Anti-platelet Aggregation Triterpene Saponins from the Galls of *Sapindus mukorossi*

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Received May 15, 2007; accepted June 4, 2007

Bioassay-directed fractionation of an ethanolic extract of the galls of *Sapindus mukorossi* has resulted in the isolation of two new tirucallane-type triterpenoid saponins, sapinmusaponins Q (1) and R (2), along with three known oleanane-type triterpenoid saponins (3—5). Their structures were elucidated on the basis of spectroscopic analysis and chemical hydrolysis. Biological evaluation showed that both sapinmusaponins Q and R demonstrated more potent anti-platelet aggregation activity than aspirin.

Key words *Sapindus mukorossi*; sapinmusaponin Q; sapinmusaponin R; saponin; anti-platelet aggregation activity

Sapindus mukorossi (Sapindaceae), better known as the soapnuts, generally grows in tropical and sub-tropical regions of Asia. It was reported that *S. mukorossi* possessed efficient natural surfactants and has served as commercial ingredient of shampoo and cosmetic cleansers.[1)] In addition, some pharmacological effects including anthelmintic,[2)] antidermatophytic,[2)] antiinflammatory,[3)] antimicrobial,[4)] antitussive,[2)] cytotoxic,[5)] haemolytic,[6)] and molluscicidal[7)] activities were found in the plant. As for the principal constitutes of this plants, various triterpenoid saponins containing dammarane-type,[8,9)] hederagenin-type,[2,10)] tirucallane-type[11,12)] as well as sesquiterpene oligoglycosides,[1,2)] have been isolated from the fruit, gall, pericarp, root, and stem. Recently, we have isolated and characterized antitumor dammarane saponins, sapinmusaponins A—E[13)] and anti-platelet aggregation tirucallane saponins, sapinmusaponins F—J[14)] from the galls of *S. mukorossi*. We report herein that two new tirucallane saponins, named sapinmusaponins Q (**1**) and R (**2**), were isolated from the other anti-platelet aggregation fraction derived from EtOH extract of galls of the titled plant. Structural elucidation of the new isolates was based on spectroscopic analysis including 1D and 2D NMR techniques (^{1}H–^{1}H COSY, HMQC, HMBC, TOCSY and NOESY) and chemical hydrolysis. Biological assay of **1**—**5** for anti-platelet aggregation, is also reported here.

The EtOH extract of the galls of *S. mukorossi* was partitioned with MeOH/$CHCl_3$/H_2O (7/10/3), then the $CHCl_3$ layer was partitioned with MeOH/*n*-hexane to give a MeOH residue. Chromatography of the MeOH layer on Diaion HP-20, Sephadex LH-20, silica gel, and then after repeated RP-HPLC purification afforded two new saponins (**1**, **2**) and three known saponins (**3**—**5**).

Compound **1** was isolated as an amorphous powder. Its molecular formula was established as $C_{43}H_{70}O_{13}$ from the HR-FAB-MS pseudomolecular ion peak at *m/z* 817.4710 $[M+Na]^+$ (Calcd 817.4714). The ^{1}H-NMR spectrum (Table 1) showed signals characteristic for five tertiary singlet methyls at δ 0.76, 0.87, 0.92, 1.01, and 1.05, two allylic-

