JOURNAL OF NATURAL PRODUCTS

Article

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Indiosides G–K: Steroidal Glycosides with Cytotoxic and Anti-inflammatory Activities from *Solanum violaceum*

Chiao-Ting Yen,[†] Chia-Lin Lee,[‡,§] Fang-Rong Chang,[†] Tsong-Long Hwang,[⊥] Hsin-Fu Yen,[∥] Chao-Jung Chen,[‡,▽] Shu-Li Chen,[†] and Yang-Chang Wu*[,†,‡,§,○]

[†]Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung 80708, Taiwan, Republic of China

[‡]School of Chinese Medicine, College of Chinese Medicine, China Medical University, Taichung 40402, Taiwan, Republic of China

[§]Natural Medicinal Products Research Center, China Medical University Hospital, Taichung 40402, Taiwan, Republic of China

[⊥]Graduate Institute of Natural Products, College of Medicine, Chang Gung University, Taoyuan 33302, Taiwan, Republic of China

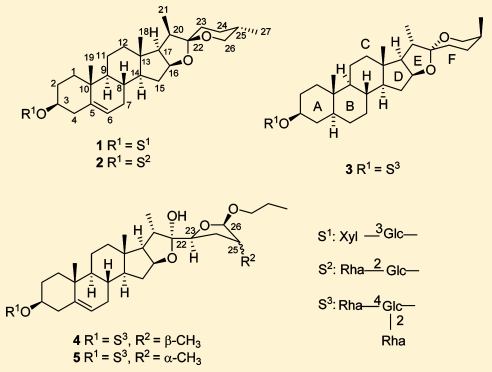
[∥]Botanical Garden, National Museum of Natural Science, Taichung, 40453, Taiwan, Republic of China

[▽]Proteomics Core Laboratory, Department of Medical Research, China Medical University Hospital, Taichung, 40402, Taiwan, Republic of China

[○]Center for Molecular Medicine, China Medical University Hospital, Taichung 40402, Taiwan, Republic of China

Supporting Information

ABSTRACT: Five new steroidal glycosides (**1**–**5**) and nine known compounds were isolated from *Solanum violaceum*. Indiosides G (**1**) and H (**2**) are spirostene saponins with an iso-type F ring, indioside I (**3**) is a spirostane saponin, and indiosides J (**4**) and K (**5**) are unusual furostanol saponins with a deformed F ring. These structures represent rare naturally occurring steroidal skeletons. The structures of the new compounds were elucidated using 1D and 2D spectroscopic techniques and acid hydrolysis. Compounds **2**, **3**, and **7**–**9** exhibited cytotoxic activity against six human cancer cell lines (HepG2, Hep3B, A549, Ca9-22, MDA-MB-231, and MCF-7) with IC_{50} values of 1.83–8.04 μg/mL. Steroidal saponins **3**, **8**, and **9** showed inhibitory effects on superoxide anion generation with IC_{50} values of 2.84 ± 0.18, 0.62 ± 0.03, and 1.62 ± 0.59 μg/mL, respectively. Saponins **8** and **9** also inhibited elastase release with IC_{50} values of 111.05 ± 7.37 and 4.04 ± 0.51 μg/mL, respectively. Structure–activity relationship correlations of these compounds with respect to cytotoxic and anti-inflammatory effects are discussed.



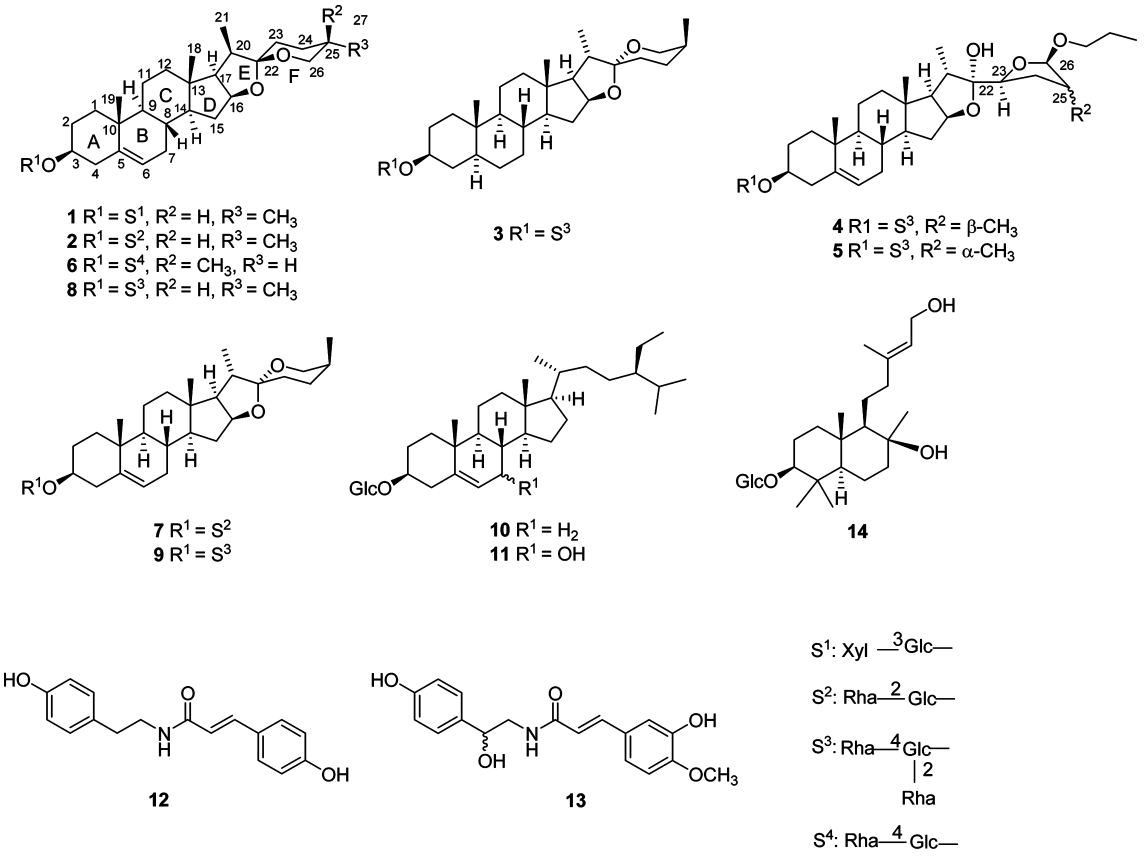
The genus *Solanum* from the family Solanaceae contains about 1500 species, which are distributed mostly in tropical and subtropical regions. An estimated 13 species of genus *Solanum* are present in Taiwan, including *Solanum violaceum* Ortega (*S. indicum auct. non* L.), called Indian Nightshade, an erect medium size shrub growing from low to medium latitudes in southern Taiwan.[1] It has been used in Chinese folk medicine to eliminate toxins from the body and as an analgesic and anti-inflammatory agent for rhinitis, toothache, and breast cancer.[2] In Thailand, the fruit of *S. violaceum* has been used as both a food and an anticarcinogen.[3] However, only a few phytochemical studies have been performed on *S. violaceum*. Thus far, the isolated active components from this plant include 10 steroidal glycosides,[2,3] one solafuranone, and three phenolic derivatives.[2] Among them, indioside D was the induced feeding preference of larvae of the moth *Mamduca sexta* (tobacco hornworm),[4] and solavetivone was cytoxic to OVCAR-3 cells.[2] The interesting biological activities and the limited number of phytochemical reports on *S. violaceum* encouraged us to pursue an intensive investigation to isolate other active components from *S. violaceum* and determine their biological effects. The EtOAc fraction of *S. violaceum* was active against various human cancer cell lines (HepG2, Hep3B, A549, Ca9-22, MDA-MB-231, and MCF-7) with IC_{50} < 20 μg/mL and also showed significant anti-inflammatory effects on superoxide anion generation and elastase release in fMLP/CB-induced human neutrophils.

