DAMMARANE-TYPE SAPONINS FROM *GYNOSTEMA PENTAPHYLLUM AND* THEIR CYTOTOXIC ACTIVITY

PHÂN LẬP VÀ HOẠT TÍNH ĐỘC TẾ BÀO CỦA CÁC SAPONIN DẠNG DAMMARANE TỪ CÂY GIẢO CỔ LAM *GYNOSTEMA PENTAPHYLLUM*

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ABSTRACT

*A new dammarane-type saponin, gypenoside VN (**1**), and a known compound, 3β,20S,21-trihydroxydammar-24-ene 3-O-{[α-L-rhamnopyranosyl(1→2)][β-D-glucopyranosyl(1→3)]-α-L-arabinopyranosyl}-21-O-β-D-glucopyranoside (**2**), were isolated from the total saponin extract of the whole plants of Gynostemma pentaphyllum. Their structures were elucidated on the basis of spectroscopic and chemical methods. The two compounds exhibited significant cytotoxic activity against four human cancer cell lines, A549 (lung), HT-29 (colon), MCF-7 (breast), and SK-OV-3 (ovary), with IC_{50} values ranging from 20.2 ± 2.1 to 39.2 ± 3.3 μM. They were weakly active against the U937 (leukemia) cell line with IC_{50} values of 89.5 ± 4.1 and 93.6 ± 3.2 μM for 1 and 2, respectively. Regarding the HL-60 (acute promyelocytic leukemia) cell line, the two compounds were less active with IC_{50} values greater than 100 μM*

TÓM TẮT

*Một saponin mới có khung dammarane, gypenoside VN (**1**), và một hợp chất đã biết 3β,20S,21-trihydroxydammar-24-ene 3-O-{[α-L-rhamnopyranosyl(1→2)][β-D-glucopyranosyl(1→3)]-α-L-arabinopyranosyl}-21-O-β-D-glucopyranoside (**2**) được phân lập từ cặn saponin tổng số của cây giảo cổ lam Gynostemma pentaphyllum. Cấu trúc hóa học của chúng được xác định bằng các phương pháp phổ. Hai hợp chất này ức chế mạnh sự phát triển của 4 dòng tế bào ung thư A549 (ung thư phổi), HT-29 (ung thư ruột), MCF-7 (ung thư vú), and SK-OV-3 (ung thư buồng trứng) với giá trị IC_{50} từ 20.2 ± 2.1 đến 39.2 ± 3.3 μM. Chúng ức chế yếu sự phát triển của tế bào ung thư U937 (ung thư bạch cầu) với giá trị IC_{50} 89.5 ± 4.1 và 93.6 ± 3.2 μM tương ứng đối với **1** và **2**. Đối với dòng tế bào ung thư HL-60 (acute promyelocytic leukemia), cả hai hợp chất đều ức chế yếu với giá trị IC_{50} > 100 μM.*

I. INTRODUCTION

Recently, dammarane-type saponins have received much attention from scientists throughout the world, especially Chinese and Japanese researchers, because of their unique structures and various biological activities. Previous investigations demonstrated that this type of compound possessed numerous, interesting biological effects, such as adjuvant potentials on the cellular and humoral immune responses of ICR mice against ovalbumin (OVA),[1—2] inhibition of NF-κB activation,[3] anti-tumor,[4—6] anti-diabetic,[7] hepatoprotective,[8] anti-atherosclerotic,[9] anti-inflammatory, and anti-oxidative effects.[10] Dammarane-type saponins were found as main constituents in the *Panax* (Araliaceae) and *Gynostemma* (Cucurbitaceae) species. However, *Gynostemma* species have the advantage of easier obtaining dammarane type saponins than *Panax* species. Thus, *Gynostemma* species, especially *G. pentaphyllum*, have attracted much interest as potential new plant drugs. There are 21 species of *Gynostemma* mostly growing in south-western China. The *G. pentaphyllum* species is the most prevalent and is dispersed throughout

India, Nepal, Bangladesh, Sri Lanka, Laos, Myanmar, Korea, and Japan. In Vietnam, there are only two species of the *Gynostemma* genus recorded to date, *G. pentaphyllum* and *G. laxum*. Of which, *G. pentaphyllum* is distributed throughout Vietnam from the plains to mountainous areas at the altitudes up to 2000 m and has been used as a folk medicine to treat cough and chronic bronchitis.[11] Previous studies on this plant have shown that all isolated dammarane-type saponins possessed numerous biological effects.[2—7] Our previous research demonstrated that the total saponin extract of *G. pentaphyllum* showed significant tumor inhibitory effects.[12] In continuation of our research on this plant, we report herein the isolation, structural elucidation, and evaluation of the *in vitro* cytotoxic activity of two dammarane-type saponins from the total saponin extract of the aerial parts of *G. pentaphyllum*.

