "Kampo" formulas. In particular, they are frequently used in "Kampo" formulas, such as Tokishakuyakusan, Shimotsuto and Keishibukuryogan, for women's hormone-related problems such as menopausal symptoms and menstrual problems.[1,2] Many monoterpene glycosides *i.e.*, paeoniflorin, albiflorin, oxypaeoniflorin and benzoylpaeoniflorin, have been isolated from Paeoniae radix.[3] However, the hormone modulators from Paeoniae radix have not been satisfactorily examined. In Japan, *shakuyaku* that are grown and processed in Nara prefecture are named *yamato-shakuyaku*; these are of the highest class and thus are more expensive than other *shakuyaku*. However, it is not known whether they actually are more potent than *shakuyaku* grown in other areas.

We have focused on hormone regulating activity and investigated the bioactive compounds from *yamato-shakuyaku*. In the course of this research, we have previously reported the isolation and androgen modulating activity of five bioactive compounds containing a new galloylated monoterpene glycoside.[4] Our continuing search for androgen modulators from *yamato-shakuyaku* has resulted in the isolation and structural determination of two new galloylated monoterpene glycosides, 4-*O*-galloylalbiflorin (**1**) and 4′-*O*-galloylpaeoniflorin (**2**). We describe in this paper their isolation, physicochemical properties, structural determination and androgen receptor (AR) binding activities.

Results and Discussion

Yamato-shakuyaku, Paeoniae radix (2000 g), grown and processed in Nara prefecture, Japan, were ground and extracted with 50% acetone at room temperature. The concentrated extract was partitioned between EtOAc and H_2O. The concentrated EtOAc extract (39.9 g) was chromatographed on a Sephadex LH-20 column (ϕ20×420 mm) with MeOH and on a reversed-phase octadecyl silica (ODS) column (Cosmosil 140-C_{18} OPN, ϕ30×70 mm) using aqueous MeOH. Final purification was achieved by reversed-phase preparative HPLC (Cosmosil 5C_{18}-MSII, ϕ20×250 mm) with aqueous acetonitrile to give compounds **1** (5.4 mg) and **2** (6.8 mg).

The molecular formula of compound **1** was found to be $C_{30}H_{32}O_{15}$ [$(M-H)^-$, *m/z* 631.16681, Δ −0.03 milli mass unit (mmu), Calcd for $C_{30}H_{31}O_{15}$ 631.16684] by HR-electrospray ionization (ESI)-MS.

The ^{1}H-, ^{13}C-NMR (Table 1) and heteronuclear multiple quantum coherence (HMQC) spectra of **1** in CD_3OD revealed that **1** contained one methyl group (C10), two methylenes (C3 and C7), two oxymethylenes (C8 and C6′), one methine (C5), five oxymethines (C4 and C2′—C5′), one anomeric carbon (C1′), seven olefinic methines (C2″—C6″, C2‴ and C6‴), two quaternary olefinic carbons (C1″ and C1‴), three quaternary carbons (δ_C C1, C2 and C6), three oxygen-bearing olefinic carbons (C3‴, C4‴ and C5‴), three ester carbons (C9, C7″ and C7‴).

A detailed analysis of the ^{1}H–^{1}H correlated spectroscopy (COSY) spectrum of **1** allowed us to elucidate the following three partial structures: C3—C5 and C5 to C7, C1′—C3′, and C2″—C6″ (Fig. 2). The heteronuclear multiple bond correlation (HMBC) correlations 10-C$\underline{H}_3$/C1, C2 and C3, 5-H/C1, C6 and C8, 7-H_b/C1 and C6, and 8-H_2/C5, C6 and C9 indicated that the partial structure of C1—C10 was the same as the corresponding section of albiflorin. The presence of a glucose unit was confirmed by HMBC correlations 3′-H/C2′

