Sapinmusaponins F−J, Bioactive Tirucallane-Type Saponins from the Galls of *Sapindus mukorossi*

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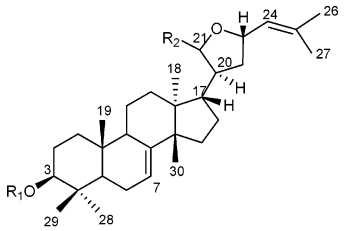
Received November 2, 2005

Five new tirucallane-type saponins, sapinmusaponins F−J (**1**−**5**), were isolated from the galls of *Sapindus mukorossi*. The structures of these saponins were elucidated on the basis of spectroscopic analysis including 1D and 2D NMR techniques (^{1}H−^{1}H COSY, HMQC, HMBC, TOCSY, and NOESY). Compounds **1**−**5** showed anti-platelet-aggregation effects, but no obvious cytotoxic activity for platelets as assayed by lactate dehydrogenase (LDH) leakage. Compounds **1**−**5** also showed moderate activity in a 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein−Barr virus early antigen (EBV-EA) activation assay.

Sapindus mukorossi Gaertn. (Sapindaceae) grows in tropical and subtropical regions of Asia, and its pericarp has been used traditionally in Japan, mainland China, and Taiwan as a source of natural surfactants, as well as an expectorant.[1−3] *S. mukorossi* also exhibits interesting pharmacological effects such as molluscicidal,[4] anti-inflammatory,[5] hemolytic,[6] antimicrobial,[7] and cytotoxic activities.[8] Phytochemical studies have identified several different types of constituents, including sesquiterpene oligoglycosides[1,2] and hederagenin-type,[1,9] tirucallane-type,[10,11] and dammarane-type saponins[12,13] in the pericarp, root, and stem. This plant family, as well as many others including the Anacardiaceae, Fagaceae, Lauraceae, Leguminosae, and Myrtaceae, develops galls.[14] Galls grow in the host plant meristematic tissues, where the gall-forming insect modifies the tissue growth processes and transforms the attacked cells into gall structures.[15]

In our continuing search for bioactive constituents from Taiwanese plants, several new cytotoxic dammarane-type saponins, sapinmusaponins A−E, were recently isolated from the galls of *S. mukorossi*.[16] Unexpectedly, during isolation of the cytotoxic constituents, we found that several noncytotoxic fractions derived from the EtOH extract of the gall inhibited the platelet aggregation induced by arachidonic acid (AA), thromboxane A_2 (TXA_2), thrombin (THB), and platelet activating factor (PAF). We report herein the isolation and characterization of five new tirucallane-type saponins, sapinmusaponins F−J (**1**−**5**), from the anti-platelet fractions of the EtOH extract. The structural elucidation of **1**−**5** was based on spectroscopic analyses including similar 1D and 2D NMR techniques (^{1}H−^{1}H COSY, HMQC, HMBC, TOCSY, and NOESY) and chemical methods. Compounds **1**−**5** and the previously isolated sapinmusaponins A−E were assayed for anti-platelet-aggregation activity, as well as cytotoxicity determined by the percentage of lactate dehydrogenase (LDH) leakage released from platelets. Moreover, some triterpenoids, such as cimigenol and related glycosides, were shown recently to be potential cancer chemopreventive agents by using the short-term 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein−Barr virus early antigen (EBV-EA) activation assay in vitro.[16] Accordingly, the newly isolated tirucallane-type saponins were evaluated in this assay.



1 R_1= Glc^6-Rha R_2= β-OCH_3
2 R_1= Glc^6-Rha R_2= α-OCH_3
3 R_1= Glc^2-Rha R_2= α-OCH_3
4 R_1= $Glc^{2,6}$-Rha R_2= β-OCH_3
5 R_1= $Glc^{2,6}$-Rha R_2= α-OCH_3

Results and Discussion

As in the previously reported extraction procedure,[17] the $CHCl_3$ layer of *S. mukorossi* was partitioned with methanol/*n*-hexane to give a MeOH extract. After evaporation of the solvent, the residue was subjected to column chromatography on Sephadex LH-20 and silica gel and then separated by HPLC, to afford **1**−**5**.