II. MATERIAL AND METHODS

Experimental

IR spectra were obtained from a Thermo Scientific Nicolet iS10 FT-IR spectrometer with KBr discs. Optical rotations were determined on a Jasco DIP-1000 KUY polarimeter. Electrospray ionization (ESI) mass spectra were obtained using an AGILENT 1200 LC-MSD Trap spectrometer. HR-FAB mass spectra were obtained using a JEOL JMS-AX700 spectrometer. The ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra were recorded on a Bruker AM500 FT-NMR spectrometer with TMS as the internal standard. Column chromatography (CC) was performed on silica gel (Kieselgel 60, 70–230 and 230–400 mesh) (Merck, Darmstadt, Germany) and YMC RP-18 resins (30–50 μm) (Fujisilisa Chemical Ltd., Aichi, Japan). Thin layer chromatography (TLC) was performed on DC-Alufolien 60 silica gel F_{254} (Merck 1.05554.0001) or DC Platen RP_{18} F_{254s} (Merck 1.15685.0001) plates. Spots were visualized by spraying 10% H_2SO_4 aqueous and heating for 5 min.

Plant Material

The aerial parts of *G. pentaphyllum* (Thunb.) Makino (Cucurbitaceae) were collected at the Caobang province, Vietnam, during September 2005, and identified by Prof. Vu Van Chuyen, Hanoi University of Pharmacy. A voucher specimen (N^{o} DK-01-DK-02) was deposited at the herbarium of Hanoi University of Pharmacy, Hanoi, Vietnam.

Extraction and Isolation

Air-dried and powdered aerial parts of *G. pentaphyllum* (5.0 kg) were defatted with petroleum ether and extracted with hot MeOH (50 ^{o}C, 3 × 5.0 L) to give the methanol extract (500 g). This extract was dissolved in hot absolute ethanol (5.0 L) and the insoluble solid was removed. The ethanol-dissolved portion was concentrated and precipitated by acetone (2.0 L), whereupon the solvent was removed by filtration to obtain the total saponin extract (200 g). A part of this extract (6.0 g) was crudely separated on a silica gel column (50 g, 3 × 50 cm) using stepwise gradient elution with chloroform–methanol–water from 4:1:0.1 to 2:1:0.1 (v/v/v) to yield six fractions, SP1—SP6 (0.2 L each). Fraction SP5 (500 mg) was further separated on a YMC RP-18 column (50 g, 2 × 50 cm), eluting with acetone–water 1:1 (0.5 L, fractions of 8.0 mL collected) to obtain the new compound **1** (30 mg) as an amorphous white powder. Compound **2** (15 mg, amorphous white powder) was purified from fraction SP6 (1.0 g) by a YMC RP-18 column (70 g, 2.5 × 50 cm) using acetone–water 1:1.5 (v/v) as an eluent (0.5 L, fractions of 8.0 mL collected).

Gypenoside VN (**1**): Amorphous white powder; $[\alpha]_D^{25}$: –8.4 (*c*= 0.25, MeOH); IR (KBr) ν_{max} (cm^{-1}): 3415.1 (OH), 2939.5 (C-H), 1694.8 (C=O); positive ESI-MS *m/z*: 1115.4 $[M + Na]^+$; negative ESI-MS *m/z*: 1091.4 $[M - H]^-$; HR-FAB-MS *m/z*: 1115.5663 $[M + Na]^+$ (Calcd for $C_{53}H_{88}O_{23}Na$: 1115.5614); ^{1}H- (CD_3OD, 500 MHz) and ^{13}C-NMR (CD_3OD, 125 MHz) are given in Table 1.