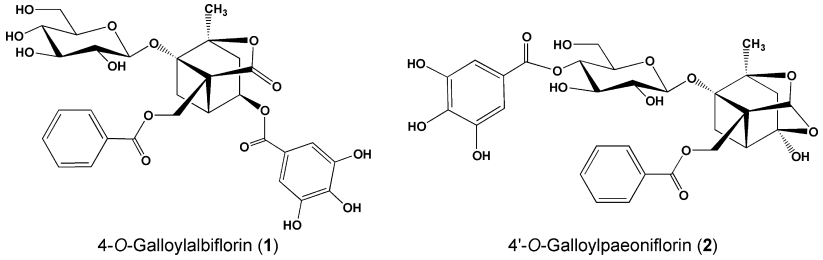

Fig. 1. Structures of Compounds **1** and **2**

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Table 1. ^{1}H- (750 MHz) and ^{13}C-NMR (187.5 MHz) Spectroscopic Data for 4-*O*-Galloylalbiflorin (**1**) in CD_3OD and 4′-*O*-Galloylpaeoniflorin (**2**) in C_5D_5N

Position	**1**		Position	**2**	
	δ_C	δ_H		δ_C	δ_H
1	86.4		1	89.1	
2	93.5		2	86.0	
3_a	39.7	2.21 (1H, d, J=16.0 Hz)	3_a	44.8	2.30 (1H, d, J=12.5 Hz)
3_b		2.68 (1H, dd, J=7.0, 16.0 Hz)	3_b		2.48 (1H, d, J=12.5 Hz)
4	71.7	5.44 (1H, m)	4	106.0	
5	39.5	3.19 (1H, m)	5	43.9	3.09 (1H, d, J=5.5 Hz)
6	57.5		6	71.8	
7_a	28.1	2.22 (1H, d, J=11.1 Hz)	7_a	23.6	2.36 (1H, d, J=10.7 Hz)
7_b		2.92 (1H, dd, J=8.0, 11.1 Hz)	7_b		2.97 (1H, m)
8_a	61.8	4.68 (1H, d, J=12.0 Hz)	8_a	61.6	5.11 (1H, d, J=12.0 Hz)
8_b		4.79 (1H, d, J=12.0 Hz)	8_b		5.28 (1H, d, J=12.0 Hz)
9	177.9		9	101.7	5.95 (1H, s)
10	20.2	1.58 (3H, s)	10	19.9	1.65 (3H, s)
1′	100.1	4.55 (1H, d, J=7.7 Hz)	1′	100.4	5.17 (1H, d, J=7.6 Hz)
2′	74.9	3.23 (1H, m)	2′	75.2	4.08 (1H, m)
3′	78.0	3.33 (1H, m)	3′	75.9	4.33 (1H, dd, J=9.0, 9.2 Hz)
4′	71.5	3.25 (1H, m)	4′	72.8	5.79 (1H, dd, J=9.2, 9.6 Hz)
5′	78.2	3.27 (1H, m)	5′	76.6	3.99 (1H, m)
$6'_a$	62.8	3.62 (1H, dd, J=5.9, 12.0 Hz)	$6'_a$	62.3	4.10 (1H, m)
$6'_b$		3.87 (1H, d, J=12.0 Hz)	$6'_b$		4.21 (1H, d, J=12.1 Hz)
1″	131.0		1″	130.7	
2″, 6″	130.7	7.90 (2H, d, J=7.3 Hz)	2″, 6″	130.0	8.13 (2H, d, J=7.6 Hz)
3″, 5″	129.6	7.33 (2H, dd, J=7.3, 7.4 Hz)	3″, 5″	128.8	7.31 (2H, dd, J=7.2, 7.6 Hz)
4″	134.3	7.53 (1H, t, J=7.4 Hz)	4″	133.4	7.49 (1H, t, J=7.2 Hz)
7″	168.0		7″	166.7	
1‴	120.9		1‴	121.1	
2‴, 6‴	110.5	7.05 (2H, s)	2‴, 6‴	110.6	7.88 (2H, s)
3‴, 5‴	146.5		3‴, 5‴	147.6	
4‴	140.3		4‴	141.2	
7‴	167.4		7‴	167.0	

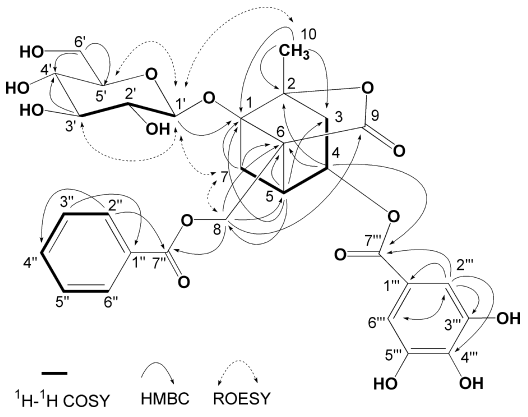

Fig. 2. The ^{1}H–^{1}H COSY, HMBC and ROESY Correlations of 4-*O*-Galloylalbiflorin (**1**)