RESULTS AND DISCUSSION

The EtOAc fraction of a MeOH extract of *S. violaceum* was subjected to CC on silica gel, C_{18}, Sephadex LH-20, and HPLC on ODS-C_{18} to give five new compounds, indiosides G–K (**1**–**5**), as well as the known borassoside D (**6**),[5] yamogenin 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranoside (**7**),[5] borassoside E (**8**),[5] 3-*O*-chacotriosyl-25(*S*)-spirost-5-en-3*β*-ol (**9**),[6] sitosterol 3-*O*-*β*-D-glucopyranoside (**10**),[5] 7-hydroxysitosterol-3-*O*-*β*-D-glucopyranoside (**11**),[7] *N*-*p*-coumaroyltyramine (**12**),[8] *trans*-*N*-feruloyloctopamine (**13**),[9] and tricalysioside U (**14**).[10]

Received: October 31, 2011
Published: March 13, 2012

Chart 1



The HRESIMS spectrum of **1** showed a molecular formula of $C_{38}H_{60}O_{12}$ with a pseudomolecular ion at m/z 731.3966 $[M + Na]^+$. The IR spectrum displayed absorption bands for hydroxy (3404 cm^{-1}) and C−O (1030 cm^{-1}) functionalities. The 1D NMR (Tables 1 and 2) and HMQC data revealed the presence of four methyl groups [δ_H 0.84/δ_C 16.4 and δ_H 0.90/δ_C 19.4 (3H each, both s, CH_3-18 and CH_3-19); δ_H 0.70/δ_C 17.3 and δ_H 1.15/δ_C 15.0 (3H each, both d, J = 6.9 Hz, CH_3-27 and CH_3-21)] and an oxymethylene [δ_H 3.51 (1H, t, J = 10.8 Hz), 3.59 (1H, br d, $J \approx$ 10.8 Hz)/δ_C 66.8, CH_2-26]. The NMR data also showed two oxymethines [δ_H 3.95 (1H, m, H-3) and 4.52 (1H, m, H-16)] along with their corresponding carbons at δ_C 78.2 and 81.1, respectively, and an olefinic proton [δ_H 5.33 (1H, d, $J \approx$ 5.4 Hz)/δ_C 121.8, CH-6], together with two anomeric signals at δ_H 5.05 (1H, d, J = 7.8 Hz, δ_C 102.7) and 5.37 (1H, d, J = 7.8 Hz, δ_C 106.5). Twenty-seven carbon signals, including four methyl, 10 methylene, nine methine, and four quaternary carbons, were observed in the ^{13}C NMR and DEPT spectra (Table 1). Among the four quaternary carbon signals, the signal at δ_C 109.2 was identified as a hemiketal (C-22) and the signal at δ_C 140.8 was assigned as an olefinic (C-5) carbon. The aforementioned data suggested that **1** is possibly a spirostanol glycoside with the aglycone having a 27-carbon skeleton along with two sugar moieties.[5] The acidic hydrolysis of **1** with HCl in 1,4-dioxane gave D-glucose and D-xylose, which were identified by GC analysis of the acetylated sugar residues.[11] In the HMBC spectrum of **1**, the xylosyl H-1″ and glucosyl H-1′ protons exhibited ^3J interactions with the glucosyl C-3′ (δ 87.8) and C-3 (δ 78.2) carbons of the aglycone moiety, respectively. These correlations led to the assignment of the glycosidic moiety as xylopyranosyl-(1→3)-glucopyranosyl, which was connected to C-3 of the aglycone moiety through an O-linkage, as shown in Figure 1. The aglycone of **1** could not be isolated due to its decomposition during acid hydrolysis.

The NMR data of **1** were similar to those of known compounds **7**[5] and **9**,[6] indicating that the three compounds shared a basic steroidal nucleus. However, differences were present in the ^1H and ^{13}C chemical shifts corresponding to the E and F rings (Table 1). In **1**, the C-26 protons appeared in close proximity at δ_H 3.51 (1H, t, J = 10.8 Hz) and 3.59 (1H, br d, $J \approx$ 10.8 Hz), indicating that **1** has an iso-type F ring.[12] The CH_3-27 protons were shifted upfield from δ_H 1.07 in **7** and **9** to δ_H 0.70 in **1**, and the corresponding carbon signal was shifted downfield from δ_C 15.8 to 17.3, indicative of an equatorial α-orientation for CH_3-27 in **1**,[13] rather than the axial β-orientation of the same group in **7** and **9**. Additionally, H-20 was observed at higher field in **1** (δ_H 1.96) compared with **7** and **9** (δ_H 2.47), suggesting that H-20 and the F ring oxygen in **1** have a *trans*-relationship.[12] The stereostructure of **1** was confirmed by ROESY analysis, as shown in Figure 2. Consequently, compound **1** was characterized as indioside G, 3-*O*-[β-D-xylopyranosyl-(1→3)-β-D-glucopyranosyl]-(22*S*,25*S*)-spirost-5-en-3β-ol. The steroidal saponin of **1** has been studied and described as having antifungal, antiviral, and hemolytic activities.[14,15] However, the glycoside indioside G (**1**) has not been isolated previously as a natural product.

Compounds **2**, **6**, and **7** have the same molecular formula, $C_{39}H_{62}O_{12}$, HRESIMS m/z 745.4241 $[M + Na]^+$. The IR spectrum of **2** displayed absorptions for hydroxy (3402 cm^{-1}) and C−O (1048 cm^{-1}) functional groups. The 1D NMR and HMQC spectra showed two anomeric proton signals at δ_H 5.05 (d, J = 7.3 Hz)/δ_C 100.4 and δ_H 6.39 (br s)/δ_C 102.1,

dx.doi.org/10.1021/np200877u | *J. Nat. Prod.* 2012, 75, 636−643

Table 1. ^{1}H and ^{13}C NMR (Pyridine-d_5) Data of Indioside G–K (1–5) (Aglycone Part)

