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SAPONINS FROM *MUSSAENDA PUBESCENS*

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Key Word Index—*Mussaenda pubescens*; Rubiaceae; saponin; mussaendosides G and K.

Abstract—Two new triterpenoid saponins, named mussaendosides G and K, were isolated from aerial parts of *Mussaenda pubescens* by normal and reverse phase chromatography. On the basis of chemical and spectroscopic methods, their structures have been elucidated as heinsiagenin A 3-*O*-{α-L-rhamnopyranosyl(1 → 2)-[β-D-glucopyranosyl(1 → 6)]-β-D-glucopyranosyl(1 → 2)}-α-L-rhamnopyranosyl(1 → 4)-*O*-β-D-glucopyranoside and 3β,19α-dihydroxyl-olean-12-en-24,28-dioic acid-24,28-di-*O*-β-D-glucopyranoside, respectively.

INTRODUCTION

Mussaenda pubescens Ait. f. is a Chinese folk medicine commonly used in diuretic, antiphlogistic and antipyretic treatments [1]. It is also used to detoxify mushroom poisoning and to terminate early pregnancy in some parts of southeast China [2, 3]. In previous papers we have reported the isolation and structural determination of several saponins and iridoids from the plant [4–6]. In our continuing investigation of this plant, the hydrophilic fractions from the aerial parts of the plant material were further investigated. In this paper we report the isolation and structural elucidation of mussaendosides G (**1**) and K (**2**), two new triterpeniod saponins from *M. pubescens*.

RESULTS AND DISCUSSION

The positive reaction of compound **1** to the Liebermann–Burchard and Molisch tests indicated this compound to be a triterpenoid saponin. The FAB-mass spectrum showed a quasimolecular ion peak at *m/z* 1367, corresponding to $[M(C_{66}H_{105}NO_{27}) + Na]^+$. The UV absorption at λ_{max} 265 nm revealed the existence of a conjugated diene ketone in its genin. The ^{1}H NMR spectrum showed the characteristic proton signals of a conjugated diene at δ 5.68 (1H, *dd*, H-22), 6.46 (1H, *dd*, H-23) and 7.39 (1H, *d*, H-24), an amide at 9.15 (1H, *d*) and a cyclopropane at 0.22 (1H, *d*) and 0.46 (1H, *d*). The above characteristic features are similar to those of heinsiagenin A, a common aglycone found in saponins of *M. pubescens*. The presence of heinsiagenin A as an aglycone in **1** was concluded based on these data and by comparison of its ^{13}C NMR chemical shifts (Table 1) with the literature values [6]. Further support was obtained by acidic hydrolysis of **1**, which yielded heinsiagenin A together with L-rhamnose and D-glucose as the sugar components. The ^{1}H NMR spectrum of **1** showed five anomeric proton signals at δ 6.43 (1H, *s*), 5.76 (1H, *s*), 5.76 (1H, *m*), 5.27 (1H, *d*, 7.5) and 4.93 (1H, *m*). In addition, two doublet methyl signals belonging to sugar units appeared at δ 1.88 (3H, *d*, $J = 4.5$ Hz) and 1.73 (3H, *d*, $J = 7.0$ Hz), which suggested the presence of two L-rhamnose moieties, and the remaining three anomeric protons were present as D-glucose units.

The structural components of **1** are indeed similar to the previously reported structure of mussaendoside O (**3**), which contains heinsiagenin A as aglycone and two glucose (G, G′) and two rhamnose (R, R′) moieties as sugar components [6]. The ^{1}H and ^{13}C NMR data for **1** are very similar to those for **3**, except for the signals from the additional glucose unit (G″). The ^{13}C NMR spectra showed two oxygen-bearing methylene carbons at δ 63.2 ($C_{G\text{-}6}$) and 61.3 ($C_{G'\text{-}6}$) for **3**, while three oxygen-bearing methylene carbons were observed at δ 70.5, 62.7 and 61.4 for **1**. The downfield shift of the oxygen-bearing methylene carbon at δ 70.5 indicates a site of glycosylation. This is presumably due to the linkage of the extra glucose unit in **1** to C-6 of G or G′. Further analyses of the ^{13}C NMR data revealed that the $C_{G'\text{-}6}$ signal remained unaffected, while the $C_{G\text{-}6}$ signal shifted from δ 63.2 in **3** to 70.5 in **1**. From the above evidence, the additional glucose unit in **1** was concluded to be linked to C-6 of the outer glucose unit G in **3**. A known saponin, mussaendoside N, with a terminal glucose unit connecting to another outer glucose unit in the sugar chain, has a reported anomeric proton signal of terminal glucose at δ 5.24 and C-6 of

