Polyhydroxysteroidal Glycosides from the Starfish *Anthenea chinensis*

Ning Ma,[†] Hai-Feng Tang,*[,†] Feng Qiu,*[,‡] Hou-Wen Lin,[§] Xiang-Rong Tian,[†,‡] and Min-Na Yao[†]

Department of Pharmacy, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, People's Republic of China, Department of Traditional Chinese Medicines, Shenyang Pharmaceutical University, Shenyang 110016, People's Republic of China, and Department of Pharmacy, Changzheng Hospital, Second Military Medical University, Shanghai 200433, People's Republic of China

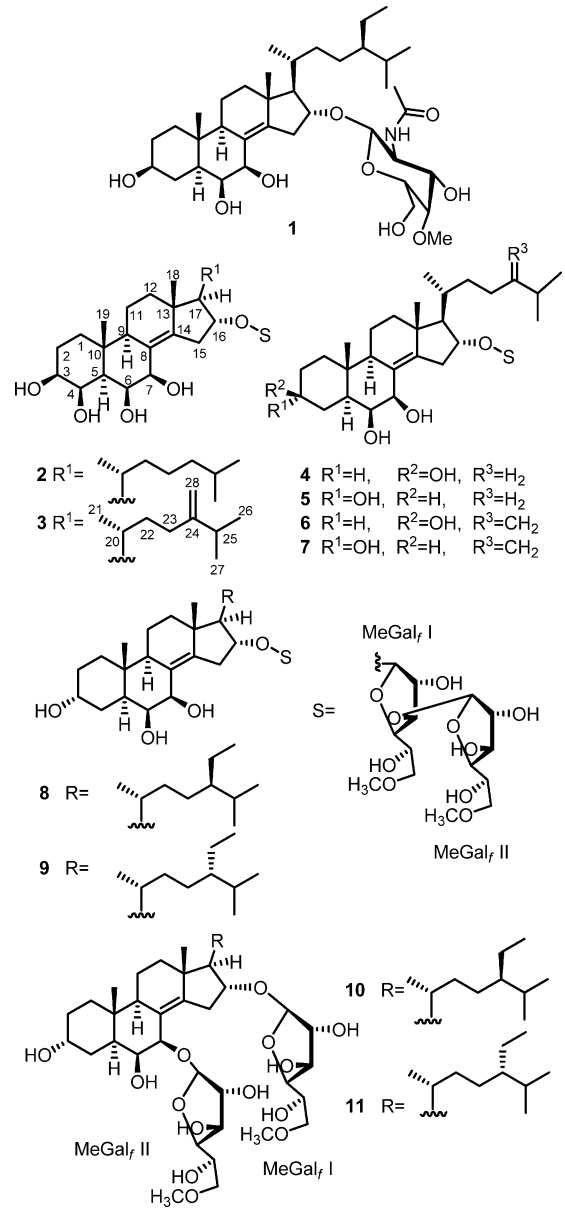
Received November 5, 2009

Ten new polyhydroxysteroidal glycosides, anthenosides B−K (**2**−**11**), were isolated from the ethanol extract of the starfish *Anthenea chinensis*. Their structures were elucidated by extensive spectroscopic studies and chemical evidence. The unprecedented carbohydrate chain 6-*O*-methyl-*β*-D-galactofuranosyl-(1→3)-(6-*O*-methyl-*β*-D-galactofuranose) was present in all the compounds except compounds **10** and **11**. Compounds **5**, **7**, a mixture of **8** and **9**, and a mixture of **10** and **11** showed inhibitory activity against human tumor K-562 and BEL-7402 cells. Furthermore, the mixture of **10** and **11** also exhibited cytotoxicity against human tumor U87MG cells and promoted tubulin polymerization.

Steroidal glycosides from starfish have been subdivided into three main groups: asterosaponins, cyclic glycosides, and glycosides of polyhydroxysteroids. These compounds are the predominant metabolites of starfish and responsible for their general toxicity.[1] However, few chemical studies have been reported on the starfish *Anthenea chinensis*, which is an abundant starfish distributed in the South China Sea and the East China Sea. In the course of searching for new bioactive compounds from starfish, we found that the EtOH extract of *A. chinensis* showed weak activity to promote polymerization of tubulin ($P_e = 21\%$ and $P_k = 78\%$ at 10 μg/mL).[2] An investigation of the bioactive fraction led to the isolation of 11 polyhydroxysteroidal glycosides. Their structures were elucidated by extensive spectroscopic studies and chemical evidence. One of them, anthenoside A (**1**), has been recently reported in a Letter.[3] We report herein the extraction, isolation, and structural elucidation of the other 10 new compounds, **2**−**11**, named anthenosides B−K, as well as the biological activity of anthenosides E (**5**) and G (**7**), a mixture of anthenosides H (**8**) and I (**9**), and a mixture of anthenosides J (**10**) and K (**11**).