Sapinmusaponin F (**1**) was obtained as a white amorphous powder. The molecular formula of **1** was determined as $C_{43}H_{70}O_{12}$ by HRFABMS, which exhibited a quasi-molecular ion peak at *m*/*z* 801.4770 $[M + Na]^+$. The IR spectrum showed the presence of hydroxyl (3388 cm^{-1}) and olefinic (1447 and 1045 cm^{-1}) groups. Acid hydrolysis of **1** with 10% HCl afforded D-glucose and L-rhammose, which were identified by GC-MS. The ^{1}H NMR spectrum of **1** (Table 1) showed the aglycon signals for a triterpene unit [five tertiary methyls (δ_H 1.01 × 2, 0.94, 0.88, 0.79), two allylic methyls (δ_H 1.72, 1.69), and three oxymethines (δ_H 4.79, 4.75, 3.18)]. In addition, signals for two sugar moieties, characterized by 10 oxygenated protons and two anomeric protons at δ_H 4.32 (d,

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10.1021/np050446z CCC: $33.50
Published on Web 04/27/2006

Table 1. ^{1}H NMR Spectroscopic Data[a] of Compounds **1**−**5** (CD_3OD, 500 MHz for **1**, **2**, and **3**, 400 MHz for **4** and **5**)

position	**1**	**2**	**3**	**4**	**5**
1	1.69[b], 1.18 m	1.71[b], 1.18 m	1.65[b], 1.19 m	1.65[b],1.14 m	1.65[b], 1.17 m
2	1.98 m, 1.68[b]	1.93 m, 1.71[b]	2.02 m, 1.66[b]	1.99 m, 1.68[b]	1.98 m, 1.65[b]
3	3.18 m	3.18 m	3.21 dd (12.5, 3.5)	3.16 dd (11.2, 3.6)	3.13 dd (11.6, 3.6)
5	1.37 dd (11.5, 5.5)	1.36 dd (11.5, 5.5)	1.35 dd (11.5, 5.5)	1.35 dd (12.0, 6.0)	1.35 dd (12.0, 6.0)
6	2.14 m, 1.96[b]	2.13 m, 1.96[b]	2.14 m, 1.96[b]	2.14 m, 1.95[b]	2.10 m, 1.96[b]
7	5.27 d (2.5)	5.29 br s	5.29 d (2.5)	5.25 d (2.8)	5.26 d (2.4)
9	2.28 m	2.26[b]	2.26[b]	2.27[b]	2.25 m
11	1.58 m	1.56 m	1.58 m	1.60 m	1.60 m
12	1.91 m, 1.40 m	1.79 m, 1.63 m	1.78 m, 1.60 m	1.89 m, 1.39 m	1.79 m, 1.62 m
15	1.62 m, 1.50 m	1.62 m, 1.50 m	1.62 m, 1.52 m	1.56 m, 1.48 m	1.54 m, 1.48 m
16	1.85 m, 1.31 m	1.98 m, 1.31 m	1.98 m, 1.31 m	1.86 m, 1.33 m	1.97 m, 1.32 m
17	2.00[b]	1.84 m	1.82m	2.00 m	1.80 m
18	0.88 s	0.89 s	0.88 s	0.86 s	0.87 s
19	0.79 s	0.79 s	0.79 s	0.77 s	0.77 s
20	2.02[b]	2.24[b]	2.25[b]	2.01 m	2.24 m
21	4.75 d (3.0)	4.82[b]	4.82[b]	4.73 d (3.2)	4.84 d (6.4)
22	1.96[b], 1.58[b]	1.83[b]	1.83[b]	1.96[b], 1.55[b]	1.83[b]
23	4.79 ddd (9.0, 3.0, 2.5)	4.84[b]	4.84[b]	4.74[b]	4.86[b]
24	5.23 d (9.0)	5.24 d (9.0)	5.23 d (9.0)	5.22 dt (8.8, 2.4, 1.2)	5.22 dt (8.8, 2.4, 1.2)
26	1.72 s	1.72 s	1.72 s	1.70 s	1.70 d (1.2)
27	1.69 s	1.69 s	1.69 s	1.68 s	1.67 d (1.2)
28	1.01 s	1.04 s	1.04 s	1.02 s	1.05 s
29	0.94 s	0.91 s	0.94 s	0.92 s	0.92 s
30	1.01 s	1.03 s	1.03 s	0.98 s	1.02 s
OCH_3	3.28 s	3.31 s	3.29 s	3.32 s	3.28 s
1′	4.32 d (8.0)	4.32 d (7.5)	4.43 d (7.5)	4.38 d (7.2)	4.39 d (7.2)
2′	3.32 m	3.31 m	3.42 dd (7.6, 7.5)	3.42 d (8.8, 7.2)	3.40 dd (8.8, 7.2)
3′	3.19 m	3.20 m	3.45 t (7.6)	3.45 t (8.8)	3.45 t (8.8)
4′	3.28 m	3.28 m	3.29 m	3.25 m	3.28 m
5′	3.39 m	3.38 m	3.25 dd (5.5, 2.5)	3.35 m	3.36 m
6′	3.98 d (11.5)	3.97 d (11.0)	3.85 dd (12.5, 2.0)	3.96 m	3.95 m
	3.60 dd (11.0, 6.5)	3.61 dd (11.0, 6.0)	3.67 dd (12.5, 5.5)	3.59 dd (11.2, 6.0)	3.58 dd (11.2, 5.6)
1″	4.75 br s	4.75 br s	5.37 br s	5.36 br s	5.37 d (1.6)
2″	3.66 m	3.67 m	3.96 dd (3.5, 2.0)	3.93 dd (3.2, 1.2)	3.94 m
3″	3.83 m	3.83 m	3.75 dd (9.5, 3.5)	3.73 dd (9.2, 3.2)	3.72 dd (9.6, 3.2)
4″	3.35 m	3.36 m	3.39 t (9.5)	3.39 t (9.2)	3.37 dd (9.6, 3.2)
5″	3.67 m	3.69 m	3.99 dd (9.5, 6.5)	3.96 dd (9.6, 6.4)	3.96 dd (9.2, 6.4)
6″	1.27 d (6.5)	1.27 d (6.5)	1.23 d (6.0)	1.22 d (6.0)	1.22 d (6.0)
1‴				4.72 d (1.2)	4.72 d (1.6)
2‴				3.82 dd (3.2, 1.6)	3.82 dd (3.6, 1.6)
3‴				3.68 dd (9.2, 3.2)	3.64 dd (9.6, 2.4)
4‴				3.37 t (9.6)	3.35 dd (9.6, 2.4)
5‴				3.67 dd (9.6, 6.4)	3.66 dd (9.6, 6.4)
6‴				1.25 d (6.4)	1.25 d (6.0)