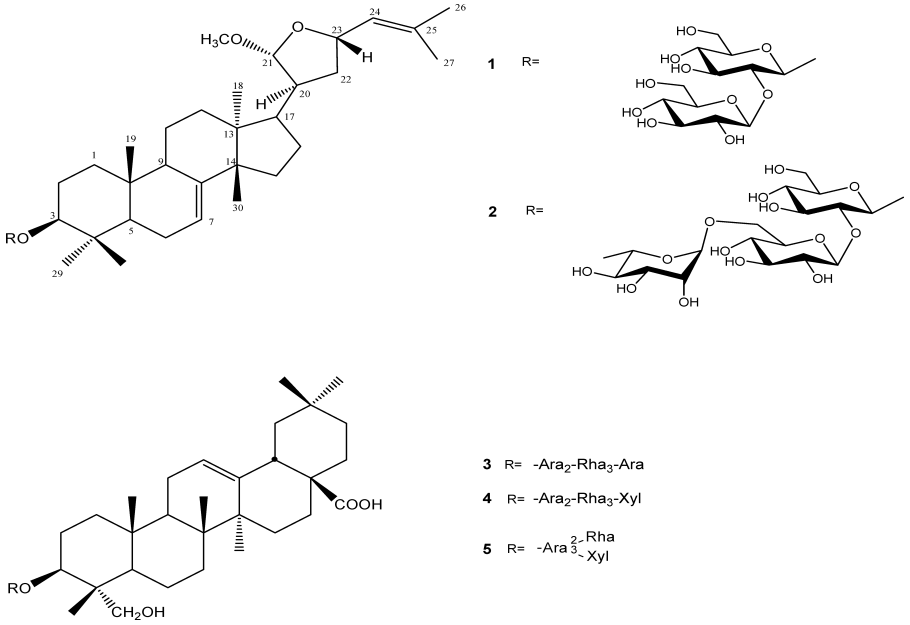

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Table 1. ^{13}C- (100 MHz) and ^{1}H-NMR (400 MHz) Spectra Data[a] of **1** and **2** in CD_3OD

Position	1		2		Position	1		2	
	δ_C	δ_H (mult, J in Hz)	δ_C	δ_H (mult, J in Hz)		δ_C	δ_H (mult, J in Hz)	δ_C	δ_H (mult, J in Hz)
1	38.4	1.67,[b] 1.13 (m)	38.3	1.67,[b] 1.16 (m)	Glc				
2	28.7	1.98 (m), 1.67[b]	27.8	1.97 (m), 1.67[b]	1′	105.3	4.45 (d, 7.2)	105.3	4.42 (d, 7.6)
3	90.3	3.18 (dd, 12.0, 4.0)	91.5	3.18 (dd, 12.4, 3.2)	2′	81.2	3.57 (dd, 7.2, 8.8)	81.0	3.57 (dd, 7.6, 8.4)
4	40.5	—	40.5	—	3′	78.4	3.55 (t, 8.8)	78.4	3.55 (t, 8.4)
5	52.4	1.34 (dd, 12.0, 4.8)	52.4	1.35 (dd, 11.6, 6.0)	4′	71.8	3.24 (m)	71.5	3.23 (t, 8.4)
6	24.8	2.10 (m), 1.94 (m)	24.9	2.10 (m), 1.95 (m)	5′	77.8	3.34 (m)	77.8	3.34 (m)
7	119.4	5.27 (br s)	119.3	5.28 (d, 2.4)	6′	63.0	3.86 (d, 10.4)	63.1	3.83 (d, 11.6)
8	146.6	—	146.6	—			3.68 (dd, 10.4, 4.8)		3.60 (dd, 11.6, 5.6)
9	50.2	2.24 (m)	50.1	2.25 (m)	Glc				
10	35.7	—	35.8	—	1″	104.5	4.76 (d, 7.6)	104.3	4.65 (d, 8.0)
11	18.9	1.58 (m)	18.9	1.60 (m)	2″	76.3	3.21 (dd, 8.8, 7.6)	76.3	3.18 (t, 8.0)
12	33.7	1.77 (m), 1.61 (m)	33.7	1.78 (m), 1.62 (m)	3″	77.6	3.38 (t, 8.8)	78.4	3.24 (t, 8.0)
13	44.8	—	44.8	—	4″	71.5	3.27 (m)	71.9	3.20 (m)
14	52.3	—	52.3	—	5″	78.2	3.23 (m)	76.3	3.38 (m)
15	34.9	1.56 (m), 1.49 (m)	34.9	1.56 (m), 1.48 (m)	6″	62.7	3.83 (d, 10.4)	67.9	3.96 (dd, 11.6, 1.2)
16	27.7	1.97 (m), 1.32 (m)	28.7	1.97 (m), 1.32 (m)			3.64 (dd, 10.4, 6.4)		3.58 (dd, 11.6, 5.6)
17	49.7	1.80 (m)	49.7	1.80 (m)	Rha				
18	23.1	0.87 (s)	23.1	0.87 (s)	1‴			102.1	4.73 (d, 1.2)
19	13.6	0.76 (s)	13.6	0.77 (s)	2‴			72.2	3.80 (dd, 3.2, 1.2)
20	49.6	2.23 (m)	49.6	2.22 (m)	3‴			72.4	3.63 (dd, 9.6, 3.2)
21	109.2	4.81[b]	109.2	4.81[b]	4‴			73.9	3.36 (t, 9.6)
22	37.4	1.83 (m)	37.3	1.83 (m)	5‴			69.7	3.66 (dd, 9.6, 6.0)
23	76.7	4.83[b]	76.8	4.83[b]	CH_3			18.1	1.25 (d, 6.0)
24	128.7	5.23 (d, 8.8)	128.7	5.23 (d, 8.8)					
25	135.4	—	135.5	—					
26	25.9	1.70 (s)	25.9	1.70 (s)					
27	17.9	1.67 (s)	17.9	1.67 (s)					
28	28.0	1.05 (s)	28.0	1.05 (s)					
29	16.0	0.92 (s)	16.0	0.92 (s)					
30	27.6	1.01 (s)	27.7	1.01 (s)					
OCH_3	55.1	3.31 (s)	55.1	3.32 (s)					