Acid Hydrolysis of 1

Compound **1** (5.0 mg) was dissolved in 1.0 N HCl (dioxane–H_2O, 1:1, v/v, 1.0 mL) and heated to 80 °C in a water bath for 3 h. The acidic solution was neutralized with silver carbonate and the solvent thoroughly driven out under N_2 overnight. After extraction with

$CHCl_3$, the aqueous layer was concentrated to dryness using N_2. The residue was dissolved in 0.1 mL of dry pyridine, followed by addition of L-cysteine methyl ester hydrochloride in pyridine (0.06 M, 0.1 mL). The reaction mixture was heated at 60 °C for 2 h. Trimethylsilylimidazole solution (0.1 mL) was then added, followed by heating at 60 °C for 1.5 h. The dried product was partitioned with *n*-hexane and water (0.1 mL each), and the organic layer was analyzed by gas chromatography (GC): column SPB-1 (0.25 mm × 30 m), detector FID, column temp 210 °C, injector temp 270 °C, detector temp 300 °C, carrier gas He (30 mL/min). Under these conditions, the standard sugars gave peaks at t_R (min) 8.55 and 9.25 for D- and L-glucose, 4.72 and 9.16 for D- and L-arabinose, and 5.31 for L-rhamnose, respectively. Peaks at t_R (min) 8.55, 9.16, and 5.31 of D-glucose, L-arabinose, and L-rhamnose for **1** were observed.

Cytotoxicity Tests

The effects of compounds **1** and **2** on the growth of human cancer cells were determined by measuring metabolic activity using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay,[20] with the six human cancer cell lines including HL-60 (acute promyelocytic leukemia), A549 (lung), HT-29 (colon), MCF-7 (breast), SK-OV-3 (ovary), and U937 (leukemia). The cell lines were obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea) and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/mL and 100 g/mL, respectively) at 37 °C in a humidified 5% CO_2 atmosphere. The exponentially growing cells were used throughout the experiments. The MTT assays were performed as follows: human cancer cell lines ($1.5 \sim 2.5 \times 10^5$ cells/mL) were treated for 3 d with 1.0, 10, 30, and 100 μM of compound **1** and **2**, as well as 1.0, 3.0, 10, and 20 μM of mitoxantrone (MX), respectively. After incubation, 0.1 mg (50 μL of a 2.0 mg/mL solution) MTT (Sigma, Saint Louis, MO, USA) was added to each well and the cells incubated at 37 °C for 4 h. The plates were centrifuged at 1000 rpm for 5 min at room temperature and the media carefully aspirated. Dimethylsulfoxide (150 μL) was then added to each well to dissolve the formazan crystals. The plates were read immediately at 540 nm on a microplate reader (Amersham Pharmacia Biotech., Uppsala, Sweden). All the experiments were performed in triplicate with the mean absorbance values calculated. The results were expressed as the percentage of inhibition that produced a reduction in the absorbance by the treatment of the compounds compared to the untreated controls. A dose-response curve was generated and the inhibitory concentration of 50% (IC_{50}) was determined for each compound as well as each cell line. Mitoxantrone (MX; ≥ 97.0%; Sigma, St. Louis, MO), an anticancer agent, was used as positive control.

II. RESULTS AND DISCUSSION

By using combined chromatographic separations, a new dammarane-type saponin, gypenoside VN (**1**), and a known compound **2**, were isolated from the total saponin extract of the aerial parts of *G. pentaphyllum*. Compound **2** was identified as 3*β*,20*S*,21-trihydroxydammar-24-ene 3-*O*-{[*α*-L-rhamnopyranosyl(1→2)][*β*-D-glucopyranosyl(1→3)]-*α*-L-arabinopyranosyl}-21-*O*-*β*-D-glucopyranoside by means of ESI-MS, 1D- and 2D-NMR experiments, and comparison of the spectral data with the literature values.[13] This is the first report of **2** from *G. pentapyllum*. Compound **1** was obtained as an amorphous white powder. The molecular formula of **1**, $C_{53}H_{88}O_{23}$, was identified by means of ESI-MS at *m/z*: 1115.4 $[M + Na]^+$ (positive), 1091.4 $[M - H]^-$ (negative), and HR-FAB-MS at *m/z*: 1115.5663 $[M + Na]^+$ (Calcd for $C_{53}H_{88}O_{23}Na$: 1115.5614). The IR spectrum of **1** was suggestive of glycosyl moieties (3415.1 and 1076.2 cm^{-1}) and a ketone group (1694.8 cm^{-1}). The NMR spectral data of **1** were similar to those of **2**, indicating that **1** was also a dammarane-type saponin, a main constituent of the *Gynostemma* species. The ^{1}H-NMR spectrum showed signals due to seven