[a] Assignments confirmed by decoupling and from the ^{1}H−^{1}H COSY, TOCSY, HMQC, and HMBC spectra. [b] Signals were not clearly observed due to the overlap of the peak or the solvent signals.

$J = 8.0$ Hz) and 4.75 (br s), were observed. The ^{13}C NMR (Table 2) and DEPT spectra indicated signals characteristic for the predicted triterpene functionality, comparing seven tertiary methyls, eight methylenes, nine methines (including two olefinic carbons at δ_C 118.1, 126.8 and three oxygenated units at δ_C 73.3, 89.8, 104.6), and six quaternary carbons, along with a methoxy group (δ_C 53.2). Moreover, carbon signals for acetal (δ_C 104.6, C-21), oxymethine (δ_C 73.3, C-23), and olefinic [δ_C 126.8 (C-24), 134.5 (C-25)] units were present in the ^{13}C NMR spectrum, and these groups were likely part of the triterpene side chain.[18] Double bonds were located at C-7 and C-24, and three secondary alcohols were assigned at C-3, C-21, and C-23, on the basis of the following long-range HMBC correlations (H-3 with C-2, 4, 5, 28, 29, Glc-1′; H-7 with C-6, 9, 30; H-21 with C-17, 20, 22, 23, OCH_3; H-23 with C-20, 22, 24, 25; H-24 with C-22, 23, 25, 26, 27). From this evidence, together with the TOCSY NMR spectroscopic data, four partial structures, drawn with bold lines (C_1−C_3, C_6−C_7, C_{11}−C_{12}, C_{15}−C_{17}, and C_{20}−C_{24}), were deduced, indicating that **1** is a tirucallane-7,24-diene-type triterpene glycoside.[19−21] Moreover, Glc-C-1′ was linked to C-3 of the aglycon and Rha-C-1″ to Glc-C-6′, on the basis of long-range correlations between δ_H 4.32 (Glc-H-1′) and δ_C 89.8 (C-3) and between δ_H 4.75 (Rha-H-1″) and δ_C 66.9 (Glc-6′) in **1** (Figure 1). Further confirmation of the stereochemistry of **1** was achieved by the 2D NOE NMR technique. In the NOESY spectrum, correlations between H-3/H-5, H-9, Me-29; H-17/Me-30; and H-21/H-12α, Me-18, H-20 suggested the following stereochemical assignments: H-3, H-17, and H-21 with α-, *β*-, and α-orientations, respectively (Figure 2). In addition, correlations between H-20/Me-18, H-21 agreed with an α-configuration of H-20 in **1**. Consequently, **1** was assigned as 21*β*-methoxy-3*β*,21(*S*),23-(*R*)-epoxytirucalla-7,24-diene-3-*O*-α-L-rhamnopyranosyl-(1→6)-*β*-D-glucopyranoside (**1**).