a) Assignments confirmed by decoupling, ^{1}H–^{1}H COSY, TOCSY, NOESY, HMQC and HMBC. *b*) Overlapped signals.

methyls at δ 1.67 and 1.70, two olefinic methene at δ 5.23 (1H, d, J=8.8 Hz) and 5.27 (1H, br s), a methoxy at δ 3.31 (3H, s), and two anomeric protons at δ 4.45 (1H, d, J=7.2 Hz) and 4.76 (1H, d, J=7.6 Hz), which gave correlations in the HMQC spectrum with carbon signals at δ 105.3 and 104.5. Additionally, the ^{13}C-NMR spectrum of the aglycone showed the presence of seven tertiary methyls, eight methylenes, nine methines, and six quaternary carbons along with a methoxy group (δ 55.1). These features indicated the aglycone of carbon with an acetal signal (δ 109.2), two oxymethine signals (δ 76.7, 90.3) and two olefinic signals (δ 119.4, 128.7, 135.4, 146.6). A detailed comparison of the ^{1}H- and ^{13}C-NMR data between **1** and the reference data of sapinmusaponin J,[14] implied that **1** possessed the aglycone of 21α-methoxy-3β,21(*R*),23(*S*)-epoxytiucall-7,24-diene.

Acid hydrolysis of **1** afforded D-glucoses, which were analyzed by gas chromatography (see Experimental section). Assignment of sugars signals (Table 1) was achieved based on their COSY, TOCSY, HMQC, and HMBC spectra, and the β-anomeric configurations of the glucose units were deduced from their $^{3}J_{H1,H2}$ coupling constants (7.2, 7.6 Hz).[15] In regard to the sugar moieties, in the HMBC spectrum (Fig. 1), H-1′ (δ 4.45) of glucose showed correlation with C-3 (δ 90.3) of aglycone, together with a correlation between H-3 (δ 3.18) of aglycone and H-1′ (δ 4.45) of glucose in the NOESY spectrum of **1**, indicating that the sugar chain was located at the C-3 position of aglycone. Moreover, a cross peak between H-1″ (δ 4.67) of glucose and C-2′ (δ 81.2) of glucose in the HMBC spectrum revealed 1→2 linkage of the terminal sugar moiety to the inner one. Accordingly, the structure of **1** was elucidated to be 21α-methoxy-3β,21(*R*),23(*S*)-epoxytirucall-7,24-diene-3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside, and named sapinmusaponin Q.

Compound **2**, an amorphous powder, had the molecular formula $C_{49}H_{80}O_{17}$ determined from its pseudomolecular ion peak at *m/z* 963.5305 $[M+Na]^+$ (Calcd 963.5293) in the HR-FAB-MS. The ^{1}H- and ^{13}C-NMR indicated that **2** had the same aglycone as that of **1**. On acid hydrolysis, **2** afforded monosaccharides that were identified to be glucose and rhamnose in the ratio 2 : 1 from GC analysis. The difference of 146 mass units compared to **1** suggested that **2** had an additional rhamnose unit compared with **1**. The 2D experiments (COSY, TOCSY, and HMQC) allowed the assignment of all the ^{1}H and ^{13}C signals of the sugars, and identified **2** to possess two β-D-glucose [the anomeric proton signals at δ 4.42 (d, J=7.6 Hz), 4.65 (d, J=8.0 Hz), the carbon signals at δ 105.3, 104.3] and one α-L-rhamnose [the anomeric proton signal at δ 4.73 (d, J=1.2 Hz), the carbon signal at δ 102.1]. The coupling constant suggested β-anomeric configuration for the glucose and α-anomeric configuration for the rhamnose.[16] As for the interglycosidic linkage positions, they were determined from the HMBC spectrum (Fig. 1), which showed correlations between H-1′ (δ 4.42) of glucose, and