*Table 1. The NMR Spectral Data of **1***

C	δ_C [a),b)]	δ_H [a),c)] mult. (*J* in Hz)	C	δ_C [a),b)]	δ_H [a),c)] mult. (*J* in Hz)
Aglycon			29	16.97	0.90 s
1	40.03	1.00 m, 2.40 m	30	17.21	0.76 s
2	27.14	1.75 m, 1.85 m	3-Ara		
3	89.25	3.17[d)]	1′	104.68	4.51 d (5.5)
4	40.51	-	2′	75.00	3.91 dd (5.5, 8.5)
5	57.49	0.85 m	3′	82.22	3.89 dd (3.0, 8.5)
6	19.31	1.60 m, 1.65 m	4′	68.52	4.05 m
7	35.35	1.45 m, 1.60 m	5′	64.71	3.53[d)], 3.89[d)]
8	41.74	-	2′-Rha		
9	52.74	1.75[d)]	1″	101.93	5.23 br s
10	38.51	-	2″	72.08	3.94 d (3.5)
11	40.51	2.17[d)]	3″	72.07	3.75 dd (3.5, 9.0)
12	215.61	-	4″	73.80	3.42 t (9.0)
13	56.70	3.19[d)]	5″	70.23	3.89 m
14	57.09	-	6″	18.01	1.24 d (6.5)
15	32.29	1.24 m, 1.83 m	3′-Glc		
16	24.14	1.75 m, 1.87 m	1‴	104.29	4.52 d (7.5)
17	40.13	2.40 m	2‴	75.25	3.32 dd (7.5, 8.5)
18	16.32	1.27 s	3‴	77.89	3.40 t (8.5)
19	16.68	1.00 s	4‴	71.17	3.37 dd (8.5, 9.0)
20	77.00	-	5‴	77.89	3.35 m
21	75.50	3.15[d)]	6‴	62.39	3.71 dd (2.5, 12.0)/3.87 dd (5.5, 12.0)
22	39.96	2.15 m, 2.51 m	21-Glc		
23	123.19	5.67 m	1⁗	105.13	4.23 d (7.5)
24	142.40	5.68 d (16.5)	2⁗	75.50	3.24 dd (7.5, 8.5)
25	71.23	-	3⁗	77.97	3.39 t (8.5)
26	29.97	1.30 s	4⁗	71.59	3.33 dd (8.5, 9.0)
27	30.00	1.29 s	5⁗	77.07	3.29 m
28	28.50	1.05 s	6⁗	62.67	3.71 dd (2.5, 12.0)/3.87 dd (5.5, 12.0)

a) recorded in CD_3OD, *b*) 125 MHz, *c*) 500 MHz, *d*) overlapped signals, assignments were confirmed by HSQC, HMBC, COSY, and ROESY experiments

Table 2. The Effects of the Dammarane-type Saponins on the Growth of Human Cancer Cells

Compounds	IC_{50} [μM][a)]					
	HL-60 (Leukemia)	A549 (Lung)	HT-29 (Colon)	MCF-7 (Breast)	SK-OV-3 (Ovary)	U937 (Leukemia)
1	>100	28.8 ± 2.4	20.2 ± 2.1	39.2 ± 3.3	27.7 ± 1.4	89.5 ± 4.1
2	>100	30.4 ± 2.2	30.0 ± 2.0	32.4 ± 1.4	30.7 ± 2.4	93.6 ± 3.2
MX[b)]	8.1 ± 0.6	9.0 ± 0.9	7.8 ± 1.0	11.0 ± 0.8	12.0 ± 1.0	6.6 ± 0.8

a) IC_{50} (Concentration that inhibits 50% of cell growth). Compounds were tested at a maximum concentration of 100 μM. Data are presented as the mean of experiments performed in triplicate, *b*) Mitoxantrone (MX), an anticancer agent, was used as reference compound