Sapinmusaponin G (**2**) was obtained as a white amorphous powder. The IR spectrum of **2** showed absorption bands at 3386, 1447, and 1097 cm^{-1}. It was assigned the same molecular formula ($C_{43}H_{70}O_{12}$) as **1**, indicated by the molecular ion (*m*/*z* 801.4760 [M + Na]$^+$) in its HRFABMS. The ^{1}H and ^{13}C NMR spectra of **2** showed characteristic signals for a substituted tirucallane-type triterpene and two sugar moieties. The proton and carbon signals of **2** were similar to those of **1**; however, the methoxy protons resonated at δ_H 3.31 in **2** as opposed to δ_H 3.28 in **1**. Thus, the methoxy group at C-21 in **2** was suspected to have an α-configuration (Figure 2). The other differences in ^{13}C NMR data between **1** and **2** included signals for C-17 (δ_C 46.0 in **1**; δ_C 48.6 in **2**), C-20 (δ_C 45.5 in **1**; δ_C 48.3 in **2**), C-21 (δ_C 104.6 in **1**; δ_C 108.1 in **2**), and C-23 (δ_C 73.3 in **1**; δ_C 75.6 in **2**) and provided evidence for an α-OCH_3 group at C-21 in **2**, by comparing with the similar differences of the epimers at C-21 found in the other tirucallane-type analogues.[18,21] Additionally, cross-peaks between H-21 and H-12*β*; H-18 and H-12α and 20; and H-23 and H-21*β* in the

Table 2. ^{13}C NMR Spectroscopic Data of Compounds **1**−**5** (CD_3OD,[a] 125 MHz for **1**, **2**, and **3**, 100 MHz for **4** and **5**)

aglycon	1	2	3	4	5	sugars	1	2	3	4	5
1	37.2	37.1	37.4	38.5	38.5	1′	105.6	105.6	104.3	105.6	105.6
2	26.5	26.5	26.5	27.8	27.9	2′	77.0	77.0	77.8	79.5	79.5
3	89.8	89.8	89.0	90.5	90.5	3′	74.4	74.4	78.3	78.8	78.8
4	39.1	39.1	39.2	40.3	40.3	4′	71.2	70.5	70.9	72.0	72.0
5	51.4	51.2	51.6	52.8	52.7	5′	75.2	75.2	76.4	76.3	76.2
6	23.7	23.7	23.7	24.9	24.9	6′	66.9	66.9	61.5	68.1	68.1
7	118.1	118.2	118.2	119.3	119.3	1″	101.0	100.9	100.7	101.8	101.8
8	145.8	145.5	145.5	147.0	146.7	2″	70.5	71.1	70.8	72.3	72.2
9	49.0	49.0	49.1	50.1	50.1	3″	71.1	71.2	70.8	72.4	72.4
10	34.6	34.6	34.6	35.8	35.8	4″	72.8	72.8	72.7	73.9	73.9
11	17.6	17.7	17.7	18.7	18.8	5″	68.6	68.6	68.8	69.8	69.8
12	31.2	32.6	32.6	32.3	33.7	6″	16.9	17.0	16.8	18.1	18.0
13	43.6	43.7	43.7	44.8	44.8	1‴				102.1	102.1
14	50.7	51.3	51.2	51.9	52.3	2‴				72.2	72.1
15	34.1	33.7	33.7	35.3	34.9	3‴				72.4	72.4
16	27.1	27.6	27.5	28.3	28.7	4‴				74.0	74.0
17	46.0	48.6	48.0	47.1	49.7	5‴				69.9	69.9
18	22.4	22.0	21.9	23.6	23.1	6‴				18.2	18.2
19	12.4	12.5	12.5	13.7	13.7						
20	45.5	48.3	48.0	46.7	49.6						
21	104.6	108.1	108.0	105.7	109.2						
22	35.5	36.2	36.2	36.7	37.3						
23	73.3	75.6	75.6	74.5	76.8						
24	126.8	127.6	127.6	128.0	128.8						
25	134.5	134.2	134.4	135.7	135.5						
26	24.6	24.8	24.7	25.8	25.9						
27	16.9	16.8	16.7	18.1	17.9						
28	26.9	26.9	26.9	28.1	28.1						
29	14.9	14.9	15.1	16.3	16.3						
30	26.6	26.6	26.6	27.9	27.7						
OCH_3	53.2	54.0	54.0	54.4	55.1						

[a] Solvent peaks for **1**−**3** were measured at 47.9 ppm, and at 49.0 ppm for **4** and **5**.

