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Immunomodulatory sesquiterpene glycosides from *Dendrobium nobile*

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Abstract

Four sesquiterpene glycosides with alloaromadendrane, emmotin, and picrotoxane type aglycones were isolated from the stems of *Dendrobium nobile* Lindl (Orchidaceae). Their structures were determined by spectroscopic methods and chemical reactions. Immunomodulatory activity of the isolates was evaluated in vitro.

Keywords: *Dendrobium nobile*; Orchidaceae; Sesquiterpene glycosides; Dendrosides D, E, F, and G; Immunomodulatory activity

1. Introduction

Several species of *Dendrobium* plants (Orchidaceae) are used in traditional Chinese medicine to nourish the stomach, promote secretion of saliva, and reduce fever (Jiangsu New Medical College, 1986). The stems of *Dendrobium nobile* Lindl (Chinese name "Jin-Chai-Shi-Hu") is one of the most famous *Dendrobium* plants in traditional Chinese medicine and is used as a Yin tonic (Jiangsu New Medical College, 1986). A series of chemical components have been identified from *D. nobile* Lindl (Chen and Chen, 1935; Hedman and Leander, 1972; Talapatra et al., 1982; Wang and Zhao, 1985; Veerraju et al., 1989; Li et al., 1991; Morita et al., 2000), and several compounds were found to possess antitumor and antimutagenic activity (Lee et al., 1995; Miyazawa et al., 1997). During a systematic study on the chemical components of *D. nobile*, several new sesquiterpene glycosides and bibenzyls were identified, among them three sesquiterpene glycosides exhibited immunomodulatory activity (Zhao et al., 2001; Ye and Zhao, 2002). As a result of further investigation of the chemical constituents of *Dendrobium* plants, the isolation, structure elucidation, and evaluation for immunomodulatory activity of four new sesquiterpene glycosides (**1–4**) from *D. nobile* is reported.

2. Results and discussion

Compound **1** was obtained as a white amorphous powder with an elemental formula of $C_{27}H_{44}O_{14}$ as determined by HRESIMS (*m*/*z* 615.2630, $[M+Na]^+$) and NMR analysis. In the ^{13}C NMR spectrum of **1**, 27 carbon signals belonging to two methyls, seven methylenes, 15 methines, and three quaternary carbons were observed. A carbonyl group in the structure of **1** was deduced according to an absorption band at 1724 cm^{-1} in its IR spectrum, which was confirmed by the carbon signal at δ_C 172.6 (*s*) in its ^{13}C NMR spectrum. Enzymatic hydrolysis of **1** gave the aglycone **1a**, and glucose was determined as its sugar moiety by co-TLC with an authentic sample. In the ^1H NMR spectrum of **1**, two anomeric proton signals were found at δ_H 6.30 (1H, d, $J=8.0$ Hz) and 4.92 (1H, *d*, $J=7.6$ Hz); while in its ^{13}C NMR spectrum, two anomeric carbon signals were observed at δ_C 95.8 (*d*) and 106.3 (*d*). The above NMR spectra data revealed that both glucose units link to the aglycone with a β configuration, and that one glucose unit should link to the aglycone via an ester linkage (Yu and Yang, 1999). Analysis of the ^1H–^1H COSY and