tertiary methyl groups (each 3H, singlet) at δ 0.76 (H-30), 0.90 (H-29), 1.00 (H-19), 1.05 (H-28), 1.27 (H-18), 1.29 (H-27), and 1.30 (H-26); a secondary methyl group at δ 1.24 (3H, d, J = 6.5 Hz, H-6″); a *trans* di-substituted double bond at δ 5.67 (1H, m, H-23)/5.68 (1H, d, J = 16.5 Hz, H-24); and four anomeric protons at δ 4.23 (d, J = 7.5 Hz, H-1‴), 4.51 (d, J = 5.5 Hz, H-1′), 4.52 (d, J = 7.5 Hz, H-1‴), and 5.23 (br s, H-1″). The ^{13}C-NMR spectrum revealed signals for 53 carbons including 8 methyl, 12 methylene, 26 methine, and 7 quaternary carbons, all detected by DEPT experiments. Of these, a *trans* di-substituted double bond, a ketone, seven tertiary, and one secondary methyl group were determined (Table 1). The site of the ketone group was identified at C-12 by comparison of the NMR spectral data of **1** with the corresponding values of 12-oxo-2*R*,3β,20(*S*)-trihydroxydammar-24-ene-3-*O*-[β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl]-20-*O*-[α-L-rhamnopyranosyl (1→6)-β-D-glucopyranoside], 12-oxo-2*R*,3β,20(*S*)-trihydroxydammar-24-ene-3-*O*-[β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl]-20-*O*-[β-D-xylopyranosyl(1→6)-β-D-glucopyranoside] (**1a**), previously isolated from *G. pentaphyllum*,[14] 3,20-di-*O*-β-D-glucopyranosyl-3β,20*S*-dihydroxydammar-24-ene-12-one,[15] and further confirmed by HMBC correlations from H-11 (δ 2.17), H-13 (δ 3.19), and H-17 (δ 2.40) to C-12 (δ 215.61). The two sugar moieties were first identified as {[α-L-rhamnopyranosyl(1→2)][β-D-glucopyranosyl(1→3)]-α-L-arabinopyranosyl}-21-*O*-β-D-glucopyranoside by comparing their NMR data with those of **2**. Of which, signals at δ 4.51 (d, J = 5.5 Hz), 4.52 and 4.23 (each doublet, J = 7.5 Hz), and 5.23 (br s) were assigned to anomeric protons of the α-arabinopyranose, two β-glucopyranoses, and rhamnose, respectively. In the HMBC spectrum, H-1′, H-1″, H-1‴, and H-1⁗ correlated with C-3, C-2′, C-3′, and with C-21, respectively. This evidence confirmed that **1** had the same constituents, linkage sites, and sequences of the four saccharides as **2** and gypenoside XLVIII.[3,16] Furthermore, acid hydrolysis of **1** gave D-glucose, L-arabinose, and L-rhamnose (see Experimental). The side chain structure was first assigned by comparison its NMR spectral data with the corresponding values of 3β,20*S*,21-trihydroxy-25-methoxydammar-23-ene 3-*O*-α-L-rhamnopyranosyl(1→2)-[β-D-xylopyranosyl(1→3)]-β-D-glucopyranosyl-21-*O*-β-D-xylopyranoside (**1b**) and gypenoside LXIX, previously isolated from *G. pentaphyllum*,[16] and further confirmed by detailed analyses of the correlations observed in the ^{1}H–^{1}H COSY and HMBC spectra of **1** (Fig. 2). From the above evidence, the planar structure of **1** was elucidated as shown in Fig. 1. The stereochemistry of **1** was assigned by comparison of its NMR spectral data with those of similar reported compounds and by a ROESY experiment. The chemical shift value of C-3 (δ 89.25), glycosylated with α-L-arabinose, whose C-1′ configuration was *R*, indicated an *S* configuration at C-3,[17] which was further confirmed by ROEs correlations from H-3 (δ 3.17) to H-28 (δ 1.05) and H-5 (δ 0.85). Proton H-17 (δ 2.40) showed ROEs correlation with H-30 (δ 0.76), but not with H-13 (δ 3.19), confirming the *S* configuration of C-17. The *S* configuration of C-20 was identified by ROEs correlations from H-13 to H-21 (δ 3.15) and H-17 to H-22 (δ 2.15), Fig. 3.[17] Thus, the structure of 3β,20*S*,21,25-tetrahydroxydammar-23-ene-12-one 3-*O*-{[α-L-rhamnopyranosyl(1→2)][β-D-glucopyranosyl(1→3)]-α-L-

arabinopyranosyl}-21-*O*-*β*-D-glucopyranoside was deduced for **1**, a new compound named gypenoside VN.