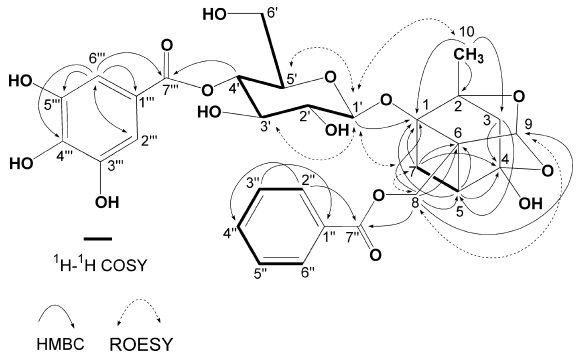

Fig. 3. The ^{1}H–^{1}H COSY, HMBC and ROESY Correlations of 4′-*O*-Galloylpaeoniflorin (**2**)

and C4′, and 6′-H_a/C4′ and C5′, and rotating frame nuclear Overhauser effect spectroscopy (ROESY) correlations 1′-H/3′-H and 5′-H. The HMBC correlations 2″-H/C7″, 3″-H/C1″, and 2‴-H/C1‴, C3‴, C4‴, C6‴ and C7‴ confirmed the presence of a benzoyl moiety and a galloyl moiety. The connection of the glucose unit and C1 *via* an oxygen atom was revealed by HMBC correlation 1′-H/C1; the connection of the benzoyl moiety and C8 *via* an oxygen atom was confirmed by HMBC correlations 8-H_2/C7″. The ROESY correlations 7-H_b/8-H_2 and 1′-H, and 10-C$\underline{H}_3$/1′-H revealed that compound **1** was an albiflorin derivative. Finally, the connection of the galloyl moiety and C4 *via* an oxygen atom was revealed by HMBC correlation 4-H/C7‴. Thus, the structure of **1** was determined as shown in Fig. 2, and named 4-*O*-galloylalbiflorin (**1**). We propose that the absolute stereochemistry of compound **1** is identical to that of albiflorin, since the ^{1}H-NMR spectroscopic data of C8, C1′—C6′ and C2″—C6″, as well as the ^{1}H–^{1}H coupling constants and ^{13}C-NMR spectroscopic data of C1—C2 and C6—C7″ in compound **1** are in good agreement with those of albiflorin.[5)]

The molecular formula of compound **2** was found to be $C_{30}H_{32}O_{15}$ [$(M-H)^-$, m/z 631.16641, Δ −0.43 milli mass unit (mmu), Calcd for $C_{30}H_{31}O_{15}$ 631.16684] by HR-ESI-MS.

Detailed analysis of 1D (shown in Table 1) and 2D NMR data including ^{1}H–^{1}H COSY, HMQC, HMBC and ROESY spectra in C_5D_5N revealed that compound **2** was a paeoniflorin derivative. The ^{1}H-NMR data (δ_H 7.88, 2H, s), ^{13}C-NMR data (δ_C 110.6, 110.6, 121.1, 141.2, 147.6, 147.6, 167.0) and molecular formula suggested the presence of a galloyl moiety in **2**. In addition, the low-field shifted signal at δ_H 5.79 was assigned to 4′-H of a glucose unit in **2**. Finally, the connection of galloyl moiety and C4′ *via* an oxygen atom was revealed by HMBC correlation of 4′-H/C7‴. Thus, the structure of **2** was determined as shown in Fig. 3, and named 4′-*O*-galloylpaeoniflorin (**2**). We propose that the absolute stereochemistry of compound **2** is identical to that of paeoniflorin, since the ^{1}H–^{1}H coupling constants, as well as the ^{1}H- and ^{13}C-NMR spectroscopic data in CD_3OD (Experimental) of C1—C2′ and C6′—C7″ in compound **2** are in good agreement with those of paeoniflorin.[5)]

Compounds **1** and **2** showed weak AR binding activity (17.8% at 50 μg/ml and 22.3% at 100 μg/ml, respectively). We have previously proposed that both the galloyl moiety and the structure of albiflorin are important for 6′-*O*-galloylalbiflorin to exhibit strong AR binding activity.[4)] However, the AR binding activity of compound **1** was much weaker than that of 6′-*O*-galloylalbiflorin (IC_{50} values 33.7 μg/ml[4)]), which strongly suggests that not only the presence but also the position of the galloyl moiety, as well as the albiflorin structure are important for strong activity.