	1		2		3		4		5	
position	δ_H (J Hz)	δ_C	δ_H (J Hz)	δ_C	δ_H (J Hz)	δ_C	δ_H (J Hz)	δ_C	δ_H (J Hz)	δ_C
1	0.94 (m)	37.0	0.98 (m)	37.5	0.82 (m)	37.2	0.99 (m)	37.0	0.99(m)	37.5
	1.70 (m)		1.75 (m)		1.59 (m)		1.75 (m)		1.73(m)	
2	1.67 (m)	30.2	1.87 (m)	31.8	1.79 (m)	29.9	1.86 (m)	29.7	1.85(m)	30.2
	2.10 (m)		2.05 (m)		2.02 (m)		2.09 (m)		2.08(m)	
3	3.95 (m)	78.2	3.95 (m)	77.9	3.92 (m)	77.2	3.88 (m)	77.5	3.91 m)	78.1
4	2.44 (m)	39.3	2.76 (m)	39.0	1.65 (m)	34.5	2.73 (m)	38.5	2.72 (m)	39.0
	2.72 (m)		2.83 (m)		1.95 (m)		2.80 (m)		2.75 (m)	
5		140.8		140.9	0.87 (m)	44.6		140.3		140.8
6	5.33 (d, 5.4)	121.8	5.32 (d, 5.1)	121.7	1.12 (2H, m)	28.9	5.31 (m)	121.3	5.30 (m)	121.8
7	1.44 (m)	32.2[a]	1.44 (m)	32.3[a]	1.53 (m)	32.4[a]	1.53 (m)	32.0[a]	1.55 (m)	32.5[a]
	1.85 (m)		1.87 (m)		2.00 (m)		2.00 (m)		2.02 (m)	
8	1.49 (m)	31.6	1.46 (m)	31.7	1.39 (m)	35.3	1.53 (m)	31.2	1.52 (m)	31.7
9	0.85 (m)	50.2	0.90 (m)	50.3	0.52 (td, 11.2, 3.9)	54.4	0.92 (m)	49.9	0.90 (m)	50.3
10		37.4		37.2		35.9		36.6		37.1
11	1.39 (m)	21.1	1.37 (m)	21.1	1.21 (m)	21.3	1.47 (2H, m)	20.6	1.52 (2H, m)	21.1
	1.44 (m)		1.40 (m)		1.43 (m)					
12	1.26 (m)	39.8	1.11 (m)	39.9	1.03 (m)	40.1	1.03 (m)	38.4	1.13 (m)	39.8
	1.67 (m)		1.73 (m)		1.77 (m)		1.77 (m)		1.76 (m)	
13		40.4		40.5		40.8		40.5		41.0
14	1.03 (m)	56.6	1.09 (m)	56.7	1.00 (m)	56.5	1.11 (m)	56.1	1.08 (m)	56.6
15	1.53 (m)	32.2[a]	1.58 (m)	32.2[a]	0.80 (m)	32.1[a]	0.80 (m)	31.8[a]	1.59 (m)	32.1
	2.07 (m)		2.15 (m)		1.39 (m)		1.39 (m)		1.90 (m)	
16	4.52 (m)	81.1	4.56 (m)	81.1	4.51 (m)	81.2	5.00 (m)	80.9	4.90 (m)	81.4
17	1.81 (m)	62.8	1.83 (m)	62.9	1.90 (m)	62.9	1.92 (m)	63.5	1.94 (m)	64.0
18	0.84 (s)	16.4	0.84 (s)	16.3	0.82 (s)	16.6	0.98 (s)	15.8	0.96 (s)	16.3
19	0.90 (s)	19.4	1.07 (s)	19.4	0.86 (s)	12.4	1.06 (s)	18.9	1.06 (s)	19.4
20	1.96 (m)	42.0	1.96 (m)	42.0	1.90 (m)	42.5	2.50 (t, 6.7)	37.6	2.40 (t, 6.5)	38.2
21	1.15 (d, 6.9)	15.0	1.15 (d, 6.9)	15.0	1.14 (d, 6.9)	14.8	1.32 (d, 6.7)	15.9	1.31 (d, 6.9)	15.8
22		109.2		109.3		109.7		108.9		109.1
23	1.51 (2H, m)	31.8	1.54 (2H, m)	29.3	1.87 (m)	26.4	4.56 (m)	82.7	4.39(m)	84.3
					2.14 (m)					
24	1.57 (2H, m)	29.2	1.46 (2H, m)	30.2	1.34 (m)	26.2	1.34 (m)	31.6	1.54 (m)	32.8[a]
					1.45 (m)		1.45 (m)		2.02 (m)	
25	1.58 (m)	30.6	1.59 (m)	30.6	1.59 (m)	27.5	2.40 (m)	39.4	2.18 (m)	39.5
26	3.51 (t, 10.8)	66.8	3.51 (t, 11.2)	66.9	3.36 (d, 11.0)	65.1	4.86 (m)	109.9	4.98 (m)	105.4
	3.59 (br d, 10.8)		3.59 (br d, 11.2)		4.05 (dd, 11.0, 7.6)					
27	0.70 (d, 6.9)	17.3	0.71 (d, 5.6)	17.3	1.08 (d, 7.1)	16.3	0.96 (d, 7.1)	16.4	1.10 (d, 6.7)	12.9
26-*O*-1							3.44 (m)	67.4	3.37 (m)	68.0
							3.92 (m)		3.86 (m)	
2							1.36 (m)	19.2	1.34 (m)	19.6
							1.38 (m)		1.38 (m)	
3							0.83 (t, 7.4)	13.5	0.81 (t, 7.4)	14.0

[a]Signals may be interchanged.

corresponding to the glycone part. Two tertiary methyl proton signals at δ_H 0.84/δ_C 16.3 and δ_H 1.07/δ_C 19.4, two secondary methyl proton signals at δ_H 0.71 (d, J = 5.6 Hz)/δ_C 17.3 and δ_H 1.15 (d, J = 6.9 Hz)/δ_C 15.0, and an olefinic proton signal at δ_H 5.32 (d, J = 5.1 Hz)/δ_C 121.7 were observed, corresponding to the aglycone part. The NMR spectra of **2** and **7** were quite similar, with the only difference evident in the signals for H_2-26. As also found in **1**, the presence of H_2-26 proton signals at δ_H 3.51 (1H, t, J = 11.2 Hz) and 3.59 (1H, br d, $J \approx$ 11.2 Hz) indicated an iso-type F ring in **2**, while the same proton signals were further apart at δ_H 3.37 (1H, d, J = 10.2 Hz) and 4.06 (1H, d, J = 10.2 Hz) in **7**, indicative of a normal-type F ring. Acidic hydrolysis of **2** afforded D-glucose and L-rhamnose. The HMBC spectrum showed a correlation between the rhamnose H-1″ (δ_H 6.38) and the glucose C-2′ (δ_C 78.0), as well as between the aglycone H-3 (δ_H 3.95) and the anomeric carbon (δ_C 100.4) of glucose (Figure 1). Consequently, **2** was identified as 3-*O*-[α-L-rhamnopyranoside-(1→2)-β-D-glucopyranosyl]-(22*S*,25*S*)-spirost-5-en-3β-ol and named indioside H.