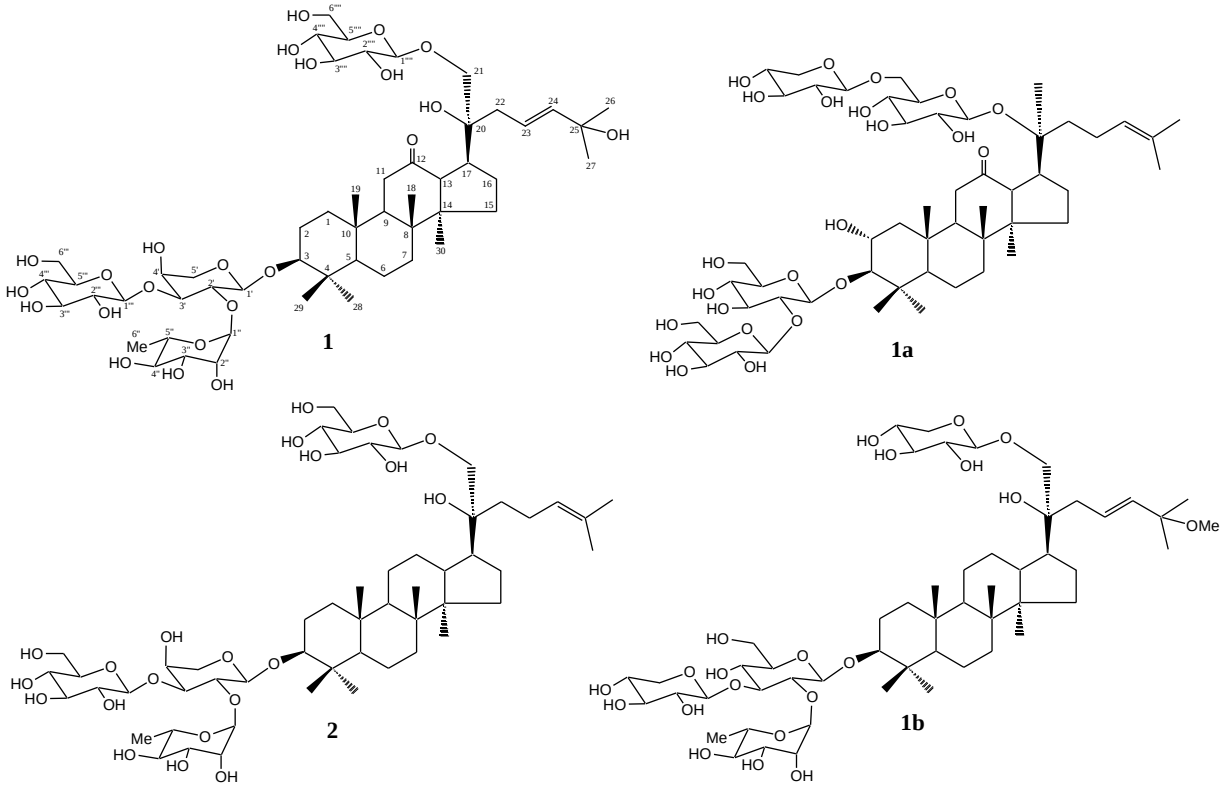

Fig. 1 Structures of Compounds 1 and 2

Compounds **1** and **2** were evaluated for their cytotoxic activity against six human cancer cell lines after continuous exposure for 72 h. The two compounds showed significant cytotoxic activity against four cell lines, A549 (lung), HT-29 (colon), MCF-7 (breast), and SK-OV-3 (ovary), and weakly active against the U937 (leukemia) cell line. However, they were ineffective against the HL-60 (acute promyelocytic leukemia) cell line, even when treated at a concentration of 100 μM. These results indicated that the compounds might possess cytotoxicity *via* changes of adherent properties in adenocarcinoma cells such as A549, HT-29, MCF-7, and SK-OV-3. Furthermore, consideration of the cytotoxicity of the two compounds, **1** showed slightly stronger effects than those of **2** (Table 2) on four of the five cell lines. This