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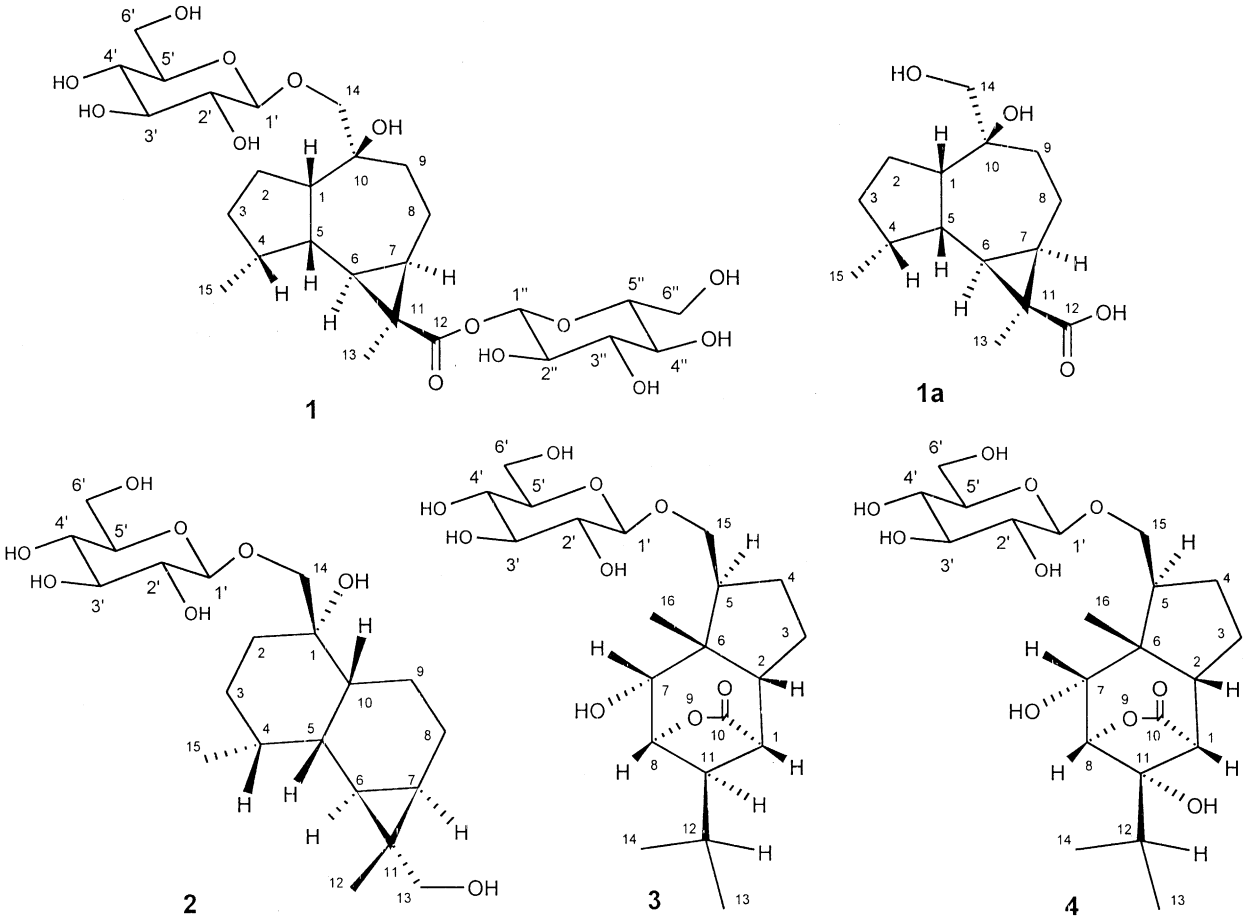