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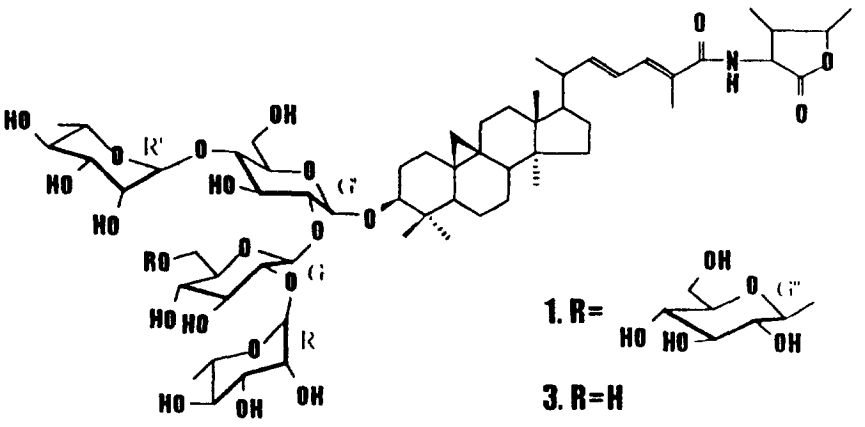

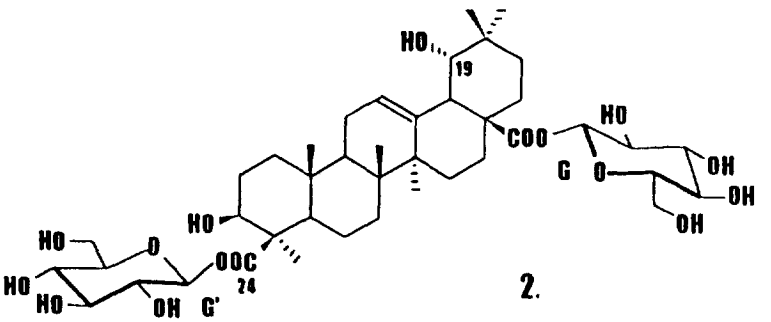

the inner glucose unit at δ 70.5, very similar to those of **1** [4].

The above conclusion was further substantiated by extensive 2D NMR experiments. First, the proton assignments of all sugar units were obtained unambiguously from anomeric proton signals and 6-Me signals of rhamnose in ^{1}H–^{1}H DQF COSY and TOCSY (Table 2). The sequence and interglycosidic linkages of sugar units were confirmed by NOESY and ROESY experiments. In the ROESY spectrum, several significant cross peaks were observed between $H_{G'-1}$ (δ 4.93) and H-3; H_{G-1} (δ 5.76) and $H_{G'-2}$ (δ 4.58); $H_{G''-1}$ (δ 5.27) and $H_{G'-6}$ (δ 4.75 and 4.35); H_{R-1} (δ 6.43) and H_{G-2} (δ 4.31); and $H_{R'-1}$ (δ 5.76) and $H_{G'-4}$ (δ 4.33). All of the above data strongly support the structure of **1** as heinsiagenin A 3-*O*-{α-L-rhamnopyranosyl(1 → 2)-[β-D-glucopyranosyl(1 → 6)]-*O*-β-D-glucopyranosyl(1 → 2)}-α-L-rhamnopyranosyl(1 → 4)-*O*-β-D-glucopyranoside, a new triterpenoid saponin now called mussaendoside G.

Table 1. ^{13}C NMR (75 MHz, pyridine-d_5) data for aglycone of **1**

No.	δ_c	DEPT	No.	δ_c	DEPT
1	32.0	*t*	19	29.7	*t*
2	29.7	*t*	20	41.3	*d*
3	89.9	*d*	21	19.7	*q*
4	41.2	*s*	22	147.8	*d*
5	47.5	*d*	23	123.5	*d*
6	21.1	*t*	24	134.8	*d*
7	26.2	*t*	25	128.9	*s*
8	47.9	*d*	26	13.3	*q*
9	19.7	*s*	27	170.7	*s*
10	26.2	*s*	28	15.4	*q*
11	26.4	*t*	29	26.0	*q*
12	33.0	*t*	30	19.3	*q*
13	45.5	*s*	1′	175.7	*s*
14	49.0	*s*	2′	55.3	*d*
15	35.6	*t*	3′	38.5	*d*
16	28.6	*t*	4′	76.9	*d*
17	51.8	*d*	3′-Me	8.0	*q*
18	18.3	*q*	4′-Me	15.4	*q*