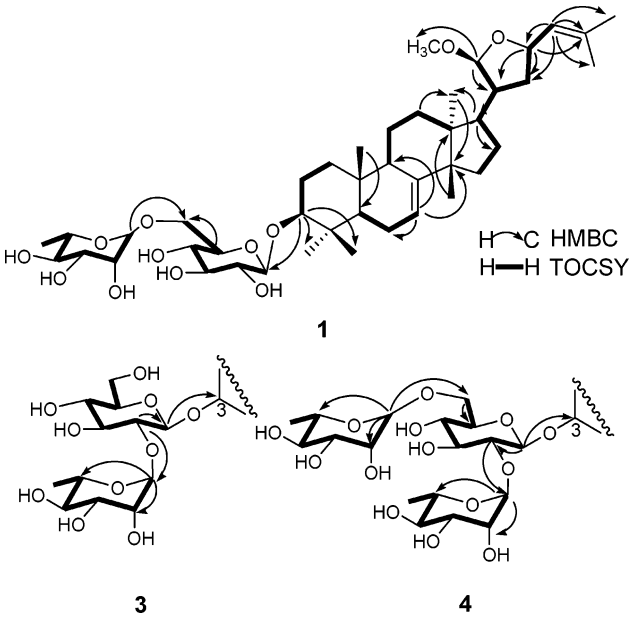

Figure 1. Key TOCSY and HMBC correlations of sugar moieties of **1**, **3**, and **4**.

NOESY spectrum of **2** were also consistent with an α-orientation of the 21-methoxy group and *β*-configuration of H-23. Accordingly, the structure of **2** was established as 21α-methoxy-3*β*,21(*R*),23-(*R*)-epoxytirucalla-7,24-diene-3-*O*-α-L-rhamnopyranosyl-(1→6)-*β*-D-glucopyranoside (**2**).

Sapinmusaponin H (**3**) was obtained as an amorphous powder. Its molecular formula was determined as $C_{43}H_{70}O_{12}$ by HRFABMS (*m*/*z* 801.4756 $[M + Na]^+$). Comparison of the ^1H and ^{13}C NMR spectroscopic data of **3** with those of **2** showed similar signals, except for the presence of an anomeric proton found at δ_H 5.37 in **3** rather than at δ_H 4.75 in **2**. Acid hydrolysis of **3** afforded D-glucose and L-rhammose, which were identified by GC-MS. Inspection of

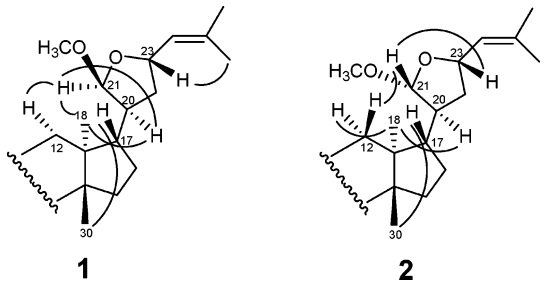

Figure 2. Main NOESY correlations for **1** and **2**.

the HMBC spectrum showed long-range correlations between δ_H 4.43 (Glc-H-1′) and δ_C 89.0 (C-3) and between δ_H 5.37 (Rha-H-1″) and δ_C 77.8 (Glc-2′), indicating the linkage of Glc-C-1′ to C-3 of the aglycon and of Rha-C-1″ to Glc-C-2′, respectively (Figure 1). An axial configuration of the glucose−aglycon linkage was deduced from the H-3 coupling constant [δ_H 3.21 (dd, J = 12.5, 3.5 Hz)]. Like **2**, the H-21 signal was correlated with H-12*β*, whereas no correlation was observed with H-18α in the 2D NOE NMR spectrum, suggesting an α-OCH_3 group at C-21 in **3**. The above evidence was used to determine **3** as 21α-methoxy-3*β*,21-(*R*),23(*R*)-epoxytirucalla-7,24-diene-3-*O*-α-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranoside.

Sapinmusaponin I (**4**) was assigned the elemental composition $C_{49}H_{80}O_{16}$ from its HRFABMS (*m*/*z* 947.5354 $[M + Na]^+$). The NMR spectroscopic data of **4** were similar to those of **1**, except for signals for one additional rhammose found in **4**. After acid hydrolysis, the three sugars were identified as one D-glucose and two L-rhammose units by GC-MS. The interglycosidic linkages were determined from the following HMBC correlations: δ_H 4.38 (H-1 of Glc)/δ_C 90.5 (C-3 of the aglycon); δ_H 5.36 and 4.72 (H-1 of the two terminal Rha)/δ_C 78.8 and 68.1 (C-2 and C-6 of the inner Glc, respectively) (Figure 1). As in compound **1**, H-21 in **4** gave an upfield ^1H NMR chemical shift (δ_H 4.73) compared with analogous data for **2** (δ_H 4.82) and **3** (δ_H 4.82), together with a correlation between H-21 and H-18α in the NOESY spectrum of

Table 3. IC_{50} Values of Sapinmusaponins F−J (**1**−**5**) on the Aggregation of Washed Rabbit Platelets Induced by PAF, U46619, THB, or AA[a]

	IC_{50} (μM)			
compound	PAF	U46619	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	4.6 ± 0.9	16.3 ± 1.3	>100	71.5 ± 2.3
4	11.8 ± 3.8	*b*	41.4 ± 1.3	2.3 ± 0.7
5	5.0 ± 0.7	6.2 ± 0.2	11.5 ± 3.8	19.4 ± 3.5

[a] PAF: platelet-activity factor; U46619: a thromboxane A2 agonist; THB: thrombin; and AA: arachidonic acid. [b] Not tested.