HMQC spectra of **1** enabled the deduction of the fragment-C-9-C-8-C-7-C-6-C-5-C-4-(C-15)-C-3-C-2-C-1-C-5- in its structure. In the HMBC spectrum of **1**, ^{13}C–^{1}H long-range correlation signals were found for C-10/H-5, 8; C-1/H-9, 14; C-11/H-5, 8, 13; C-13/H-6, 7; C-12/H-6, 7, 13, 1″; H-15/C-3, 5, and H-14/C-9, 1′, which enabled the establishment of an alloaromadendrane-type sesquiterpene skeleton for the aglycone of **1** (Lima et al., 1999). In the NOESY spectrum of **1**, correlation signals were found between H-6 and H-7, H-15; H-1 and H-4, H-5; H-4 and H-5; and H-1′ and H-14. The relative configuration of the aglycone of **1** could be defined from the above information, except for C-10 and C-11. In order to elucidate the stereochemistry of C-10 and C-11, the NMR spectra of **1a** were acquired using DMSO-d_6 as solvent. All of the proton and carbon signals of **1a** could be assigned on the basis of ^{1}H–^{1}H COSY, HMQC, and HMBC spectra. In the ^{1}H NMR spectrum of **1a**, the 10-OH proton signal was observed at δ_H 3.52 (1H, *s*). Correlation signals between the 10-OH and H-1 and between the 10-OH and H-5 in the ROESY spectrum suggested that 10-OH was in the β configuration. Furthermore, in the ROESY spectrum of **1a**, correlation signals were found between H-6 and H-7, H-13, H-15; and H-7 and H-13, which indicated an α configuration for C-13. The relative configuration of **1a** could thus be fully established. To the best of our knowledge, aglycone **1a** is a new compound. Furthermore, the two glucose units were deduced to connect to C-12 and C-14 of the aglycone according to the above mentioned correlation signals in the NOESY and HMBC spectra of **1**. Consequently, the structure of **1** was identified to be 10β,14-dihydroxy-alloaromadendran-12-oic acid 12,14-di-*O*-β-D-glucopyranoside, and was given the name dendroside D (Zhao et al., 2001; Ye and Zhao, 2002).

Compound **2** was obtained as a white amorphous powder with an elemental formula of $C_{21}H_{36}O_8$ as determined by HRESIMS (*m*/*z* 439.2327, $[M+Na]^+$) and NMR analysis. In the ^{13}C NMR spectrum of **2**, 21 carbon signals belonging to two methyls, seven methylenes, 10 methines, and two quaternary carbons were observed. From the enzymatic hydrolysis of **2**, glucose was identified as its sugar component. In the ^{1}H NMR spectrum of **2**, one anomeric proton signal was found at δ_H 4.95 (1H, *d*, $J=7.7$ Hz), the glucose unit was therefore in a β configuration (Yu and Yang, 1999). Analysis of

the ^{1}H–^{1}H COSY and HMQC spectra of **2** enabled the deduction of fragments-C-2-C-3-C-4-(C-15)-C-5- and -C-7-C-6-C-5-C-10-C-9-C-8- in its structure. In the HMBC spectrum of **2**, ^{13}C–^{1}H long-range correlation signals were found for C-11/H-5, 8, 12, 13; C-1/H-3, 9, 10, 14; H-15/C-3, 4, 5; H-14/C-2, 10, 1′, which enabled establishment of planar structure of its aglycone. Relative configuration of aglycone of **2** was characterized on the basis of NOE correlation signals between H-7 and H-6, H-13; H-6 and H-13, H-15; H-4 and H-5, H-10; and H-10 and H-5, H-14 in its NOESY spectrum. The aglycone of **2** was thus deduced to possess an emmotin-type sesquiterpene skeleton (Oliveira et al., 1974). The glucose unit was shown to connect to C-14 of the aglycone according to the correlation signals in the NOESY and HMBC spectra mentioned above. The structure of **2** was thus established to be 1α,13,14-trihydroxyemmotin 14-*O*-β-D-glucopyranoside, and was assigned the name dendroside E.