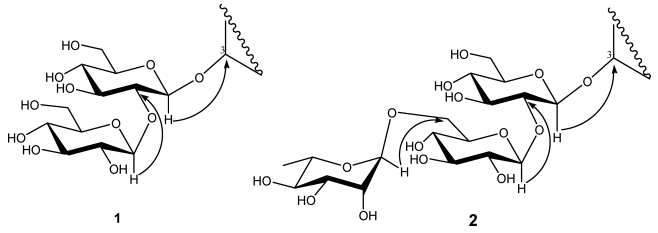

Fig. 1. Key HMBC Correlations of Compounds **1** and **2**

Table 2. The IC_{50} (μM) Values of Compounds **1**—**5** on of Washed Rabbit Platelets Induced by PAF, TXA_2, THB or AA[a)]

Compound	IC_{50} (μM)			
	PAF	TXA_2	THB	AA
1	7.7±0.2	3.4±0.7	8.4±0.2	6.7±0.4
2	13.5±0.3	5.4±0.2	8.9±0.4	12.5±0.1
3	b)	b)	b)	b)
4	b)	b)	b)	b)
5	b)	b)	b)	b)
Aspirin	c)	c)	c)	30.5±5.7

a) PAF: platelet-activity factor, TXA_2=U46619, a thromboxane A2 agonist, THB: thrombin, and AA: arachidonic acid. *b*) Inactive (IC_{50} >100 μM). *c*) No test.

C-3 (δ 91.5) of aglycone, H-1″ (δ 4.65) of glucose and C-2′ (δ 81.0) of glucose, and H-1‴ (δ 4.73) of rhamnose and C-6″ (δ 67.9) of glucose. Based on these findings, the structure of **2** was elucidated to be 21α-methoxy-3β,21(*R*),23(*S*)-epoxytirucall-7,24-diene-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside, and designated sapinmusaponin R.

Compounds **3**—**5** were identified to be the known saponins A (**3**),[17] sapindoside B (**4**),[17] and hederagenin-3-*O*-[β-D-xylopyranosyl-(1→3)]-[α-L-rhamnopyranosyl-(1→2)]-α-L-arabinopyranoside (**5**).[18]

Biological evaluation showed that **1** and **2** had more potent anti-platelet aggregation (induced by PAF, TXA_2, THB, or AA; IC_{50} *ca.* 3.4—13.5 μM) than aspirin (IC_{50} *ca.* 30.5 μM) (Table 2). In contrast, the IC_{50} values for compounds **3**—**5** all exceeded 100 μM. By measuring the percentage of LDH leakage as released from the platelets, compounds **1** and **2** had no obvious cytotoxicity (LDH <10.0%). These results, together with the previously reported data,[14] suggest that the saponins that possess tirucallane-type triterpenoids might play a significant role for the anti-platelet aggregation activities.

Experimental

Optical rotations were measured by JASCO P-120 polarmeter. Infrared (IR) spectra were measured with a Mattson Genesis II (by a KBr disk method) spectrophotometer. FAB-MS data were performed on a Jeol SX-102A instrument. High-resolution FAB-MS were measured on a Finnigan/Thermo Quest MAT mass spectrometer. 1D and 2D NMR spectra were performed on a Bruker NMR spectrometer (Avance 400 MHz) using CD_3OD as solvent for measurement. Gas chromatorgaphy was performed on an Agilent Technologies 6890N Network GC System. Diaion HP-20, Sephadex LH-20 and silica gel (Merck 70—230 mesh and 230—400 mesh) were used for column chromatography, and pre-coated silica gel (Merck 60 F-254) plates were used for TLC. The spots on TLC were detected by spraying with 95% H_2SO_4 then heating on a hotplate. HPLC separations were performed on a Shimadzu LC-6AD series apparatus with a RID-10A Refractive Index, equipped with a 250×20 mm i.d. preparative Cosmosil 5C18-AR II column.

Plant Material The galls of *Sapindus mukorossi* were collected in Taipei County in October 2001, and identified by Prof. Muh-Tsuen Kao of the National Institute of Chinese Medicine. A voucher specimen (NRICM20011007A2 for galls) was deposited in the National Institute of Chinese Medicine, Taipei, Taiwan.