Table 4. Relative Ratio of EBV-EA Activation with Respect to the Positive Control TPA (100%) in the Presence of Compounds **1**−**5**[a]

	percentage EBV-EV-positive cells			
	compound concentration (mol ratio/TPA[b]) =			
compound	1000	500	100	10
1	9.1 (60)[c]	53.6	82.6	100
2	9.7 (60)	53.9	83.7	100
3	11.0 (60)	54.4	83.7	100
4	14.3 (60)	60.5	82.6	100
5	8.2 (60)	52.3	81.6	100

[a] Values represent percentages relative to the positive control value (100%). [b] TPA concentration = 20 ng/mL (32 pmol/mL). [c] Values in parentheses are viability percentages of Raji cells.

4, suggesting that H-21 in **4** has an α-configuration. On the basis of the above evidence, the structure of **4** was determined as 21β-methoxy-3β,21(*S*),23(*R*)-epoxytirucalla-7,24-diene-3-*O*-α-L-dirhamnopyranosyl-(1→2,6)-β-D-glucopyranoside.

Sapinmusaponin J (**5**) was obtained as white amorphous powder. Its HRFABMS showed a molecular ion (*m*/*z* 947.5359 $[M + Na]^+$) corresponding to the molecular formula $C_{49}H_{80}O_{16}$, as also found for **4**. A careful comparison of the ^1H and ^{13}C NMR spectra of **5** with those of **4** revealed that these two compounds are similar, except for the methoxy group at C-21. While this proton signal was present at 4.73 (d, 3.0) in **4**, it was found at 4.84 (d, 6.4) in **5**, suggesting a α-OCH_3 at C-21 in **5**. On the basis of TOCSY, HMQC, and NOESY experiments, the complete ^1H and ^{13}C NMR resonances of **5** were assigned, and its configuration was confirmed unambiguously as shown. Consequently, **5** was identified as 21α-methoxy-3β,21(*R*),23(*R*)-epoxytirucalla-7,24-diene-3-*O*-α-L-dirhamnopyranosyl-(1→2,6)-β-D-glucopyranoside.

Biological evaluation (Table 3) of sapinmusaponins F−J (**1**−**5**) showed potent anti-platelet aggregation [induced by PAF, TXA_2, THB, or AA], while the IC_{50} values for sapinmusaponins A−E in this assay were greater than 100 μM. Compounds **1**, **2**, **4**, and **5** also showed promising effects against thrombin (THB)-induced aggregation. Moreover, by measuring the percentage of LDH leakage, released from the platelets, compounds **1**−**5** showed no obvious cytotoxicity (LDH < 10.0%). In contrast, the dammarane-type saponins, sapinmusaponins A−E, exhibited significant cytotoxicity, as indicated by high leakage (LDH 62.3%).

Table 4 shows the results for compounds **1**−**5** as the inhibition of TPA-induced EBV-EA activation, and these substances possessed moderate inhibitory effects in this assay. At the highest tested concentration (1×10^3 mol ratio/TPA), 86−92% inhibitions were found. These findings imply that anti-platelet-aggregative agents, such as tirucallane-type saponins, could also have cancer chemopreventive effects.

Experimental Section

General Experimental Procedures. Melting points were determined using a Fisher-Johns melting point apparatus and are uncorrected. Optical rotations were measured on a JASCO P-1020 polarimeter equipped with a sodium lamp (589 nm). Infrared (IR) spectra were measured on a Mattson Genesis II spectrophotometer using a KBr matrix. NMR spectra were performed on a Bruker NMR spectrometer (Unity Plus 400 MHz) and Varian NMR spectrometers (Unity Plus 500 MHz) using CD_3OD as solvent for measurement. JEOL FABMS data were performed on a SX-102A instrument. High-resolution FABMS were measured on a Finnigan/Thermo Quest MAT mass spectrometer. GC-MS was performed on a Thermo Finnigan TRACE GC apparatus using a HP-5MS column (30 m × 0.25 mm i.d., 0.25 μm), with an initial temperature of 150 °C for 2 min, and then temperature programming to 300 °C at a rate of 15 °C/min. HPLC separations were performed on a Shimadzu LC-6AD series apparatus with a RID-10A refractive index, equipped with a preparative Cosmosil 5SL-II column (25 cm × 20 mm i.d.). Diaion HP-20, Sephadex LH-20, and silica gel (Merck 70−230 mesh and 230−400 mesh) were used for column chromatography, and precoated silica gel (Merck 60 F-254) plates were used for TLC. TLC detection was carried out by spraying with 50% H_2SO_4 and then heating on a hot plate.