Compound **3** has the molecular formula $C_{45}H_{74}O_{16}$, as established by HRESIMS (m/z 893.4862 [M + Na]$^+$). IR absorptions at 3445 and 1054 cm^{-1} indicated the presence of hydroxy and ether functionalities. The ^{1}H NMR spectrum displayed signals corresponding to four steroidal methyl groups [δ_H 0.82, 0.86 (3H each, both s, H_3-18, H_3-19), 1.08, 1.14 (3H each, both d, J = 7.1, 6.9 Hz, H_3-27, H_3-21)], a methylene [δ_H 3.36 (1H, d, J = 11.0 Hz), 4.05 (1H, dd, J = 11.0, 7.6 Hz), H_2-26], and two oxymethines [δ_H 3.92 (1H, m, H-3) and 4.51 (1H, m, H-16)]. D-Glucose and L-rhamnose in a 1:2 ratio were obtained upon acidic hydrolysis of **3**, as indicated by GC.

dx.doi.org/10.1021/np200877u | J. Nat. Prod. 2012, 75, 636–643

Table 2. ^{1}H and ^{13}C NMR (Pyridine-d_5) Data of Indioside G–K (1–5) (Sugar Part)

	1		2		3		4		5	
position	δ_H (J Hz)	δ_C	δ_H (J Hz)	δ_C	δ_H (J Hz)	δ_C	δ_H (J Hz)	δ_C	δ_H (J Hz)	δ_C
3-*O*-Glc-1′	5.05 (d, 7.8)	102.7	5.05 (d, 7.3)	100.4	4.96 (d, 7.8)	99.9	5.00 (overlap)	99.8	4.92 (m)	100.3
2′	4.10 (m)	74.4[b)]	4.28 (m)	78.0	4.21 (m)	78.1	4.24 (overlap)	77.3	4.15 (m)	77.9
3′	4.31 (m)	87.8	4.30 (m)	79.7	3.72 (m)	77.0	3.66 (overlap)	76.5	3.71 (m)	77.0
4′	4.23 (m)	70.9	4.18 (t, 8.9)	71.9	4.38 (m)	78.8	4.41 (m)	78.2	4.28 (m)	78.7
5′	4.17 (m)	78.2	3.90 (m)	78.3	4.28 (m)	78.0	4.24 (m)	77.6	4.16 (m)	77.8
6′	4.36 (m)	62.4	4.37 (m)	62.7	4.11 (m)	61.4	4.11 (m)	60.8	4.08 (m)	61.3
	4.56 (m)		4.53 (m)		4.26 (m)		4.22 (m)		4.22 (m)	
3′-*O*-Xyl-1″	5.37 (d, 7.8)	106.5								
2″	4.09 (m)	75.5 [b)]								
3″	4.17 (m)	78.2								
4″	4.30 (m)	69.5								
5″	3.74 (m)	67.4								
	4.34 (m)									
2′-*O*-Rha-1″			6.39 (s)	102.1	6.27 (s)	102.1	6.42 (s)	101.6	6.42 (s)	102.0
2″			4.81 (m)	72.6 [b)]	4.83 (m)	72.5	4. 85 (m)	72.0	4. 69 (m)	72.5
3″			4.64 (dd, 3.3, 9.2)	72.9 [b)]	4.61 (br s)	72.7	4. 53 (br s)	72.3	4. 33 (br s)	74.1
4″			4.35 (m)	74.2	4.34 (m)	73.9	4.35 (m)	73.4	4.35 (m)	72.7
5″			4.91 (m)	69.5	4.91 (m)	69.5	4.94 (m)	69.9	5.00 (overlap)	69.5
6″			1.79 (d, 6.2)	18.7	1.75 (d, 6.2)	18.5	1.65 (d, 5.9)	18.0	1.78 (d, 6.1)	18.6
4′-*O*-Rha-1‴					5.85 (s)	102.9	5.88 (s)	102.4	5.88 (s)	102.4
2‴					4.69 (m)	72.5	4. 70 (m)	72.0	4.69 (m)	72.5
3‴					4.55 (br s)	72.8	4. 64 (br s)	72.3	4.64 (br s)	73.9
4‴					4.35 (m)	74.1	4.37 (m)	73.7	4.56 (m)	72.8
5‴					4.92 (m)	70.4	4.94 (m)	69.0	5.00 (overlap)	70.4
6‴					1.63 (d, 6.2)	18.6	1.78 (d, 5.9)	18.1	1.64 (d, 6.2)	18.5

Analysis of NMR data indicated the presence of three sugar units in a β-chacotriosyl moiety, with three anomeric protons [δ_H 4.96 (1H, d, J = 7.8 Hz, H-1′ of β-D-glucopyranosyl), 5.85 (1H, br s, H-1‴ of α-L-rhamnopyranosyl) and 6.37 (1H, br s, H-1″ of α-L-rhamnopyranosyl)] with correlations to carbon signals at δ_C 99.9, 102.9, and 102.1, respectively.[5] In the HMBC spectrum of **3**, a correlation between H-1′ of the β-D-glucopyranosyl moiety at δ_H 4.96 and C-3 at δ_C 77.2 indicated that the aglycone is glycosylated at C-3. The ^{1}H and ^{13}C NMR spectra of **3** were similar to those of immunoside,[12,16] with the exception of the A/B ring carbon signals, leading to a molecular structure as shown. The α-orientation of H-5 was determined by ROESY analysis (Figure 2). Finally, the full structure of **3** (indioside I) was elucidated as 3-*O*-{α-L-rhamnopyranoside-(1→2)-*O*-[α-L-rhamnopyranoside-(1→4)]-β-D-glucopyranosyl}-(22*R*,25*S*)-spirost-5α-an-3β-ol.

Compounds **4** and **5** were separated readily by HPLC with distinctly different retention times (**4**, t_R = 32.90 min; **5**, t_R = 50.75 min, 80% MeOH(aq)), although they showed similar spectroscopic data. Compounds **4** and **5** have the same molecular formula of $C_{48}H_{78}O_{18}$ (**4**: m/z 965.5055 [M + Na]$^+$; **5**: m/z 965.5102 [M + Na]$^+$) based on HRESIMS analysis. Similar specific rotations (**4**: $[\alpha]_D^{26}$ −68.9 (c 0.47, MeOH); **5**: $[\alpha]_D^{26}$ −65.2 (c 0.37 MeOH) were also observed. The IR spectra of the two compounds also had similar absorption bands [**4**: 3438 (OH), 1038 (C–O) cm^{-1}; **5**: 3438 (OH), 1038 (C–O) cm^{-1}].

The ^{1}H NMR spectrum of **4** showed two tertiary methyl groups (δ_H 0.99 and 1.06), two secondary methyl groups [δ_H 0.96 (d, J = 7.1 Hz) and 1.32 (d, J = 6.7 Hz)], one olefinic proton (δ_H 5.31), and three monosaccharide moieties; the sugar signals were essentially identical to those of **3**. Analysis of the ^{13}C NMR spectrum of **4** indicated 48 carbon signals corresponding to five methyl groups [δ_C 13.5 ($OCH_2CH_2\underline{C}H_3$), 15.8 (C-18), 15.9 (C-21), 16.4 (C-27), 18.9 (C-19)], one trisubstituted double bond (δ_C 140.3 and 121.3), one oxymethylene (δ_C 67.4), three oxymethines (δ_C 77.6, 80.9, and 82.7), one hemiketal (δ_C 108.9), one hemiacetal (δ_C 109.9), and three anomeric carbons (δ_C 99.8, 101.6, and 102.4). The HMBC data (Figure 1) revealed that the triglycoside unit [δ_H 5.00 (1H, overlapped with proton signals of water, inner glucosyl H-1′)] was connected to the aglycone at C-3 (δ_C 77.5), a double bond was located between C-5 and C-6 [H_2-4/C-5, C-6; H-6/C-4, C-10; H_3-19/C-1, C-5, C-9], and hemiketal and hemiacetal carbons were present at C-22 [H-20/C-13, C-17, C-22; H_3-21/C-17, C-20, C-22] and C-26 [H-26/C-24, C-25; H_3-27/C-24, C-25, C-26], respectively. Moreover, ROESY correlations (Figure 2) were observed between H-23 (δ_H 4.56) and H_3-27 (δ_H 0.96) and between H-26 (δ_H 4.86) and H_3-27 (δ_H 0.96). The 1D NMR assignments were analogous to those of anguivioside XI[17] and solasodoside F;[18] however, the *O*-glucosyl group attached to C-26 in solasodoside F was absent, while an *n*-propyl group was present in **4**. Accordingly, the structure of compound **4** (indioside J) was elucidated as 3-*O*-{α-L-rhamnopyranoside-(1→2)-*O*-[α-L-rhamnopyranoside-(1→4)]-β-D-glucopyranosyl}-(22*S*,23*S*,25*R*,26*S*)-23,26-epoxy-26β-propoxy-3β,22α,26-trihydroxyfurost-5-ene.