An ethanolic extract of *A. chinensis* was suspended in H_2O and partitioned successively with petroleum ether and *n*-BuOH. The *n*-BuOH extract was subjected to several chromatographic purification steps to afford **2**−**11**.

Anthenoside B (**2**), a colorless, crystalline powder, gave an $[M + Na]^+$ pseudomolecular ion at *m/z* 825.4611 by HRESITOFMS, which was consistent with the molecular formula $C_{41}H_{70}O_{15}Na$. The ^1H NMR spectrum contained signals for five methyl groups, consistent with the sterol carbon skeleton (Table 1): singlets at δ_H 0.92 and 1.16 (H_3-18 and H_3-19), a doublet at δ_H 1.04 (H_3-21), and a pair of doublets at δ_H 0.90 (H_3-26 and H_3-27). Standard NMR analysis using a combination of ^1H, ^{13}C, and DEPT experiments quickly established that **2** was a steroidal glycoside containing signals with two acetal methines (δ_H 4.95, δ_C 107.7 and δ_H 5.04, δ_C 109.4) and one tetrasubstituted double bond (δ_C 128.3 and 147.2). The assignments of the NMR signals associated with the aglycone moiety (Table 1) were derived from $^1H-^1H$ COSY, TOCSY, HSQC, and HMBC experiments, as shown in Figure 1. The double bond could be located only at the $\Delta^{8(14)}$ position, which was identified by the cross-peaks in the HSQC and HMBC spectra (Figure 1). The HMBC correlations between the olefinic quaternary carbon C-8 at δ_C 128.3 and the allylic oxymethine protons H-6 (δ_H 3.97, m) and H-7 (δ_H 4.23, d, $J = 2.0$ Hz), as well as between C-14 at δ_C 147.2 and the methyl protons H_3-18 (δ_H 0.92, s) and H-7 (δ_H 4.23, d, $J = 2.0$ Hz), allowed us to locate the double bond



* Corresponding authors. (H.-F.T.) Tel and Fax: +86-29-84775471. E-mail:tanghaifeng71@163.com. (F.Q.) Tel and Fax: +86-24-23986463. E-mail:fengqiu2000@tom.com.
[†] Fourth Military Medical University.
[‡] Shenyang Pharmaceutical University.
[§] Second Military Medical University.

10.1021/np9007188
Published on Web 02/25/2010

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for Anthenoside B (**2**)[a] (δ in ppm)