Plant Material. The galls of *Sapindus mukorossi* were collected in October 2001 in the northern mountains of Taiwan, in Taipei County, and identified by Professor Muh-Tsuen Kao, National Institute of Chinese Medicine. A voucher specimen (No. NRICM20011007A2) has been deposited in the National Research Institute of Chinese Medicine, Taipei, Taiwan.

Extraction and Isolation. The dried galls of *S. mukorossi* (8.5 kg) were extracted three times with EtOH. After removal of solvent 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 dried $CHCl_3$ layer (190 g) was partitioned with MeOH/*n*-hexane three times to give MeOH (100 g) and *n*-hexane layers. After the MeOH layer was evaporated in vacuo, the residue was further purified by chromatography on a silica gel column (41 × 10.5 cm) with $CHCl_3$/MeOH (1:0 → 0:1) to give 13 fractions, 1−13. Fraction 12 ($CHCl_3$/MeOH, 2:1) was subjected to column chromatography over a 23 × 4.5 cm Diaion HP-20 porous polymer resin column, eluting with 10, 40, 60, 80, and 100% aqueous MeOH, successively, to yield six fractions, 12.1−12.6. Fraction 12.3 (60% MeOH, 3.0 g) was further separated by chromatography on a 30 × 2.8 cm Sephadex LH-20 with MeOH to yield four fractions, 12.3.1−12.3.4. Using repeated purification by the same method as fraction 12.3, fraction 12.3.2 (2.0 g) was separated into four fractions, 12.3.2.1−12.3.2.4. Fraction 12.3.2.2 (1.1 g) was further purified by HPLC (ODS, 250 × 20.0 mm, 80% aqueous MeOH) to afford **1** (17 mg, t_R = 26.3 min) and **4** (7.4 mg, t_R = 28.8 min). Fraction 12.3.3 (0.3 g) was chromatographed using HPLC (ODS, 250 × 20.0 mm) with 75% aqueous MeOH as the eluent to afford **5** (21 mg, t_R = 46.5 min). Using HPLC (ODS, 250 × 20.0 mm) with 60% aqueous MeOH as solvent, **2** (23 mg, t_R = 55.5 min) and **3** (6 mg, t_R = 62.1 min) were obtained from fraction 12.3.4 (0.7 g).

Sapinmusaponin F (1): white amorphous powder; mp 166−168 °C; $[\alpha]_D^{24}$ −16.3 (*c* 0.4, MeOH); IR ν_{max} (KBr) 3388, 2946, 2859, 1447, 1367, 1045, 1015 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRFABMS *m*/*z* 801.4770 (calcd 801.4764 for $C_{43}H_{70}O_{12}Na$ $[M + Na]^+$).

Sapinmusaponin G (2): white amorphous powder; mp 171−172 °C; $[\alpha]_D^{24}$ −36.2 (*c* 0.2, MeOH); IR ν_{max} (KBr) 3386, 2884, 2947, 1447, 1384, 1097, 1069 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRFABMS *m*/*z* 801.4760 (calcd 801.4764 for $C_{43}H_{70}O_{12}Na$ $[M + Na]^+$).

Sapinmusaponin H (3): white amorphous powder; mp 224−225 °C; $[\alpha]_D^{24}$ −19.7 (*c* 0.1, MeOH); IR ν_{max} (KBr) 3382, 2947, 2883, 1446, 1384, 1165, 1045 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRFABMS *m*/*z* 801.4756 (calcd 801.4764 for $C_{43}H_{70}O_{12}Na$ $[M + Na]^+$).

Sapinmusaponin I (4): white amorphous powder; mp 195−197 °C; $[\alpha]_D^{24}$ −21.0 (*c* 0.2, MeOH); IR ν_{max} (KBr) 3401, 2945, 2360, 1635, 1448, 1384, 1046 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRFABMS *m*/*z* 947.5354 (calcd 947.5344, $C_{49}H_{80}O_{16}Na$ $[M + Na]^+$).

Sapinmusaponin J (5): white amorphous powder, mp 186−188 °C; $[\alpha]_D^{24}$ −65.7 (*c* 0.2, MeOH); IR ν_{max} (KBr) 3404, 2931, 2360, 1635, 1448, 1384, 1045 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRFABMS *m*/*z* 947.5359 (calcd 947.5344, $C_{49}H_{80}O_{16}Na$ $[M + Na]^+$).