Compound **3** was obtained as a white amorphous powder with an elemental formula of $C_{21}H_{34}O_9$ as determined by HRESIMS (*m*/*z* 453.2124, $[M+Na]^+$) and NMR analysis. In the ^{13}C NMR spectrum of **3**, 21 carbon signals belonging to three methyls, four methylenes, 12 methines, and two quaternary carbons were observed. Absorption bands at 3410 and 1770 cm^{-1} in the IR spectrum of **3** indicated the presence of hydroxyl and lactonic carbonyl groups in its structure, respectively. Enzymatic hydrolysis of **3** yielded glucose as its sugar component. Elucidation of the ^{1}H–^{1}H COSY and HMQC spectra of **3** enabled a deduction of the fragment-C-14-C-12-(C-13)-C-11-(C-8-C-7)-C-1-C-2-C-3-C-4-C-5-C-15- in its aglycone. The planar structure of the aglycone of **3** could be deduced on the basis of the ^{13}C–^{1}H long-range correlation signals at C-6/H-1, 2, 3, 4, 8, 15; C-11/H-7, 13, 14; C-16/H-2, 5, 7; C-10/H-1, 8, 11; and H-15/C-4, 6, 1′ in its HMBC spectrum. The relative configuration of the aglycone of **3** was characterized on the basis of a NOESY experiment, in which NOE correlation signals were found between H-7 and H-8, H-16; H-2 and H-1, H-12, H-16; and H-15 and H-1′, H-16. Compound **3** was thus deduced to possess an aglycone with a picrotoxane-type sesquiterpene skeleton (Sha et al., 1997; Morita et al., 2000). The glucose unit was identified to link to C-15 of the aglycone according to the above mentioned signals in its NOESY and HMBC spectra. The structure of **3** was thus established to be 7-hydroxy-5-hydroxymethyl-11-isopropyl-6-methyl-9-oxatricyclo[6.2.1.$0^{2,6}$]undecan-10-one-15-*O*-β-D-glucopyranoside, and was assigned the name dendroside F.

Compound **4** was obtained as a white amorphous powder with an elemental formula of $C_{21}H_{34}O_{10}$ as determined by HRESIMS (*m*/*z* 469.2035, $[M+Na]^+$) and NMR analysis. Absorption bands at 3410 and 1760 cm^{-1} in the IR spectrum of **4** indicated the presence of hydroxyl and lactonic carbonyl groups in its structure, respectively. Enzymatic hydrolysis of **4** yielded glucose as its sugar component. In the ^{13}C NMR spectrum of **4**, 21 carbon signals belonging to three methyls, four methylenes, 11 methines, and three quaternary carbons were observed. The ^{13}C NMR spectroscopic data of **4** were similar to those of **3** except for an additional hydroxy-substituted quaternary carbon at δ_C 81.5 and the disappearance of a methine group. Analysis of the ^{1}H–^{1}H COSY, HMQC, and HMBC spectra of **4** also led to the deduction of a picrotoxane-type skeleton similar to that of **3**. The difference lay in the substitution of a hydroxyl group at C-11 in the aglycone of **4**. The relative configuration of **4** was further characterized on the basis of a NOESY experiment, in which NOE correlation signals were found between H-1 and H-13, H-14; H-7 and H-8, H-16; H-2 and H-1, H-16; H-15 and H-4β, H-16; H-5 and H-4α; and H-15 and H-1′. The glucose unit was also deduced to link to C-15 according to above mentioned NOE evidence, and the result was further confirmed by a ^{13}C–^{1}H long-range correlation signal between C-15 and H-1′ in the HMBC spectrum of **4**. The structure of **4** was thus established to be 7,11-dihydroxy-5-hydroxymethyl-11-isopropyl-6-methyl-9-oxatricyclo[6.2.1.$0^{2,6}$]undecan-10-one-15-*O*-β-D-glucopyranoside, and was assigned the name dendroside G.

Previously, polysaccharides and sesquiterpene glycosides from *Dendrobium* plants were reported to stimulate the proliferation of T and B lymphocytes in mice (Zhao et al., 1994, 2001). In preliminary in vitro biological tests, it was found that dendrosides D–G (**1–4**) stimulated significantly the proliferation of mouse T and/or B lymphocytes in vitro (Table 3) (Li et al., 1990; Xiang and Li, 1993).