	in CD_3OD		in pyridine-d_5	
position	δ_C (125 MHz), mult.	δ_H (500 MHz, *J* in Hz)	δ_C (150 MHz)	δ_H (600 MHz, *J* in Hz)
1	33.8, CH_2	α 1.35, m; *β* 1.56, m	33.6	α 1.62, m; *β* 2.19, br t (11.8)
2	25.1, CH_2	α 2.02, m; *β* 1.55, m	25.4	α 2.53, br t (11.8); *β* 2.01, br d (12.3)
3	70.9, CH	3.75, m	70.2	4.46, m
4	78.3, CH	3.90, br s	78.4	4.69, m
5	41.0, CH	2.02, br s	40.7	3.05, br s
6	79.5, CH	3.97, m	79.4	4.78, m
7	71.4, CH	4.23, d (2.0)	70.8	4.86, d (2.0)
8	128.3, qC		129.3	
9	46.4, CH	2.22, br t (8.5)	45.7	2.86, br t (8.4)
10	38.4, qC		38.3	
11	18.9, CH_2	α 1.61, m; *β* 1.54, m	17.7	α 1.72, m; *β* 1.40, m
12	37.1, CH_2	α 1.25, m; *β* 1.83, br d (12.0)	35.9	α 1.09, m; *β* 1.79, m
13	44.8, qC		43.5	
14	147.2, qC		144.0	
15	33.8, CH_2	α 2.44, dd (17.0, 3.0) *β* 2.94, ddd (17.0, 9.0, 3.0)	33.5	α 2.82, dd (16.8, 4.8) *β* 3.10, ddd (16.8, 8.4, 6.0)
16	77.3, CH	4.46, td (9.0, 5.0)	76.0	4.72, m
17	62.8, CH	1.48, dd (9.5, 5.0)	61.4	1.49, dd (9.6, 3.6)
18	20.2, CH_3	0.92, s	19.6	1.02, s
19	17.8, CH_3	1.16, s	18.1	1.78, s
20	32.9, CH	1.67, m	31.5	1.74, m
21	21.8, CH_3	1.04, d (6.5)	21.4	1.21, d (6.6)
22	35.3, CH_2	1.35, m; 1.60, m	34.1	1.79, m; 1.67, m
23	26.7, CH_2	1.22, m; 1.50, m	33.5	2.19, m; 1.63, m
24	40.7, CH_2	1.23, m	39.5	1.36, m
25	29.4, CH	1.56, m	28.3	1.64, m
26	23.3, CH_3	0.90, d (7.0)	22.8	0.93, d (7.8)
27	23.2, CH_3	0.90, d (7.0)	22.6	0.93, d (7.8)
MeGal$_f$ I				
1′	107.7, CH	4.95, d (1.0)	107.6	5.48, br s
2′	83.7, CH	4.03, m	80.8	4.68, m
3′	82.2, CH	4.04, m	83.7	5.02, dd (3.0, 4.8)
4′	84.3, CH	4.15, dd (6.5, 3.5)	84.3	4.74, dd (6.0, 3.6)
5′	69.8, CH	4.01, m	69.6	4.66, m
6′	75.8, CH_2	3.51, d (7.0)	75.5	3.98, m
6′-OCH_3	59.4, CH_3	3.38, s	58.7	3.47, s
MeGal$_f$ II				
1″	109.4, CH	5.04, d (2.0)	109.2	5.88, d (1.5)
2″	83.6, CH	3.97, m	83.6	4.91, m
3″	78.2, CH	4.02, m	77.8	5.06, m
4″	84.1, CH	3.88, dd (7.0, 3.0)	84.1	4.70, m
5″	70.0, CH	3.83, m	68.8	4.53, m
6″	75.4, CH_2	3.49, d (7.0)	75.4	3.94, m
6″-OCH_3	59.5, CH_3	3.39, s	58.6	3.40, s

[a] Assignments aided by ^{1}H−^{1}H COSY, TOCSY, DEPT, HSQC, and HMBC experiments.

between the C-8 and C-14 positions. The relative configuration of the aglycone was elucidated by analysis of NOESY data and coupling constants in both CD_3OD and pyridine-d_5 (Figure 1). In the CD_3OD NOESY spectrum, the NOE correlation of H-5 with H-9 indicated the A/B-*trans* ring fusion. The α configuration for H-7 was deduced from the cross-peaks H-7/H-9 and H-5/H-7. The coupling constant $J_{H\text{-}6/H\text{-}7}$ (2.0 Hz), which suggested the ax/eq coupling relationship between H-6 and H-7, was strongly indicative of the α configuration for H-6. The correlation between H-16 and Me-18 indicated the axial orientation of H-16, so 16-OH was confirmed with the α configuration. There was no NOE correlation between H-17 and Me-18, which was strongly indicative of the α configuration for H-17. The 20*R* configuration was assigned on the basis of the NOE cross-peak Me-18/H-20 and the large coupling constant $J_{H\text{-}17/H\text{-}20}$ (9.5 Hz), which suggested the *anti* relationship between H-17 and H-20. On the other hand, a NOE in pyridine-d_5 was observed between H-3 and H-5, which indicated the axial orientation of H-3. The NOE cross-peaks H-4/H-6, 4-OH/CH_3-19, and 6-OH/CH_3-19 indicated the *β* configuration of 4-OH and 6-OH. Therefore, the structure of the aglycone was established as (20*R*)-5α-cholest-8(14)-en-3*β*,4*β*,6*β*,7*β*,16α-pentol. Additional signals were attributed to two hexose units and two methoxy groups (δ_C 59.4 and 59.5, in CD_3OD).