Further studies of structure–activity relationships and mechanisms of action for these compounds are in progress.

Experimental

General Optical rotation was measured with a DIP-1000 digital polarimeter (Jasco). UV spectrum was measured with a V-630 spectrophotometer (Jasco). IR spectrum was recorded on an IR Prestige-21 FTIR-8400S (Shimadzu). NMR (^{1}H-NMR: 750, 400 MHz, ^{13}C-NMR: 187.5 MHz) spectra were measured on AVANCE-750 (Bruker Biospin) and JNM-GSX-400 (JEOL). MS was obtained on an Apex-Qe 9.4T FT-ICR-MS (Bruker Daltonics). The radioactivity was measured in a wallac 1450 microbeta TRILUX (PerkinElmer).

Plant Material *Yamato-shakuyaku*, Paeoniae radix grown and processed in Nara, Japan, were purchased from Ruta Corporation (Osaka, Japan). Voucher specimens were deposited in the core laboratory of Nara Prefectural Small and Medium-sized Enterprises Support Corporation (Kashihara, Nara, Japan).

Chemicals [H^3]-Mibolerone and unlabeled mibolerone were purchased from PerkinElmer, U.S.A. The other reagents were analytical-grade products from Wako Pure Chemical Industries, Japan.

Extraction and Isolation *Yamato-shakuyaku* (2000 g), Paeoniae radix grown and processed in Nara, Japan, were ground and extracted with 50% acetone. The concentrated extract was partitioned between EtOAc and H_2O. The concentrated EtOAc extract (39.9 g) was chromatographed on a Sephadex LH-20 column (ϕ20×420 mm) with MeOH to give seven fractions; F1, F2, F3, F4, F5, F6 and F7. The fraction F2 was subjected to chromatography on a reversed-phase ODS (Cosmosil 140-C_{18} OPN, ϕ30×70 mm) using aqueous MeOH (10—100%) to give four fractions; F2A (10%), F2B (20%), F2C (30%) and F2D (100%). The fraction F2C was chromatographed on a reversed-phase preparative HPLC (Cosmosil 5C_{18}-MSII, ϕ20×250 mm) using 20% acetonitrile to give 20 fractions; F2C-1 to F2C-20. The fraction F2C-15 was chromatographed on a reversed-phase preparative HPLC (Cosmosil 5C_{18}-MSII, ϕ20×250 mm) with 15% acetonitrile to give compound **1** (5.4 mg). The fraction F2C-13 was twice chromatographed on reversed-phase preparative HPLC (Cosmosil 5C_{18}-MSII, ϕ20×250 mm) with 15% acetonitrile to give compound **2** (6.8 mg).

4-*O*-Galloylalbiflorin (**1**): A yellow-brown amorphous powder: $[\alpha]_D^{25}$ −63.5° (c=0.24, MeOH); UV_{max} (MeOH, ε) 389.6 nm (69700), 275.8 nm (85100), 220.8 nm (110000); IR (film) 3397, 1753 and 1707 cm^{-1}. HR-ESI-MS *m/z*: 631.16681 [(M−H)$^-$, 631.16684 for $C_{30}H_{31}O_{15}$]; The ^{1}H-(750 MHz) and ^{13}C-NMR (187.5 MHz) spectral data are shown in Table 1.