might be explained by the presence of the 12-keto-group in the molecule of **1** as most dammarane tetracyclic triterpenoids and their glucosides, which bear a ketone group instead of a hydroxyl group at the C-3 of ring A or at C-12 in ring C, possess greater toxicity toward tumor cells than other compounds and have weaker hemolytic effects.[15] Thus, the previous reports for gypenosides[5,18,19] and our findings, suggest that the effect of compound **1** on the induction of apoptosis in adenocarcinoma cells requires further investigations for medicinal purposes directed towards antitumor activity.

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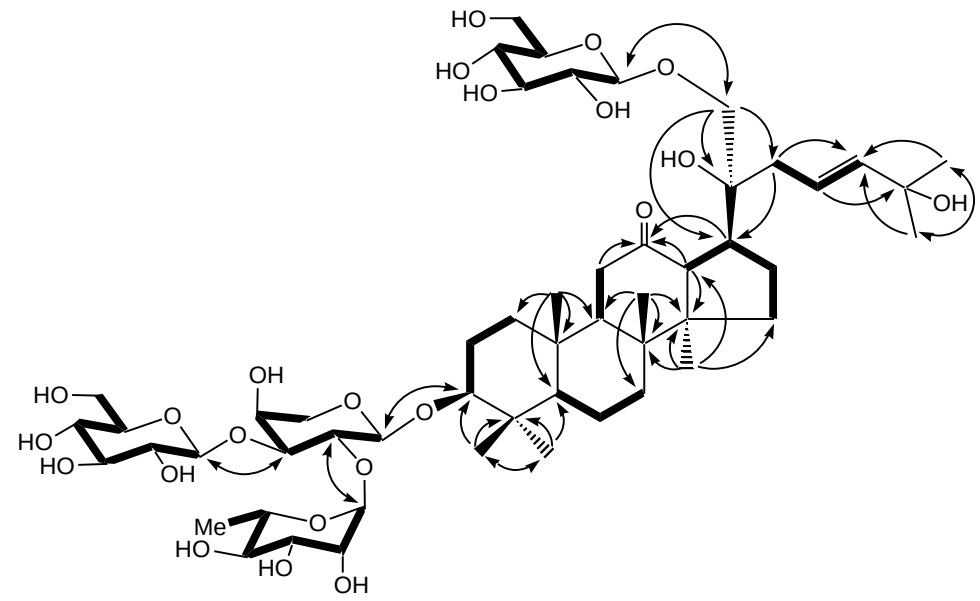

Fig. 2 Key HMBC and $^{1}H–^{1}H$ COSY Correlations of 1

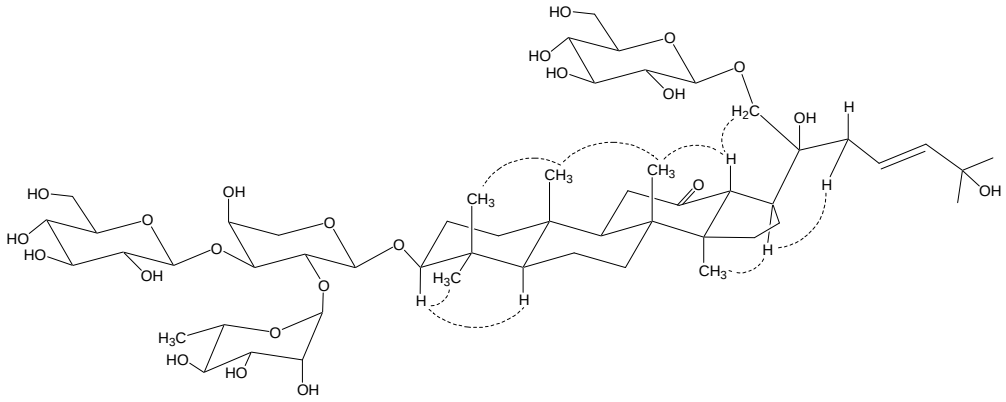

Fig. 3 Probable Configuration and Key ROEs Correlations of 1

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