The sugar moiety of **2** was determined to be galactose or its derivative by methanolysis followed by GC-MS analysis of the corresponding methylated hydrolysate comparing with those of the authentic samples prepared in the same manner.[4] After demethylation followed by hydrolysis of **2** with 1 N HCl, the hydrolysate was trimethylsilated, and GC retention times of the sugar were compared with D-galactose and L-galactose prepared in the same manner; the two carbohydrate units were confirmed as the common D-configurations.[5] The ^{1}H NMR spectrum showed signals for two anomeric protons at δ_H 4.95 and 5.04, each a doublet with $J_{H\text{-}1/H\text{-}2}$ = 1.0 and 2.0 Hz, which were correlated in the HSQC experiment with the corresponding carbons at δ_C 107.7 and 109.4, respectively. The signals of the carbohydrate chain were assigned on the basis of ^{1}H−^{1}H COSY, TOCSY, HSQC, and HMBC spectra. The presence of the methoxy groups at C-6′ and C-6″ was indicated by

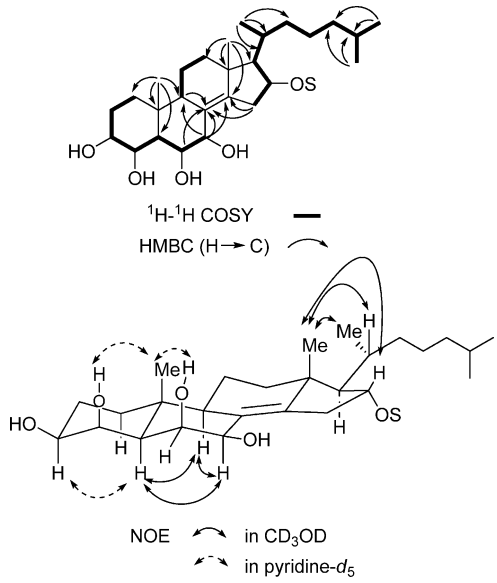


Figure 1. Key correlations observed in the ^{1}H−^{1}H COSY, HMBC, and NOESY spectra of anthenoside B (**2**).

the HMBC correlations from the methyl protons at δ_H 3.38 and 3.39 to C-6′ and C-6″ at δ_C 75.8 and 75.4, respectively. Data from the above experiments (all carbon chemical shifts were downfield relative to the shifts in galactopyranose, such as $\delta_{C\text{-}1} < 105$ and $\delta_{C\text{-}2 \text{ and } 4} < 80$ in galactopyranose) indicated that the two sugar residues were in their furanose forms, and they had similar structures by comparing their NMR data and the results of the GC-MS analysis. Furthermore, all the signals of the sugar residues were assigned by analysis of the ^{13}C NMR spectrum and by comparison with known compounds with a galactofuranose (Gal*f*) such as antarcticoside I,[6a] which was isolated from the Antarctic starfish of the family Echinasteridae. These data indicated that **2** had two 6-*O*-methyl-*β*-galactofuranosyl residues, MeGal*f* I and MeGal*f* II (Table S1, Supporting Information, $\delta_{C\text{-}6'}$ 75.8 and $\delta_{C\text{-}6''}$ 75.4 vs $\delta_{C\text{-}6}$ 64.8 in Gal*f*, and $\delta_{C\text{-}5'}$ 69.8 and $\delta_{C\text{-}5''}$ 70.0 vs $\delta_{C\text{-}5}$ 72.6 in Gal*f*). The structure of the carbohydrate chain of **2** was determined by an HMBC spectrum, which showed a cross-peak between C-16 of the aglycone and H-1 of MeGal*f* I, indicating that MeGal*f* I was connected to C-16 of the aglycone, and the linkage of the terminal MeGal*f* II at C-3 of MeGal*f* I was indicated by cross-peak MeGal*f* II H-1/MeGal*f* I C-3. On the basis of these findings, the structure of **2** was elucidated as (20*R*)-16-*O*-[6-*O*-methyl-*β*-D-galactofuranosyl-(1→3)-(6-*O*-methyl-*β*-D-galactofuranosyl)]-5α-cholest-8(14)-en-3*β*,4*β*,6*β*,7*β*,16α-pentol. Polyhydroxysteroidal glycosides with a 3*β*,4*β*,6*β*,7*β*,16α-pentol have not been previously reported. Moreover, only a few polyhydroxysteroidal glycosides with Gal*f* or MeGal*f* sugars have been identified,[6] and the disaccharide chain of MeGal*f* II(1→3)MeGal*f* I has not been reported. The linkage of the saccharide chain at C-16 of the aglycone has not been observed before in starfish.