4′-*O*-Galloylpaeoniflorin (**2**): A yellow-brown amorphous powder: $[\alpha]_D^{26}$ −18.3° (c=0.23, MeOH); UV_{max} (MeOH, ε) 389.8 nm (69600), 275.0 nm (85500), 220.4 nm (111200); IR (film) 3404, 1707 and 1609 cm^{-1}. HR-ESI-MS *m/z*: 631.16641 [(M−H)$^-$, 631.16684 for $C_{30}H_{31}O_{15}$]; The ^{1}H- (750 MHz) and ^{13}C-NMR (187.5 MHz) spectral data in C_5D_5N are shown in Table 1. ^{1}H-NMR (CD_3OD 750 MHz) δ: 1.83 (1H, d, J=12.5 Hz, 3-H$_a$), 2.20 (1H, d, J=12.5 Hz, 3-H$_b$), 2.61 (1H, d, J=5.1 Hz, 5-H), 1.98 (1H, d, J=10.6 Hz, 7-H$_a$), 2.53 (1H, m, 7-H$_b$), 4.74 (1H, d, J=12.0 Hz, 8-H$_a$), 4.79 (1H, d, J=12.0 Hz, 8-H$_b$), 5.43 (1H, s, 9-H), 1.39 (3H, s, 10-H), 4.63 (1H, d, J=7.5 Hz, 1′-H), 3.34 (1H, m, 2′-H), 3.59 (1H, m, 3′-H), 4.86 (1H, m, 4′-H), 3.52 (1H, m, 5′-H), 3.52 (1H, m, 6′-H$_a$), 3.59 (1H, m, 6′-H$_b$), 8.07 (2H, d, J=7.3 Hz, H-2″, H-6″), 7.51 (2H, dd, J=7.3, 7.3 Hz, H-3″, H-5″), 7.64 (1H, t, J=7.3 Hz, H-4″), 7.07 (2H, s, H-2‴, H-6‴); ^{13}C-NMR (CD_3OD, 187.5 MHz) δ: 89.5 (C1), 87.3 (C2), 44.6 (C3), 106.4 (C4), 44.0 (C5), 72.3 (C6), 23.5 (C7), 61.8 (C8), 102.3 (C9), 19.6 (C10), 100.2 (C1′), 75.3 (C2′), 75.9 (C3′), 72.7 (C4′), 76.3 (C5′), 62.5 (C6′), 131.3 (C1″), 130.8 (C2″, C6″), 129.7 (C3″, C5″), 134.5 (C4″), 168.1 (C7″), 121.1 (C1‴), 110.4 (C2‴, C6‴), 146.5 (C3‴, C5‴), 140.2 (C4‴), 167.9 (C7‴).

Measurement of Androgen Receptor (AR) Binding Activity Compounds **1** and **2** and testosterone (positive control) were evaluated for their AR binding activity by measuring the binding [H^3]-mibolerone to AR. Membrane (rat) preparation obtained from Panvera (Cat # P2719) was prepared in modified triphosphate pH 7.4 buffer using standard techniques. An aliquot (78 ng) of membrane preparation was incubated with 1.5 nM [H^3]-mibolerone in either the presence or absence of a test sample for 4 h at 4 °C. Non-specific binding was estimated in the presence of 10 μM mibolerone. The reaction mixture was incubated with a hydroxyapatite slurry over 15 min and filtered. The filters were washed 3 times and counted to determine [H^3]-mibolerone specifically bound. AR binding activity was calculated by the equation as described below:

AR binding activity (%)=100×[1−([H^3]-miborelone specifically bound in the presence of test sample−non specific binding in the presence of test sample)/([H^3]-miborelone specifically bound in the control−non specific binding in the control)]

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References

1) Kumagai Y., Hyuga S., Hyuga M., Watanabe K., Kawanishi T., Hanawa T., *J. Trad. Med.*, **22**, 228—236 (2005).
2) Watanabe K., Hyuga S., Hyuga M., Kawanishi T., Hanawa T., *J. Trad. Med.*, **23**, 203—207 (2006).
3) Kaneda M., Iitaka Y., Shibata S., *Tetrahedron*, **28**, 4309—4317 (1972).
4) Washida K., Itoh Y., Iwashita T., Nomoto K., *Chem. Pharm. Bull.*, **57**, 971—974 (2009).
5) Yen P. H., Kiem P. V., Nhiem N. X., Tung N. H., Quang T. H., Minh C. V., Kim J. W., Choi E. M., Kim Y. H., *Arch. Pharm. Res.*, **30**, 1179—1185 (2007).