Saponin **2**, an amorphous powder, gave positive reactions to the Liebermann–Burchard and Molisch tests. In the positive mode, its liquid secondary ion (LSI) mass spectrum showed a quasimolecular ion peak at *m/z* 849 $[M+Na]^+$ and a fragment arising from aglycone (A) at *m/z* 503 $[A+H]^+$. The CID-mass spectrum of 2 yielded fragments at *m/z* 687 (loss of one sugar from $[M+Na]^+$), 485 $[A-H_2O+H]^+$, 467 $[A-2H_2O+H]^+$ and 421 $[M-2H_2O-HCO_2H+H]^+$. In the negative mode, the LSI-mass spectrum exhibited ion peaks of $[M-H]^-$ at *m/z* 825, $[M-H-162]^-$ at *m/z* 663 (loss of one sugar), and aglycone $[A-H]^-$ at *m/z* 501. The ESI (electrospray) mass spectrum confirmed the quasimolecular ion peak at *m/z*

Table 2. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data for sugar units in **1** (pyridine-d_5)

No.	δ_H	J (Hz)	δ_C	DEPT
G-1	5.76	*m*	101.9	*d*
2	4.31	*m*	78.1	*d*
3	4.22	*m*	78.1	*d*
4	4.08	*m*	72.5	*d*
5	4.04	*m*	76.7	*d*
6a	4.75	*m*	70.5	*t*
6b	4.35	*m*		
G′-1	4.93	*m*	104.7	*d*
2	4.58	*m*	79.2	*d*
3	4.51	*m*	77.6	*d*
4	4.33	*m*	79.2	*d*
5	3.67	*m*	76.1	*d*
6a	4.20	*m*	61.4	*t*
6b	4.08	*m*		
G″-1	5.27	*d*, $J = 7.5$ Hz	105.1	*d*
2	4.07	*m*	75.2	*d*
3	4.25	*m*	78.7	*d*
4	4.38	*m*	71.5	*d*
5	4.08	*m*	78.0	*d*
6a	4.59	*m*	62.7	*t*
6b	4.42	*m*		
R-1	6.43	*s*	101.9	*d*
2	4.78	*m*	72.2	*d*
3	4.68	*m*	72.5	*d*
4	4.37	*m*	74.1	*d*
5	5.02	*m*	69.4	*d*
6	1.88	*d*, $J = 4.5$ Hz	19.0	*q*
R′-1	5.76	*s*	102.6	*d*
2	4.62	*m*	72.4	*d*
3	4.55	*m*	72.4	*d*
4	4.30	*m*	73.8	*d*
5	4.89	*m*	70.5	*d*
6	1.73	*d*, $J = 7.0$ Hz	18.5	*q*

849 $[M + Na]^+$. In combination the mass spectral and ^{13}C NMR data for **2** gave the molecular formula $C_{42}H_{66}O_{16}$.

The ^{1}H NMR spectrum showed the signals of six tertiary methyls at δ 0.95, 1.12, 1.18, 1.21, 1.60 and 1.74, one trisubstituted olefinic proton at δ 5.49 (*br s*), and two esterified anomeric protons at δ 6.32 (*d*, J = 7.7 Hz) and 6.30 (*d*, $J = 8.0$ Hz). The ^{13}C NMR spectrum revealed the presence of six sp3 quaternary carbons at δ 50.2, 40.1, 38.2, 42.3, 46.6 and 35.6, a pair of olefinic carbons at δ 123.6 and 144.3, two ester carbonyls at δ 176.2 and 177.2, and two anomeric carbons at δ 96.0 and 95.8. The NMR spectral data and the molecular formula suggested that **2** was an olean-12-ene-type dicarboxylic acid saponin having two sugar units with two ester glycosidic-linkages.