3. Experimental

3.1. General

Optical rotations were measured with a Perkin-Elmer 241MC polarimeter or Perkin-Elmer 341 polarimeter. IR spectra were recorded using a Perkin-Elmer 577 spectrometer. LRESIMS were measured using a Finnigan LCQ-DECA instrument, and HRESIMS data were obtained on Micromass LCT and Mariner spectrometers. FABMS measurements were made with a Varian MAT 212 instrument. NMR spectra were run on a Bruker AM 400 spectrometer with TMS as internal standard. Column chromatographic separations were carried out using silica gel H60 (Qingdao Haiyang Chemical Group Corporation, Qingdao, People's Republic of China), Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden), and RP-18 (100–200 mesh, Tianjin No. 2 Chemical Reagent Factory, Tianjin, People's Republic of China) as packing materials, and a LiChroprep RP-18 (40–63 μm, Merck). HSGF254 silica gel TLC plates (Yantai Chemical Industrial Institute, Yantai, People's Republic of China) were

Table 1
^{1}H NMR (400 MHz) spectra data of **1–4** (C_5D_5N) and **1a** (DMSO-d_6) (ppm, J in Hz)

No	1	1a	2	3	4
1	2.32 *m*	1.78 *m*		2.40 *dd* (3.7, 3.7)	2.75 *d* (3.5)
2	1.48 *m*; 1.75 *m*	1.35 *m*; 1.52 *m*	2.08 *m*; 2.20 *m*	2.08 *m*	2.22 *m*
3	1.22 *m*; 1.70 *m*	1.20 *m*; 1.70 *m*	1.38 *m*; 1.70 *m*	1.99 *m*	2.11 *m*
4	1.86 *m*	1.85 *m*	1.97 *m*	2.29 *m*	1.65 *m*; 2.41 *m*
5	3.02 *m*	2.32 *m*	1.68 *m*	3.32 *m*	3.55 *m*
6	0.80 *t* (9.2, 9.2)	0.55 *t* (9.2, 9.2)	0.68 *dd* (9.3, 9.3)		
7	1.20 *m*	1.04 *m*	1.10 *m*	3.90 *m*	4.17 *s*
8	1.66 *m*; 2.98 *m*	1.44 *m*; 2.15 *m*	1.22 *m*; 1.88 *m*	4.76 *d* (5.5)	5.02 *s*
9	1.55 *dd* (13.5, 13.5) 1.95 *dd* (13.5, 6.8)	1.25 *m*; 1.42 *m*	1.98 *m*; 2.12 *m*		
10			2.35 *m*		
11				2.05 *m*	
12			1.22 *s*	1.65 *m*	2.05 *t* (6.6, 6.6)
13	1.44 *s*	1.15 *s*	3.50 *d* (10.7); 3.78 *d* (10.7)	0.80 *d* (5.8)	1.15 *d* (6.2)
14	3.70 *d* (10.4); 3.96 *d* (10.4)	3.00 *dd* (10.8, 5.5) 3.10 *dd* (10.8, 5.5)	3.88 *d* (10.3); 4.10 *d* (10.3)	0.80 *d* (5.8)	1.13 *d* (6.2)
15	1.08 *d* (6.4)	0.88 *d* (6.6)	1.02 *d* (6.6)	4.09 *dd* (8.6, 8.6); 4.36 *dd* (8.6, 5.6)	4.21 *m* 4.50 *dd* (8.5, 5.7)
16				1.12 *s*	1.25 *s*
1′	4.92 *d* (7.6)		4.95 *d* (7.7)	4.78 *d* (7.5)	4.90 *d* (7.8)
2′	4.04 *m*		4.09 *m*	3.92 *m*	4.03 *m*
3′	4.22 *m*		4.25 *m*	4.10 *m*	4.24 *m*
4′	4.20 *m*		4.24 *m*	4.12 *m*	4.24 *m*
5′	3.94 *m*		3.99 *m*	3.80 *m*	3.94 *m*
6′	3.99 *m*; 4.58 *dd* (11.7, 2.2)		4.38 *dd* (11.8, 5.4); 4.58 *dd* (11.8, 2.4)	4.30 *dd* (11.8, 5.3); 4.45 *dd* (11.8, 2.3)	4.41 *dd* (11.6, 4.6); 4.57 *dd* (11.6, 1.6)
1″	6.30 *d* (8.0)				
2″	4.15 *m*				
3″	4.22 *m*				
4″	4.28 *m*				
5″	4.00 *m*				
6″	3.97 *m*; 4.44 *dd* (12.0, 2.3)				
10-OH		3.52 *s*			
12-OH		11.84 *brs*			
14-OH		4.28 *dd* (5.5, 5.5)			