The ^{1}H and ^{13}C NMR spectra of **5** were similar to those of **4** (Tables 1 and 2), except the CH_3-27 proton signal was shifted downfield from δ_H 0.96 to 1.10 and the corresponding carbon signal was shifted upfield from δ_C 16.4 to 12.9. The molecular structure of **5** was determined on the basis of analysis of ^{1}H–^{1}H COSY and HMBC experiments (Figure 1). Cross-peaks between H-23 (δ_H 4.39) and H-26 (δ_H 4.98) and between H-26 and H_3-27 (δ_H 1.10, d, J = 6.7 Hz) were

dx.doi.org/10.1021/np200877u | J. Nat. Prod. 2012, 75, 636–643

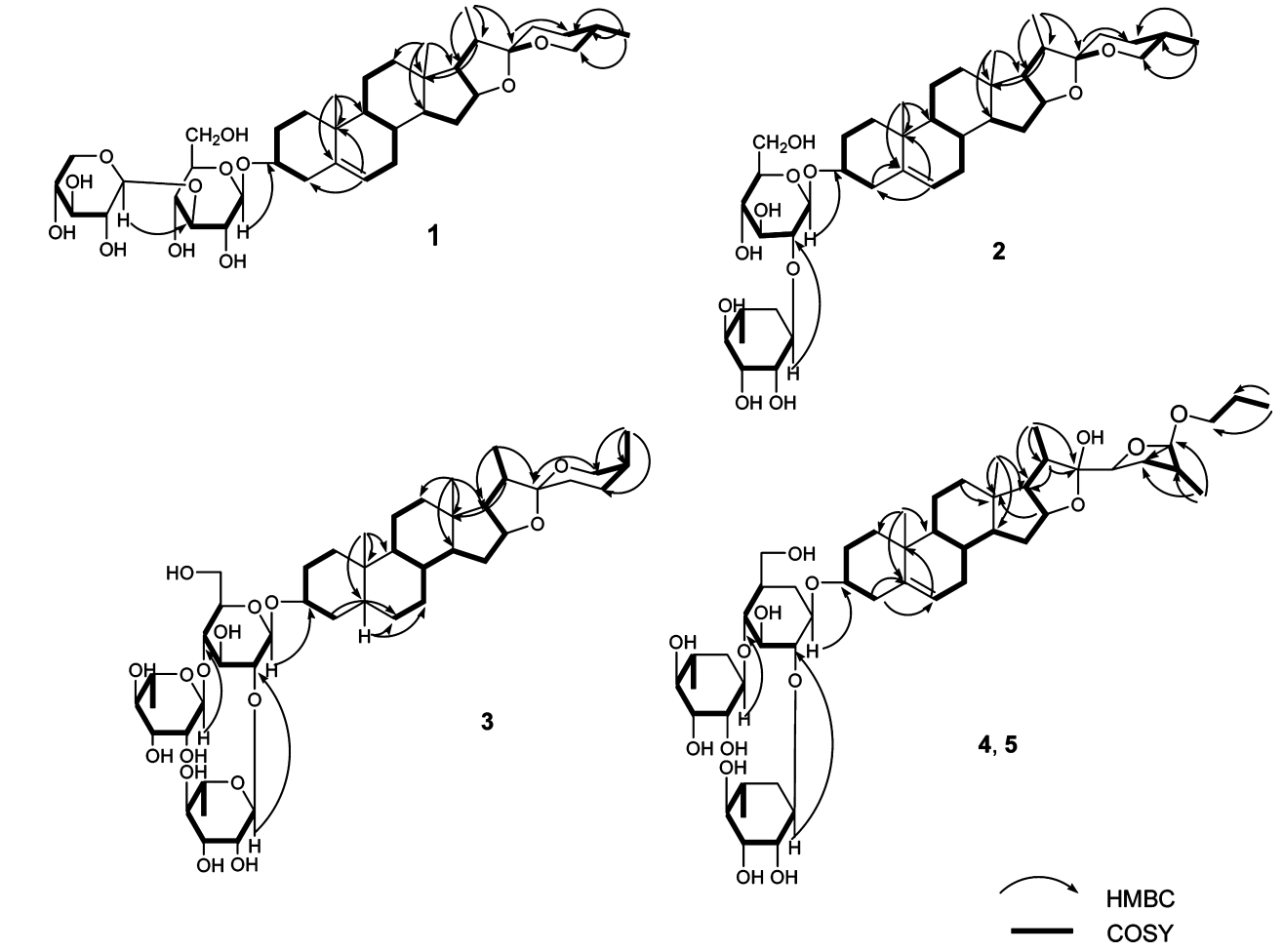


Figure 1. Key HMBC and COSY correlations of compounds **1**−**5**.

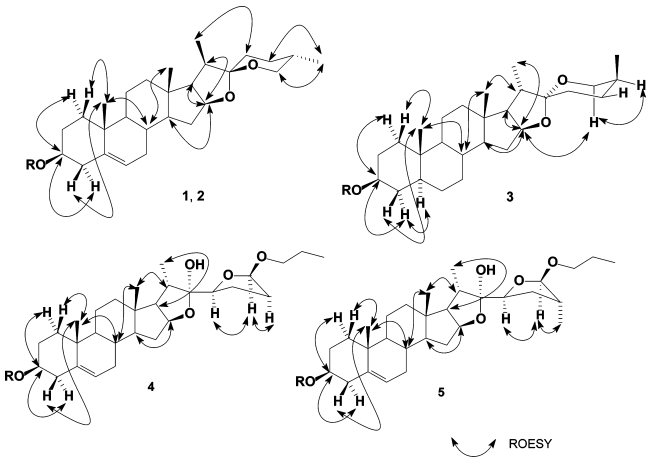


Figure 2. Key ROESY correlations of compounds **1**−**5** (aglycone part).

identified in the ROESY experiments. On the basis of the above data, indioside K (**5**) is a C-25 epimer of **4**. It was characterized as 3-*O*-{α-L-rhamnopyranoside-(1→2)-*O*-[α-L-rhamnopyranoside-(1→4)]-β-D-glucopyranosyl}-(22*S*,23*S*,25*S*,26*S*)-23,26-epoxy-26β-propoxy-3β,22α,26-trihydroxyfurost-5-ene.