Acid Hydrolysis of 1−5. Compounds **1**−**5** (4.0 mg) were each treated with 2 N methanolic HCl (2 mL) under reflux at 90 °C for 1 h,

respectively. The mixture obtained was extracted with CH_2Cl_2. The aqueous hydrolysate was neutralized with Na_2CO_3 and filtered. L-Rhamnose and D-glucose in the filtrate were acetylated with pyridine/Ac_2O. The acetylated sugar residues were compared with the acetylated reference L-rhamnose and D-glucose by GC-MS analysis, and the results showed that peracetylrhamnose and peracetylglucose derived from **1**−**5** were identical to the reference samples used.

Evaluation of Anti-Platelet-Aggregation Activity.[22] Preparation of Rabbit Washed Platelet Suspension. Washed platelets were obtained from rabbits as previously described.[23] Briefly, rabbit blood was collected from the marginal ear vein into tubes containing one-sixth volume of acid-citrate-dextrose as anticoagulant. The blood was centrifuged at 200*g* for 15 min at room temperature. The platelet-rich plasma was mixed with 1/40 volume of EDTA (final concentration 5 mM) and recentrifuged at 1000*g* for 12 min. The supernatant was discarded, and the platelet pellet was suspended in modified Ca^{2+}-free Tyrode's buffer (137 mM NaCl, 2.8 mM KCl, 2 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 5 mM glucose, 10 mM HEPES) with 0.35% bovine serum albumin, heparin (50 unit/mL), and apyrase (1 unit/mL). Following incubation at 37 °C for 20 min, the washed platelet pellet was resuspended in Tyrode's buffer containing 1 mM Ca^{2+}. The platelet numbers were counted by hemacytometer and adjusted to 3.5×10^8 platelets/mL. To eliminate or minimize any possible effects of the solvent, the final concentration of the vehicle dimethyl sulfoxide (DMSO) in the platelet suspension was fixed at 0.5%.

Measurement of Platelet Aggregation. Aggregation was measured by a turbidimetric method.[24] The PACKS-4 aggregrometer (Helena Laboratories, Beaumont, TX) was used. Transmission of washed platelet suspension was assigned 0% aggregation, while transmission through Tyrode's buffer was assigned 100% aggregation. Platelets (0.5 mL) were preincubated with 2.5 μL of the vehicle DMSO (0.5%) to serve as control or saponins for 2 min and then stimulated with 2.5 μL of aggregation inducers such as AA, TXA_2, THB, or PAF.

Lactate Dehydrogenase (LDH) Leakage. Leakage of LDH from platelets was measured as described by Bergmeyer et al.[25] LDH leakage was expressed as percentage of total enzyme activity.

Inhibition of EBV-EA Activation Assay.[26] Raji (virus nonproducer type) and EBV genome-carrying human lymphoblastoid cells were used for assaying the inhibition of EBV-EA activation as described previously.[27,28] The indicator cells (1×10^6 /mL) were incubated at 37 °C for 48 h in medium (1 mL) containing *n*-butyric acid (4 mM) as trigger, TPA [32 pM = 20 ng in 2 μL of dimethyl sulfoxide (DMSO)] as inducer, and various amounts of the test compounds dissolved in 5 μL of DMSO. Smears were made from the cell suspension. The EBV-EA-inducing cells were stained with high-titer EBV-EA-positive serum from NPC patients and detected by an indirect immunofluorescence technique.[29] In each assay, at least 500 cells were counted, and the number of stained cells (positive cells) was recorded. Triplicate assays were performed for each data point. The average EBV-EA induction of the test compound was expressed as a relative ratio to the positive control experiment (100%), which was carried out with *n*-butyric acid (4 mM) plus TPA (32 pM). In the experiments, the EBV-EA induction was ordinarily around 35%, and this value was taken as the positive control (100%). *n*-Butyric acid (4 mM) alone induced 0.1% EA-positive cells. The viability of treated Raji cells was assayed by the Trypan blue staining method. The cell viability of the TPA-positive control was greater than 80%. Therefore, only the compounds that induced less than 80% (% of control) of the EBV-activated cells (those with a cell viability of more than 60%) were considered able to inhibit the activation caused by promoter substances. The student's *t*-test was used for all statistical analyses.

Acknowledgment. Financial support of this work by the National Science Council (NSC94-2313-B-077-002) and the National Research Institute of Chinese Medicine (NRICM-94-DHM-02) of the Republic of China (Y.-H.K.) is gratefully acknowledged, as well as support from NIH CA17625 (K.-H.L.).

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NP050446Z