used for analytical TLC. β-cellulase was manufactured by Lizhu Dongfeng Bio-Tech Co. Ltd., Shanghai, People's Republic of China.

3.2. Plant material

The fresh stems of *D. nobile* were collected in the suburb of Chongqing in December, 1999 and identified by Professor Ming Zhang of Chongqing Institute of Traditional Chinese Medicine, Chongqing, Sichuan Province, People's Republic of China. A voucher specimen (No. SIMMW9912) is deposited in the herbarium of Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, People's Republic of China.

3.3. Extraction and isolation

Powdered air-dried stems of *D. nobile* (2.5 kg) were refluxed three times with 95% aq. EtOH. After evaporation of the EtOH extract in vacuo, the residue was partitioned between water and petroleum ether, EtOAc, and *n*-BuOH, successively. The *n*-BuOH extract (40 g) was subjected to cc over RP-18 eluted with a MeOH–water step gradient (in ratios, 3:7, 1:1, 3:2, successively). The fraction from the MeOH–H_2O (3:7) eluent (0.53 g) was filtered through a Sephadex LH-20 column using EtOH as eluent, then applied to a silica gel column eluted with a $CHCl_3$–MeOH–water step gradient (5:1: 0.1, 4:1: 0.1, and 7:3:0.5, respectively), and then over Lobar RP-18 columns eluted with a MeOH–water step gradient (1:1 and 3:2) to give compounds **1** (25 mg), **2** (15 mg), **3** (40 mg), and **4** (15 mg).

3.4. Characterization

3.4.1. Dendroside D (1)

White amorphous powder; $[\alpha]_D^{20}$ −31.3° (*c* 0.5, MeOH); IR (KBr) ν_{max} 3403 (OH), 2927, 2890, 1724

Table 2
^{13}C NMR (100 MHz) spectroscopy data for **1–4** (C_5D_5N) and **1a** (DMSO-d_6) (ppm)

No.	1	1a	2	3	4
1	54.3 *d*	52.9 *d*	75.2 *s*	46.2 *d*	54.8 *d*
2	24.9 *t*	23.9 *t*	34.6 *t*	45.3 *d*	47.9 *d*
3	29.8 *t*	28.9 *t*	30.1 *t*	26.5 *t*	26.9 *t*
4	38.4 *d*	37.3 *d*	39.3 *d*	28.6 *t*	29.0 *t*
5	39.4 *d*	38.3 *d*	39.8 *d*	43.9 *d*	44.4 *d*
6	30.4 *d*	27.5 *d*	19.9 *d*	49.5 *s*	48.8 *s*
7	34.8 *d*	32.4 *d*	24.0 *d*	73.2 *d*	75.6 *d*
8	17.7 *t*	17.1 *t*	19.9 *t*	84.7 *d*	90.1 *d*
9	33.0 *t*	31.8 *t*	24.6 *t*		
10	75.4 *s*	74.5 *s*	51.0 *d*	179.4 *s*	179.7 *s*
11	28.5 *s*	27.7 *s*	25.7 *s*	52.3 *d*	81.5 *s*
12	172.6 *s*	174.5 *s*	12.4 *q*	25.2 *d*	29.4 *d*
13	23.6 *q*	23.7 *q*	72.1 *t*	20.6 *q*	16.6 *q*
14	80.0 *t*	69.9 *t*	78.8 *t*	19.7 *q*	15.6 *q*
15	16.9 *q*	16.3 *q*	16.5 *q*	73.2 *t*	73.6 *t*
16				24.1 *q*	24.9 *q*
1′	106.3 *d*		106.1 *d*	104.5 *d*	105.0 *d*
2′	75.4 *d*		75.2 *d*	74.8 *d*	75.2 *d*
3′	78.8 *d*		78.6 *d*	78.3 *d*	78.7 *d*
4′	71.8 *d*		71.7 *d*	71.4 *d*	71.8 *d*
5′	78.7 *d*		78.5 *d*	78.3 *d*	78.7 *d*
6′	62.8 *t*		62.8 *t*	62.6 *t*	63.0 *t*
1″	95.8 *d*				
2″	74.2 *d*				
3″	78.9 *d*				
4″	71.3 *d*				
5″	79.5 *d*				
6″	62.4 *t*				