Two different structures, normal- and iso-type F rings, occur in steroidal saponins.[13] The normal-type F ring is found most commonly in naturally occurring spirostanols, and thus far, only a few iso-type substances have been identified.[12,16] Our investigation of *S. violaceum* has led to the isolation of two new spirostene saponins, **1** and **2**, with an iso-type F ring, and a sprirostane saponin, **3**, with a normal-type F ring. Interestingly, new compounds **4** and **5** possess an unusual furostanol saponin skeleton with a deformed F ring. Only two additional furostanols, anguivioside XI[17] and solasodoside F,[18] with a deformed F ring have been reported previously. The nine known constituents were isolated previously from different genera of Solanaceae (*Lycium chinense*),[9] Arecaceae (*Borassus flabellifer*[5] and *B. decumbens*[6]), Rubiaceae (*Tricalysia dabia*),[10] and Urticaceae (*Urtica dioica*)[7] families, but are first reported herein from *S. violaceum*.

Several prior studies have highlighted a close relationship between cytotoxic and anti-inflammatory effects.[19] Kawabata and his co-workers[20] proposed that steroidal saponins can prevent production of inflammatory cytokines, such as TNF-α, in a monocytic cell line and melanin synthesis in melanoma cells. In the present investigation, the bioassay-guided fractionation of the EtOAc fraction of *S. violaceum* led to the isolation of compounds **1**−**14**, which were evaluated for cytotoxic activity against six human cancer cell lines [HepG2 and Hep3B (hepatoma), A549 (lung), Ca9−-22 (oral), and MDA-MB-231 and MCF-7 (breast)] and anti-inflammatory effects on superoxide anion generation and elastase release induced by fMLP/CB in human neutrophils.

dx.doi.org/10.1021/np200877u | *J. Nat. Prod.* 2012, 75, 636−643

Table 3. MTT Cytotoxicity of Compounds 1−14

	cancer cell line[a] (IC_{50} value, μg/mL)					
compound	HepG2	Hep3B	MCF7	A549	Ca9-22	MDA-MB-231
1	NT	NT	NT	NT	NT	NT
2	2.22 ± 0.01	2.95 ± 0.02	4.78 ± 0.02	3.09 ± 0.02	2.95 ± 0.07	6.12 ± 0.15
3	5.33 ± 0.16	3.32 ± 0.42	11.57 ± 0.70	7.27 ± 0.07	6.76 ± 0.15	8.04 ± 0.12
4	NT[c]	NT	NT	NT	NT	NT
5	NT	NT	NT	NT	NT	NT
6	>20	>20	>20	>20	>20	>20
7	6.48 ± 0.01	6.98 ± 0.05	5.84 ± 0.04	4.26 ± 0.02	4.51 ± 0.24	7.25 ± 0.15
8	1.83 ± 0.12	2.03 ± 0.03	2.61 ± 0.10	2.34 ± 0.02	2.33 ± 0.02	2.75 ± 0.10
9	6.44 ± 0.45	2.87 ± 0.04	8.84 ± 0.12	4.09 ± 0.08	3.77 ± 0.02	5.84 ± 0.06
10	>20	>20	>20	>20	>20	>20
11	>20	>20	>20	>20	>20	>20
12	9.57 ± 0.26	11.23 ± 0.02	>20	15.21 ± 0.12	12.28 ± 0.07	>20
13	8.18 ± 0.01	15.31 ± 0.21	>20	17.53 ± 0.01	16.84 ± 0.12	>20
14	>20	>20	>20	>20	>20	>20
doxorubicin[b]	0.18 ± 0.00	1.31 ± 0.12	0.80 ± 0.03	1.40 ± 0.02	0.31 ± 0.01	1.39 ± 0.00

[a]Human cancer cell lines used were HepG2 and Hep3B (liver), A549 (lung), Ca9-22 (oral), MDA-MB-231 and MCF-7 (breast). Results are presented as mean ± SEM (n = 3). [b]Doxorubicin was used as positive control. [c]"NT" means not tested.

The cytotoxicity data are shown in Table 3. Compound **2** showed moderate activity against all six cancer cell lines (HepG2, Hep3B, A549, Ca9-22, MCF-7, and MDA-MB-231 with IC_{50} values of 2.22 ± 0.01, 2.95 ± 0.02, 3.09 ± 0.07, 2.95 ± 0.07, 4.78 ± 0.02, and 6.12 ± 0.15 μg/mL, respectively). Compound **8** showed moderate cytotoxicity against six cancer cell lines with IC_{50} values of 1.83−2.75 μg/mL. Compounds **3** and **9** showed selective cytotoxicity against the Hep3B cancer cell line (**3**: 3.32 ± 0.42 μg/mL; **9**: 2.87 ± 0.04 μg/mL), which is a p53 mutant cell line.[21] Compounds **2** and **7**, as well as **8** and **9**, have the same sugar moieties, but differ in the spirostanol F ring. Comparison of the data for the paired compounds (**2**/**7** and **8**/**9**) showed that the normal spirostanols (**7** and **9**) were less active than the iso-compounds (**2** and **8**). Among the nonsteroidal saponins, compounds **12** and **13** exhibited weak cytotoxic activity with IC_{50} values of 8.18−17.53 μg/mL against four cancer cell lines (HepG2, Hep3B, A549, Ca9-22), but not MCF7 and MDA-MB-231 breast cancer cell lines.

Regarding the anti-inflammatory data (Table 4), steroidal saponins **3**, **8**, and **9** showed selective inhibition of superoxide anion generation with IC_{50} values of 2.84 ± 0.18, 0.62 ± 0.03, and 1.62 ± 0.59 μg/mL, respectively. In the human neutrophil elastase release assay, the normal spirostanol **9** was considerably more active (IC_{50}: 4.04 ± 0.51 μg/mL) than the iso-spirostanol **8** (IC_{50}: 111.05 ± 7.37 μg/mL) and also was 5-fold more active than phenylmethylsulfonyl fluoride, the positive control. However, compounds **3**, **8**, and **9** also reduced the cell viability, as measured by LDH release (see Supporting Information). In contrast, treatment with compounds **12** and **13** resulted in only slight inhibition of superoxide anion generation and elastase release, but did not increase LDH release compared to control values. Thus, phenolic components **12** and **13** exhibited mild anti-inflammatory effects without toxicity toward human neutrophils.

To date, the major constituents of *S. violaceum* had not been evaluated previously for cytotoxic and anti-inflammatory activities. Thus, our study provides the first scientific evidence to support the traditional use of *S. violaceum* in Chinese folk medicine. Most importantly, unique spirostanol and unusual furostanol saponins were characterized, and the stereoisomeric compounds showed distinct differences in bioactivity.