The sugars of the other compounds **3**−**9** were also determined to be galactose or its derivative by methanolysis followed by GC-MS analysis of the corresponding methylated hydrolysate prepared in the same manner as for **2**. A comparison of the NMR data of the carbohydrate chain of **2**−**9** showed that compounds **2**−**9** had the same disaccharide chain, 6-*O*-methyl-*β*-D-galactofuranosyl-(1→3)-(6-*O*-methyl-*β*-D-galactofuranose).

The molecular formula of anthenoside C (**3**) was established as $C_{41}H_{70}O_{14}$ on the basis of the HRESITOFMS and ^{13}C NMR spectroscopic data (*m*/*z* 837.4617 [M + Na]$^+$). The ^{1}H, and ^{13}C NMR and DEPT spectra of **3** as well as an HSQC experiment revealed mainly the presence of one tetrasubstituted double bond (δ_C 128.4 and 147.1), two quaternary sp^3 carbons (δ_C 38.4 and 46.4), one terminal double bond (δ_C 157.6, 107.2, and $\delta_{H\text{-}28}$ 4.77, 4.74), two acetal methines (δ_C 107.6, δ_H 4.96 and δ_C 109.4, δ_H 5.03), and five methyl groups (Tables 2 and 3). Comparing the NMR data of compounds **3** and **2** revealed a close similarity and indicated that both have the same steroidal skeleton, with the only difference being in the side chain. The chemical shift of C-24 in **3** was shifted downfield to δ_C 157.6, indicating that the terminal double bond was at C-24. This deduction was further supported by HSQC and HMBC spectra. HMBC correlations from H_2-28 to C-23, C-24, and C-25 and from H-25 to C-24 and C-28 could be observed in Figure 2. Thus, the structure of **3** was elucidated as (20*R*)-16-*O*-[6-*O*-methyl-*β*-D-galactofuranosyl-(1→3)-(6-*O*-methyl-*β*-D-galactofuranosyl)]-24-methyl-5α-cholest-8(14),24(28)-diene-3*β*,4*β*,6*β*,7*β*,16α-pentol.

Anthenoside D (**4**) was isolated as a colorless, crystalline powder. The exact mass of the [M + Na]$^+$ ion (*m*/*z* 809.4661) matched well with the calculated mass for $C_{41}H_{70}O_{14}Na$. The ^{1}H and ^{13}C NMR and DEPT spectra as well as an HSQC experiment displayed resonances due to one tetrasubstituted double bond (δ_C 128.3 and 147.2), two quaternary sp^3 carbons (δ_C 38.1 and 44.9), two acetal methines (δ_C 107.7, δ_H 4.95 and δ_C 109.4, δ_H 5.04), and five methyl groups (Tables 2 and 3). The assignments of the NMR signals associated with **4** were derived from ^{1}H−^{1}H COSY, TOCSY, HSQC, and HMBC experiments. The aglycone was determined to be (20*R*)-5α-cholest-8(14)-en-3*β*,6*β*,7*β*,16α-tetrol, whose relative configuration was confirmed by the NOESY spectrum as shown in Figure S1, Supporting Information. In the NOESY spectrum, the NOE correlation of H-5 with H-9 indicated the A/B-*trans* ring fusion. The NOE cross-peaks H-7/H-9 and H-5/H-7 indicated the α configuration of H-7. The coupling constant $J_{H\text{-}6/H\text{-}7}$ (2.5 Hz) was strongly indicative of the α configuration for H-6. The NOE correlations of H-3 with H_{ax}-1 and H-5 indicated the *β* configuration for 3-OH, and this deduction for 3*β*-OH was confirmed by comparing the carbon chemical shifts for the A ring of **4** with those for certonardosterol E isolated from the starfish *Certonardoa semiregularis*[7] and comparing the H-3 and C-3 NMR data for **4** with those for other 3-OH steroidal compounds having A/B-*trans* or A/B-*cis* connection of the rings (Tables S2 and S3, Supporting Information). There was no NOE correlation between H-17 and Me-18, which was strongly indicative of the α configuration for H-17. The NOE correlation of H-16 with Me-18 indicated the α configuration for 16-OH. The 20*R* configuration was assigned on the basis of the NOE cross-peak Me-18/H-20 and the large coupling constant $J_{H\text{-}17/H\text{-}20}$ (9.0 Hz) that suggested the *anti* relationship between H-17 and H-20. Accordingly, compound **4** was established as (20*R*)-16-*O*-[6-*O*-methyl-*β*-D-galactofuranosyl-(1→3)-(6-*O*-methyl-*β*-D-galactofuranosyl)]-5α-cholest-8(14)-en-3*β*,6*β*,7*β*,16α-tetrol.