Table 3
Effect of compounds **1–4** on murine lymphocyte proliferation induced by concanavalin A (ConA) (5 μg/ml) or lipopolysaccharide (LPS) (10 μg/ml)

Samples	Concentration (M)	[^{3}H] TdR incorporation $\times 10^{-3}$ (cpm)	
Compounds		ConA-induced T cell proliferation	LPS-induced B cell proliferation
Negative control		3.48±0.19	8.68±0.49
Positive control (Con A or LPS)		18.14±0.83	35.05±0.30
1	10^{-7}	19.75±1.89	36.37±1.00
	10^{-6}	20.18±0.72*	34.49±0.35
	10^{-5}	19.56±0.37	34.44±0.91
2	10^{-7}	19.60±0.48	36.06±1.08
	10^{-6}	20.12±0.58*	34.47±1.89
	10^{-5}	20.21±0.22*	37.80±0.77*
3	10^{-7}	20.20±0.13*	35.47±0.65
	10^{-6}	20.19±0.86*	34.43±1.82
	10^{-5}	20.14±0.41*	36.21±0.52*
4	10^{-7}	20.96±0.56**	36.30±0.99
	10^{-6}	19.76±0.31	35.92±0.91
	10^{-5}	19.30±0.92	37.03±0.96

Results are represented as mean±S.D. based on three independent experiments. ($n=3$; $^*P<0.05$; $^{**}P<0.01$ compared with control group).

(C=O), 1635, 1456, 1379, 1263, 1163, 1076, 1039, 880, and 606 cm^{-1}; ESI–MS *m/z* 615 $[M+Na]^+$, 1207 $[2M+Na]^+$; HR-ESI–MS *m/z* 615.2630 $[M+Na]^+$ (calc. for $C_{27}H_{44}O_{14}Na$, 615.2629); for ^1H and ^{13}C NMR spectra data, see Tables 1 and 2.

3.4.2. Compound 1a

Colorless gum; $[\alpha]_D^{20}+6.5^\circ$ (*c* 0.48, MeOH); IR (KBr) ν_{max} 3412 (OH), 2926, 2879, 1725 (C=O), 1660, 1464, 1379, 1279, 1167, 1067, 1030, and 843 cm^{-1}; ESI–MS *m/z* 267 $[M-H]^-$; for ^1H and ^{13}C NMR spectra data, see Tables 1 and 2.

3.4.3. Dendroside E (2)

White amorphous powder; $[\alpha]_D^{20}-38.3^\circ$ (*c* 0.4, MeOH); IR (KBr) ν_{max} 3382 (OH), 2928, 2872, 1643, 1454, 1384, 1165, 1078, 1034, 880, and 631 cm^{-1}; ESI–MS *m/z* 417 $[M+H]^+$, 439 $[M+Na]^+$; HR-ESI–MS *m/z* 439.2327 $[M+Na]^+$ (calc. for $C_{21}H_{36}O_8Na$, 439.2302); ^1H and ^{13}C NMR spectra data, see Tables 1 and 2.