Table 4. Effects of Compounds 1−14 on Superoxide Anion Generation and Elastase Release in fMLP/CB-Induced Human Neutrophils

	superoxide anion	elastase release
compound	IC_{50} (μg/mL) or (Inh %)	IC_{50} (μg/mL) or (Inh %)[a]
1	NT	NT
2	NT	NT
3	2.84 ± 0.18	(38.90 ± 3.34)[d]
4	NT	NT
5	NT	NT
6	NT	NT
7	NT	NT
8	0.62 ± 0.03	111.05 ± 7.37[e]
9	1.62 ± 0.59	4.04 ± 0.51
10	(24.86 ± 6.08)[b]	(1.58 ± 1.72)
11	NT	NT
12	(23.24 ± 3.66)[c]	(−0.17 ± 3.13)
13	(22.58 ± 4.22)[c]	(24.05 ± 5.59)[b]
14	NT	NT
DPI[f]	0.22 ± 0.13	
PMSF[f]		22.80 ± 5.07

[a]Percentage of inhibition (Inh %) at 10 μg/mL concentration. Results are presented as mean ± SEM (n = 3). [b]$p < 0.05$. [c]$p < 0.01$. [d]$p < 0.001$ compared with the control value. NT means not tested. [e]Compound **8** alone induced elastase release by human neutrophils. The results are expressed as a percentage of the fMLP/CB-activated data. [f]Diphenyleneiodonium (DPI) and phenylmethylsulfonyl fluoride (PMSF) were used as positive controls for superoxide anion generation and elastase release, respectively.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured with a JASCO P-1020 digital polarimeter (JASCO Inc., Tokyo, Japan). The IR spectra were measured on a Perkin-Elmer 2000 FT-IR spectrophotometer (Perkin-Elmer, Norwalk, CT, USA). 1D (^{1}H, ^{13}C, DEPT) and 2D (COSY, HSQC, HMBC, ROESY) NMR spectra using pyridine-d_5 as solvent were obtained on a Bruker Magnet System 500 MHz Ultrashield Plus 600 NMR spectrometer (Bruker

dx.doi.org/10.1021/np200877u | J. Nat. Prod. 2012, 75, 636−643

Instruments, Billerica, MA, USA). Chemical shifts are reported in parts per million (δ), and coupling constants (J) are expressed in Hz and were internally referenced to the solvent signals in pyridine-d_5 (^{1}H, δ_H 7.21, 7.58, 8.74; ^{13}C, δ_C 123.5, 136.0, 150.3). Low-resolution ESIMS were measured on a Bruker Daltonics EsquireHCT ultra high capacity trap mass spectrometer (Bruker Instruments, Billerica, MA, USA). HRESIMS were obtained on a Bruker Daltonics APEX II 30e spectrometer (Bruker Instruments, Billerica, MA, USA). A Shimadzu LC-20AT pump, a Shimadzu RID-10A refractive index detector (Shimadzu Inc., Kyoto, Japan), and a Supelco Ascentis ODS 5 μm (250 × 10 mm i.d.) column were used for HPLC (Supelco, Bellefonte, PA, USA).

Plant Material. Whole plants of *S. violaceum* Ortega were collected from Yunlin, Taiwan (Sept 2010), and identified by one of the authors (H.-F.Y.). The samples (SIEM 201009) were authenticated and deposited at the Natural Medicinal Products Research Center, Taichung.

Extraction and Isolation. The air-dried whole plants (5 kg) were extracted with MeOH (3 × 20 L) overnight at room temperature. The combined MeOH extracts were evaporated under vacuum to give 243.9 g of dried crude extract. The MeOH extract was partitioned between EtOAc and H_2O (1:1, v/v) to give an EtOAc-soluble fraction (103.9 g) and an aqueous phase (140 g). The EtOAc-soluble fraction was further extracted with *n*-hexane and 90% MeOH (1:1, v/v) to yield *n*-hexane (15.7 g) and 90% MeOH (88.2 g) fractions. The 90% MeOH fraction was separated on silica gel column chromatography (840 g, 10 × 25 cm, 70−230 mesh) and eluted with a gradient mixture of $CHCl_3$/MeOH (50:1 → 0:1, v/v) to give 10 fractions (A1−A10). Fraction A3 (8.9 g) was recrystallized from MeOH and filtered to afford sitosterol 3-*O*-β-D-glucopyranoside (**10**, 2.5 g). The mother liquid was concentrated under reduced pressure and chromatographed on silica gel (500 g, 8 × 17 cm, 230−400 mesh) with a gradient elution of $CHCl_3$/MeOH (50:1 → 0:1, v/v) to provide seven subfractions (A3.1−A3.7, 2 L/each). Subfraction A3.4 (1.35 g) was subjected to column chromatography on Sephadex LH-20 (820 g, 5 × 57 cm) with $CHCl_3$/MeOH (1:1, v/v) and further purified by silica gel column chromatography (120 g, 3.5 × 27 cm, 230−400 mesh, EtOAC) to give *N*-*p*-coumaroyltyramine (**12**, 324.7 mg). Subfraction A3.5 (1.1 g) was chromatographed on Sephadex LH-20 (820 g, 5 × 57 cm) with $CHCl_3$/MeOH (1:1, v/v) to furnish *trans*-*N*-feruloyloctopamine (**13**, 14.1 mg). Fraction A4 (3.8 g) was chromatographed on silica gel with a gradient of $CHCl_3$/MeOH (25:1 → 15:1 → 0:1, v/v) to give four subfractions (A4.1−A4.4, 1.2 L/each). Subfractions A4.2 (2.3 g) and A4.3 (1.2 g) were combined and chromatographed repeatedly on Sephadex LH-20 [820 g, 5 × 45 cm, $CHCl_3$/MeOH (1:1)], silica gel [300 g, 2 × 24 cm, 230−400 mesh, $CHCl_3$/MeOH (20:1, v/v)], and RP-RI-HPLC (MeOH/H_2O, 9:1, v/v) to give indioside G (**1**, 4.0 mg), borassoside D (**6**, 8.5 mg), and 7-hydroxysitosterol-3-*O*-β-D-glucopyranoside (**10**, 2.2 mg). Fraction A5 (4.0 g) was chromatographed on Sephadex LH-20 (820 g, 5 × 50 cm) with MeOH to give four subfractions (A5.1−A5.4, 1.5 L/each). Subfraction A5.2 (1.8 g) was purified using ODS HPLC to furnish tricalysioside U (**14**, 2.1 mg), borassoside E (**8**, 43.4 mg), 3-*O*-chacotriosyl-25(*S*)-spirost-5-en-3β-ol (**9**, 25.4 mg), indioside H (**2**, 4.4 mg), indioside I (**3**, 8.1 mg), and yamogenin 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (**7**, 2.2 mg). Fraction A6 (8.13 g) was chromatographed on Sephadex LH-20 (820 g, 5 × 57 cm) with $CHCl_3$/MeOH (1:1, v/v) to give four subfractions (A6.1−A6.4, 2.0 L/each). Subfraction A6.3 (591.4 mg) was chromatographed on RP-18 (50 g, 40−63 μm, 2 × 26 cm, 90% $MeOH_{aq}$, v/v) and further purified using ODS HPLC (MeOH/H_2O, 4:1, v/v) to give indioside J (**4**, 3.5 mg) and indioside K (**5**, 2.2 mg).

*Indioside G (**1**):* white, amorphous powder; $[\alpha]_D^{26}$ −92.4 (*c* 0.3, MeOH); IR (neat) ν_{max} 3402 (OH), 2922, 2841, 1274, 1259, 1030 cm^{-1}; ^{13}C and ^{1}H NMR data, see Tables 1 and 2; ESIMS *m*/*z* 731 [M + Na]$^+$; HRESIMS *m*/*z* 731.3966 [M + Na]$^+$ (calcd for $C_{38}H_{60}O_{12}Na$, 731.4085).