Acidic hydrolysis of **2** yielded D-glucose only, which indicated the presence of two glucose units connected to two different carboxyls in ester glycosidic linkage. In the ^{1}H NMR spectrum measured at 20°, a doublet signal at δ 6.00 (*d*, $J = 6.0$ Hz) was observed, which broadened with rising temperature from 20° to 25° and disappeared with addition of D_2O. This was deduced to be an active hydroxyl proton. In the ^{1}H–^{1}H DQF-COSY spectrum, the hydroxyl proton showed a cross peak with a doublet methine signal at δ 3.56, which further correlated with a broad singlet methine signal at δ 3.49. In the NOESY spectrum, the signals at δ 3.56 and 3.49 both exhibited cross peak with olefinic protons H-12 at δ 5.49. From the above evidence, the two protons at δ 3.56 and 3.49 were assigned at H-19 and H-18, respectively. Consequently, the hydroxyl group was placed at C-19 with the α-configuration according to the small J value between H-19β and H-18.

In a further study of the ^{1}H NMR spectrum, another hydroxyl was considered to be at C-3 with a β-configuration according to the presence of a methine signal at δ 3.77 (*dd*, $J = 4.1$ and 11.8 Hz). In the ROESY experiment, H-3 exhibited a cross peak with a methyl signal at relative low field (δ 1.74), which was suggested to be Me-23 from modelling. Therefore, a carboxyl was deduced to be derived from C-24.

By comparison of the ^{13}C NMR data for **2** with those for compounds with similar structures, another carboxyl was suggested to be at C-17. To confirm its substitution pattern, the ^{13}C NMR data for two known triterpenoids, ilexgenin and trachelosperoside E-1, were picked out as references for comparison of the ^{13}C NMR data. The signals of C-1–C-15 and C-23–C-27 were in good agreement with those of ilexgenin, i.e. 3β,19α-dihydroxy-urs-12-en-24β,28-dioic acid, in which the structural parts in rings A, B and C were similar to those of **2**. Meanwhile, the signals of C-12–C-22 and C-26–C-30 were in good agreement with those of trachelosperoside E-1, in which the structural parts in rings C, D and E were similar to those of **2** [7, 8]. Therefore, the structure of **2** was elucidated to be 3β,19α-dihydroxy-olean-12-en-24,28-dioic acid-24,28-di-*O*-β-glucopyranoside as a new saponin now named mussaendoside K.

EXPERIMENTAL

General. Optical rotations were recorded on a JASCO DIP-181 polarimeter; IR spectra were collected using a Perkin-Elmer 599B spectrometer; EI mass spectra were collected using a Finnigan MAT-8430; ^{1}H and ^{13}C NMR spectra were recorded with Bruker AM-300 and AM-400 and Varian Unity 500 Instruments. Residual proton signals from the solvent (pyridine-d_5) were used as int. standard. Samples for 2D NMR were prepd by dissolving the compound in 600 μl pyridine-d_5. All 2D homonuclear experiments were carried out at 25° using a Varian Unity 500. Standard pulse sequences and phase cycling schemes were used for the DQF-COSY (Rance, 1983 #5401), NOESY (Jeener, 1979 #5404), ROESY [Kessler, 1987 #5400] and TOCSY (Bax, 1985 #5402) experiments. TOCSY experiments were performed with a spin-lock period of 80 msec with a $2KH_2$ field strength. ROESY experiments were performed with a mixing time of 300 msec. The NOESY experiment was performed with a mixing time of 400 msec. Spectra were recorded in the phase-

sensitive mode with quadrature detection in F1 dimension using the methods of States and Haberkorn (States, 1982 #1456).

Plant materials. The aerial parts of *M. pubescens* were collected in December, 1993, at Yongtai County, Fujian Province, People's Republic of China. A voucher specimen was identified by Prof. Rentong Chen of Fujian Institute of Traditional Chinese Medicines.

Extraction and isolation. Dried aerial parts of the plant (4.0 kg) were percolated 4× with 95% EtOH at room temp. After evapn of EtOH at 50° *in vacuo*, the residual aq. soln was extracted with EtOAc and *n*-BuOH to yield 250 and 150 g residues of each fr., respectively. The *n*-BuOH fr. was subjected to chromatography on DA-201 and eluted with H_2O and 40 and 90% EtOH. After concn to dryness, 70, 40 and 40 g of corresponding residues were obtained. The 90% EtOH fr. (40 g) was further subjected to silica gel chromatography with a $CHCl_3$–MeOH–H_2O gradient. The crude saponins were purified by HPLC on a Lobar RP-18 column with MeOH–H_2O or CH_3CN–H_2O gradients to afford pure **1** (300 mg) and **2** (50 mg).