3.4.4. Dendroside F (3)

White amorphous powder. $[\alpha]_D^{20}-30.6^\circ$ (*c* 0.5, MeOH); IR (KBr) ν_{max} 3410 (OH), 2960, 2950, 2890, 1770 (C=O), 1641, 1467, 1384, 1150, 1076, 1040, and 640 cm^{-1}; ESI–MS *m/z* 431 $[M+H]^+$, 453 $[M+Na]^+$. HR-ESI–MS *m/z* 453.2124 $[M+Na]^+$ (calc. for $C_{21}H_{34}O_9Na$, 453.2095). For ^1H and ^{13}C NMR spectra data, see Tables 1 and 2.

3.4.5. Dendroside G (4)

White amorphous powder. $[\alpha]_D^{20}-24.9^\circ$ (*c* 0.6, MeOH); IR (KBr) ν_{max} 3410 (OH), 2968, 2950, 2888, 1760 (C=O), 1639, 1383, 1159, 1078, 1043, and 650 cm^{-1}; FAB–MS *m/z* 469 $[M+Na]^+$. HR-ESI–MS *m/z* 469.2035 $[M+Na]^+$ (calc. for $C_{21}H_{34}O_{10}Na$, 469.2044). For ^1H and ^{13}C NMR spectra data, see Tables 1 and 2.

3.5. Enzymatic hydrolysis

Compound **1** (15 mg) was dissolved in H_2O (7 ml), and β-cellulase (105 mg) was added to the solution and kept at 37 °C for 7 days. The aqueous solution was then extracted with EtOAc three times, and the EtOAc extract was concentrated to dryness to afford **1a** (5 mg). The aqueous residue was compared with authentic sugar samples by co-TLC (EtOAc–MeOH–H_2O–HOAc, 13:3:3:4, R_f 0.46 for glucose). Compounds **2–4** (5 mg) were hydrolyzed with β-cellulase using the same method described above.

3.6. Configuration of D-glucose

A solution of the hydrolyzed sugar from **1** to **4** (0.04 M) in pyridine (100 μl) and L-cysteine methyl ester hydrochloride (0.06 M) were mixed, and warmed at

60 °C for 1 h. Acetic anhydride (150 μl) was then added, and the mixture was then warmed at 90 °C for a further 1 h. After evaporation of the solvents in vacuo, the residue was dissolved in acetone (350 μl) and the solution (1 μl) was subjected to GLC (Hara et al., 1987; Zhao et al., 2001). A peak for a peracetylated thiazolidine derivative with a retention time at 9.39 min was observed for all five samples, which was identical to the derivative of authentic D-glucose prepared in the same manner.

3.7. Lymphocyte proliferation test

The prepared spleen cells of mice (4×10^6) were seeded into each well of a 96-well microplate and various concentrations of compounds **1–4** and 5 μg/mL of concanavalan A (Con A, from *Canavalia ensiformis* Type III, Sigma) or lipopolysaccharide (LPS, from *Escherichia coli*, Sigma) were added alone or in combination. The plates were cultured at 37 °C with 5% CO_2 in a humidified atmosphere for 48 h. For the last 6 h, each well was pulsed with 0.25 μ*Ci*/well ^{3}H-TdR (thymidine, [methyl-^{3}H], ICN Pharmaceuticals, Inc., Irvine, CA). The cells were harvested and the radioactivity incorporated was counted by a liquid scintillation counter. All counts/min values shown were the mean of triplicate sample±S.D. Statistical analysis was carried out by Student *t*-test. ConA or LPS was used as a positive control (Li et al., 1990; Xiang and Li, 1993).

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