*Indioside H (**2**):* white, amorphous powder; $[\alpha]_D^{26}$ −89.2 (*c* 0.52, MeOH); IR (neat) ν_{max} 3402 (OH), 2915, 1048 cm^{-1}; ^{13}C and ^{1}H NMR data, see Tables 1 and 2; ESIMS *m*/*z* 722 [M + Na]$^+$; HRESIMS *m*/*z* 722.4241 [M + Na]$^+$ (calcd for $C_{38}H_{62}O_{12}Na$, 722.9024).

*Indioside I (**3**):* white, amorphous powder; $[\alpha]_D^{27}$ −68.9 (*c* 0.47, MeOH); IR (neat) ν_{max} 3445 (OH), 2931, 1054 cm^{-1}; ^{13}C and ^{1}H NMR data, see Tables 1 and 2; ESIMS *m*/*z* 893 [M + Na]$^+$; HRESIMS *m*/*z* 893.4862 [M + Na]$^+$ (calcd for $C_{45}H_{74}O_{16}Na$, 893.0413).

*Indioside J (**4**):* white, amorphous powder; $[\alpha]_D^{26}$ −68.9 (*c* 0.47, MeOH); IR (neat) ν_{max} 3438 (OH), 2929, 1258, 1271, 1039 cm^{-1}; ^{13}C and ^{1}H NMR data, see Tables 1 and 2; ESIMS *m*/*z* 965 [M + Na]$^+$; HRESIMS *m*/*z* 965.5055 [M + Na]$^+$ (calcd for $C_{48}H_{78}O_{18}Na$, 965.1040).

*Indioside K (**5**):* white, amorphous powder; $[\alpha]_D^{26}$ −65.2 (*c* 0.37, MeOH); IR (neat) ν_{max} 3438 (OH), 2929, 1258, 1271, 1039 cm^{-1}; ^{13}C and ^{1}H NMR data, see Tables 1 and 2; ESIMS *m*/*z* 965 [M + Na]$^+$; HRESIMS *m*/*z* 965.5102 [M + Na]$^+$ (calcd for $C_{48}H_{78}O_{18}Na$, 965.1040).

Acid Hydrolysis. A solution of steroidal glycoside (**8**, 2 mg; **1**−**5**, 1 mg) was dissolved in 2 M HCl/1,4-dioxane (1:1, v/v, 1.0 mL) and stirred overnight at 90 °C. After cooling, the mixture was partitioned with CH_2Cl_2/H_2O (1:1, v/v), and the aqueous layer was neutralized with Na_2CO_3 and filtered. The filtrate was dissolved in pyridine (0.2 mL), and Ac_2O was added. The mixture was stirred overnight at 60 °C. After cooling, the reaction mixture was dried under vacuum, and the residue was extracted with CH_2Cl_2 and H_2O. The CH_2Cl_2 layer was subjected to GC analysis (DSQ II Single Quadrupole GC/MS, Thermo Fisher Scientific Inc., Rockford, IL, USA) under the following conditions: H_2 flame ionization detector; DB-5MS capillary column (Agilent J&W Scientific, Palo Alto, CA, USA) (30 m × 0.25 mm × 0.25 μm); column temperature, 100−200 °C increased at a rate of 4 °C/min; carrier gas, He (1 mL/min); injector temperature, 200 °C; ion source temperature, 200 °C; EI, 70 eV; injection volume, 5 μL; mass range, *m*/*z* 50−1000. The acetate derivatives of D-glucose, L-rhamnose, and D-xylose had the following retention times, t_R (min): D-glucose (27.32), L-rhamnose (20.76), and D-xylose (20.70). Identification of the sugar derivative was based on a comparison of mass spectra with those of authentic samples, data from Wiley/NBS Registry of Mass Spectral Data (V. 5.0)/National Institute of Standards and Technology (NIST) MS Search V. 2.0.

MTT Cytotoxicity Assay. Fractions and isolates were tested against six human cancer cell lines [HepG2 and Hep3B (hepatoma), A549 (lung), Ca9-22 (oral), MDA-MB-231 and MCF-7 (breast)]. The inhibitory effect of test drug on the cell viability was measured by the MTT colorimetric method as described previously.[22] Cells were seeded at densities of 5000−10 000 cells/well in 96-well tissue culture plates. On day 2, cells were treated with test drug for various time periods. After drug treatment, attached cells were incubated with MTT (0.5 mg/mL, 1 h) and subsequently solubilized in DMSO. The absorbency at 550 nm was then measured using a microplate reader. The IC_{50} is the concentration of agent that reduced the cell viability by 50% under the experimental conditions. Experiments were performed in triplicate, and the values are the averages of three (n = 3) independent experiments. Doxorubicin was used as the positive control.

Human Neutrophil Superoxide Anion Generation. Human neutrophils were obtained by means of dextran sedimentation and Ficoll centrifugation. Superoxide anion production was assayed by monitoring the superoxide dismutase-inhibitable reduction of ferricytochrome *c*.[23,24] In brief, after supplementation with 0.5 mg/mL ferricytochrome *c* and 1 mM Ca^{2+}, neutrophils were equilibrated at 37 °C for 2 min and incubated with drug for 5 min. Cells were incubated with cytochalasin B (CB) (1 μg/mL) for 3 min, before activation by the tripeptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) (100 nM) for 10 min. Changes in absorbance with the reduction of ferricytochrome *c* at 550 nm were continuously monitored in a double-beam, six-cell positioner spectrophotometer (Hitachi U-3010, Tokyo, Japan) with constant stirring. Calculations were based on differences in the reactions with and without superoxide dismutase (100 U/mL) divided by the extinction coefficient for the reduction of ferricytochrome *c* (ε) 21.1 mM/10 mm). Diphenyle-

neiodonium (a NADPH oxidase inhibitor) was used as the positive control.

Measurement of Elastase Release. Degranulation of azurophilic granules in human neutrophils was determined by elastase release as described previously.[25] Experiments were performed using MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide as the elastase substrate. After supplementation with MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide (100 μM), neutrophils (6×10^5/mL) were equilibrated at 37 °C for 2 min and incubated with each test compound for 5 min. Cells were activated by fMLP (100 nM)/CB (0.5 μg/mL), and changes in absorbance at 405 nm were monitored continuously for elastase release. The results were expressed as the percentage of the initial rate of elastase release in the fMLP/CB-activated, test compound-free (DMSO) control system. Phenylmethylsulfonyl fluoride (a serine protease inhibitor) was used as the positive control.

Lactate Dehydrogenase (LDH) Release. LDH release was determined by a commercially available method (Sigma). Cytotoxicity was represented by LDH release in the cell-free medium as a percentage of the total LDH release. The total LDH release was determined by lysing cells with 0.1% Triton X-100 for 30 min at 37 °C.[26]

■ ASSOCIATED CONTENT

Supporting Information

The ^{1}H, ^{13}C, and 2D NMR data of all new compounds and LDH experiment are available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +886-4-22057153. Fax: +886-4-22060248. E-mail: yachwu@mail.cmu.edu.tw.

Funding

The authors declare no competing financial interest.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We gratefully acknowledge financial support for this project from the Department of Health, Executive Yuan, Taiwan (DOH101-TD-C-111-004), and sincerely appreciate Dr. S. L. Morris-Natschke for English revision (Natural Products Research Laboratories, UNC).

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