Extraction and Isolation The dried galls of *S. mukorossi* (8.5 kg) were refluxed three times with 95% EtOH. After concentration *in vacuo*, the EtOH extract was obtained and partitioned with MeOH/$CHCl_3$/H_2O (7/10/3) three times to give $CHCl_3$ and H_2O layers. The $CHCl_3$ layer (190 g) was partitioned with MeOH/*n*-hexane three times to give MeOH (100 g) and *n*-hexane layers. After the MeOH was evaporated in vacuum, the residue was further chromatographed over a silica gel column (41×10.5 cm i.d.), which was eluted with $CHCl_3$–MeOH (1 : 0→0 : 1) to give 13 fractions. The 10th and 11th fractions were combined and further purified by chromatography on a Sephadex LH-20 column (35×2.5 cm) with MeOH; 5 fractions (fr. 10.1 to 10.5) were obtained. Fr. 10.4 was further purified by HPLC (Cosmosil 5C18-AR II, 250×20.0 mm i.d., flow rate: 5 min/ml, 80% MeOH) to give **3** (8.6 mg). Using the same column as that of fraction 10.4 on HPLC with 75% MeOH, **4** (6.5 mg) and **5** (4.3 mg) were furnished from fr. 10.5. The 12th fraction was active for anti-platelet aggregation and was subjected to column chromatography over a 23×4.5 cm i.d. Diaion HP-20 porous polymer resin column, eluting with 10, 40, 60, 80, and 100% aq. MeOH, successively, to yield 6 fractions, fr. 12.1—12.6. Fr. 12.3 was further separated by chromatography on a 30×2.8 cm i.d. Sephadex LH-20 with MeOH to yield 4 fractions, fr. 12.3.1—12.3.4. Fr. 12.3.4 was further purified by HPLC (Cosmosil 5C18-AR II, 250×20.0 mm i.d., flow rate: 5 min/ml, 70% MeOH) to afford **1** (10.4 mg) and **2** (9.4 mg).

Sapinmusaponin Q (**1**): White amorphous powder, $[\alpha]_D^{24}$ −24.6° (*c*=0.4, MeOH). IR (KBr) ν_{max}: 3372, 2937, 1647, 1451, 1384, 1033 cm^{-1}. HR-FAB-MS: *m/z* 817.4710 $[M+Na]^+$ (Calcd for $C_{43}H_{70}O_{13}Na$: 817.4717). ^{1}H-NMR (CD_3OD, 400 MHz) and ^{13}C-NMR (CD_3OD, 100 MHz) spectra data, see Table 1.

Sapinmusaponin R (**2**): White amorphous powder, $[\alpha]_D^{24}$ −21.2° (*c*=0.2, MeOH). IR (KBr) ν_{max}: 3407, 2932, 1641, 1450, 1384, 1045 cm^{-1}. HR-FAB-MS: *m/z* 963.5305 $[M+Na]^+$ (Calcd for $C_{49}H_{80}O_{17}Na$: 963.5293). ^{1}H-NMR (CD_3OD, 400 MHz) and ^{13}C-NMR (CD_3OD, 100 MHz) spectra data, see Table 1.

Acid Hydrolysis of 1 and 2 Saponins **1** and **2** (each 2.0 mg) were refluxed with 1 N HCl (1,4-dioxane : H_2O, 1 : 1, 2 ml) at 80 °C for 3 h, respectively. The respective mixture was extracted with CH_2Cl_2 to afford the aglycone part, and the aqueous layer was neutralized with NaOH then filtered. The filtrates were evaporated to dryness, then 1-(trimethylsilyl)imidazole and pyridine (0.2 ml) were added. After reacting for 1 h, the mixture was dried by a stream of N_2, and partitioned with *n*-hexane and water. The *n*-hexane layer was analyzed by GC with the following conditions: CP-Chirasil-L-Val column (25 m×0.25 mm); injection temperature: 200 °C; column temperature: 100—200 °C; rate: 4 °C/min. Peaks of trimethylsilyl derivatives derived from **1** [D-glucose (21.26 min)] and **2** [L-rhamnose (12.23 min) and D-glucose (21.26 min)] were detected by comparison with retention times of authentic samples [L-rhamnose (12.22 min) and D-glucose (21.25 min)] treated with 1-(trimethylsilyl)imidazole.

Bioassay The antiplatelet aggregation,[19] and lactate dehydrogenase (LDH) leakage[20] activities of saponins were evaluated according to the same protocol as reported in the literature.

Acknowledgements Financial support of this work by the National Science Council and the National Research Institute of Chinese Medicine of the Republic of China to Y. H. Kuo is gratefully acknowledged.

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