Saponin **1** (*mussaendoside G*). Amorphous powder, $[\alpha]_D^{24}$ +13.6° (MeOH, *c* 0.09). IR γ_{max}^{KB} cm^{-1}: 3400 (OH), 1765 (COO), 1640 (CONH), 1600 (C=C). FAB-MS *m/z*: 1367 $[M(C_{66}H_{105}NO_{27}) + Na]^+$ (matrix: G + HOAc + SG + NaCl). UV λ_{max}^{MeOH} 265 nm. ^1H NMR (500 MHz, pyridine-d_5): δ 9.15 (1H, *d*, NH), 7.39 (1H, *d*, H-24), 6.46 (1H, *dd*, H-23), 5.68 (1H, *dd*, H-22), 2.94 (1H, *m*, H-3′), 2.24 (3H, *s*, H-26), 1.42 (3H, *s*), 1.24 (3H, *s*), 1.20 (3H, *d*), 1.02 (3H, *d*), 1.01 (3H, *s*), 0.91 (3H, *s*), 0.89 (3H, *d*), 0.46 (1H, *d*, H-19β), 0.22 (1H, *d*, H-19α). For sugar protons: see Table 2. ^{13}C NMR (125 MHz, pyridine-d_5): see Tables 1 and 2.

Acidic hydrolysis of **1**. Saponin **1** (10 mg) dissolved in 2 N HCl (3 ml) was heated at 90° for 4 hr. The soln was treated with $CHCl_3$ and the aq. layer was neutralized with Ag_2CO_3 and filtered. The filtrate was concd and identified by silica gel TLC in comparison with authentic sugar samples.

Saponin **2** (*mussaendoside K*). Amorphous powder, $[\alpha]_D^{24}$ +36.0° (pyridine-d_5, *c* 0.21). IR ν_{max}^{KBr}, cm^{-1}: 3400 (OH), 1725 (COO), 1630 (C=C). Positive LSI-MS *m/z*: 849 $[M(C_{42}H_{66}O_{16}) + Na]^+$, 503 [aglycone + H]$^+$. CID of *m/z* 849 $[M(C_{42}H_{66}O_{16}) + Na]^+$ yield *m/z* 687, CID of *m/z* 503 yielded *m/z* 485, 467 and 421. Negative LSI-MS *m/z*: 825 $[M(C_{42}H_{66}O_{16}) - H]^-$, 663 $[M - H - 162]^-$, 501 [aglycone − H]$^-$. EIS-MS *m/z*: 849 $[M(C_{42}H_{66}O_{16}) + Na]^+$. ^1H NMR (400 MHz, pyridine-d_5): δ 6.32 (1H, *d*, *J* = 7.7 Hz), 6.30 (1H, *d*, *J* = 8.0 Hz), 6.00 (1H, *d*, *J* = 6.0 Hz, OH), 5.49 (1H, *br s*, H-12), 3.56 (1H, *m*, H-19), 3.49 (1H, *br s*, H-18), 3.37 (1H, *dd*, *J* = 11.8, 4.1 Hz, H-3), 1.74 (3H, *s*, H-23), 1.60 (3H, *s*), 1.21 (3H, *s*), 1.18 (3H, *s*), 1.12 (3H, *s*), 0.95 (3H, *s*). ^{13}C NMR (75 MHz, pyridine-d_5): see Table 3.

Table 3. ^{13}C NMR data for **2** (pyridine-d_5)

No.	δ_c	DEPT	No.	δ_c	DEPT
1	39.4	*t*	19	81.3	*d*
2	29.1	*t*	20	35.6	*s*
3	78.5	*d*	21	29.0	*t*
4	50.2	*s*	22	33.0	*t*
5	57.5	*d*	23	24.5	*q*
6	20.9	*t*	24	176.2	*s*
7	33.5	*t*	25	14.0	*q*
8	40.1	*s*	26	17.3	*q*
9	48.0	*d*	27	24.7	*q*
10	38.2	*s*	28	177.2	*s*
11	24.4	*t*	29	28.8	*q*
12	123.6	*d*	30	24.8	*q*
13	144.3	*s*	Glu(or GLu′)-1	96.0, 95.8	*d,d*
14	42.3	*s*	Glu(or GLu′)-2	74.3, 74.2	*d,d*
15	28.8	*t*	Glu(or GLu′)-3	79.4, 79.2	*d,d*
16	28.3	*t*	Glu(or GLu′)-4	71.5, 71.1	*d,d*
17	46.6	*s*	Glu(or GLu′)-5	78.9, 78.9	*d,d*
18	44.7	*d*	Glu(or GLu′)-6	62.6, 62.3	*t,t*

Acidic hydrolysis of **2**. Same procedure as in hydrolysis of **1**.

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