Anthenoside E (**5**) gave a pseudomolecular ion peak at *m*/*z* 809.4660 [M + Na]$^+$ ($C_{41}H_{70}O_{14}Na$) in the HRESITOFMS spectrum. The ^{1}H and ^{13}C NMR and DEPT spectra of **5** as well as an HSQC experiment revealed the presence of one tetrasubstituted double bond (δ_C 128.4 and 147.1), two quaternary sp^3 carbons (δ_C 38.7 and 44.8), two acetal methines (δ_C 107.7, δ_H 4.95 and δ_C 109.3, δ_H 5.05), and five methyl groups (Tables 2 and 3). The ^{1}H and ^{13}C NMR signals of **5** were assigned by the application of 2D NMR experiments including ^{1}H−^{1}H COSY, TOCSY, HSQC, and HMBC. The NMR spectroscopic data showed that **4** and **5** had the same steroidal aglycone except for the configuration for the 3-OH ($\delta_{C\text{-}3}$: 72.5 vs 67.4 in **5**). In the NOESY spectrum, the NOE correlation from H-3 to H-5 was not observed, which suggested that the configuration for 3-OH should be α. This proposal was confirmed by the width of the multiplet of the H-3 signal (about 10 Hz for **5**, while the corresponding value was approximate 35 Hz for **4**). Thus, the structure of **5** was defined as (20*R*)-16-*O*-[6-*O*-methyl-*β*-D-galactofuranosyl-(1→3)-(6-*O*-methyl-*β*-D-galactofuranosyl)]-5α-cholest-8(14)-en-3α,6*β*,7*β*,16α-tetrol.

Table 2. ^{13}C NMR Spectroscopic Data (125 MHz, CD_3OD) for Anthenosides C−K (**3**−**11**)[a] (δ in ppm)

position	**3**	**4**	**5**	**6**	**7**	mixture of **8** and **9**	mixture of **10** and **11**
1	33.8	39.3	34.6	39.3	34.6	34.6	34.5
2	25.1	32.2	29.5	32.3	29.5	29.6	29.7
3	70.9	72.5	67.4	72.5	67.4	67.4	67.5
4	78.2	35.7	33.4	35.8	33.4	33.5	33.3
5	41.0	43.3	37.7	43.3	37.7	37.7	38.0
6	79.5	77.1	77.4	77.2	77.3	77.2	75.2
7	71.4	71.9	72.0	72.0	72.0	72.1	78.4
8	128.4	128.3	128.4	128.4	128.4	128.4	127.0
9	46.4	45.6	45.6	45.7	45.7	45.7	45.9
10	38.4	38.1	38.7	38.1	38.7	38.7	38.8
11	18.9	19.8	19.4	19.9	19.5	19.5	19.5
12	37.1	37.2	37.2	37.3	37.2	37.2	37.2
13	44.8	44.9	44.8	44.9	44.9	44.8	45.0
14	147.1	147.2	147.1	147.2	146.9	146.9	147.3
15	33.8	33.9	33.8	33.9	33.8	33.8	33.8
16	77.3	77.4	77.3	77.6	77.4	77.3	77.5
17	62.8	62.9	62.8	63.0	62.8	62.8	63.1
18	20.2	20.2	20.2	20.3	20.3	20.3	20.2
19	17.8	16.3	15.4	16.3	15.4	15.4	15.4
20	32.7	33.0	32.9	32.8	32.7	33.3	33.6
21	21.7	21.8	21.8	21.6	21.7	21.9 22.0	21.6 21.8
22	33.6	35.4	35.4	33.7	33.6	33.0 33.1	33.1 33.2
23	33.6	26.7	26.7	33.8	33.6	29.3 29.5	29.4 29.6
24	157.6	40.7	40.7	157.8	157.6	47.1 47.2	47.0 47.2
25	35.0	29.4	29.4	35.1	35.0	30.3 30.2	30.3 30.1
26	22.6	23.3	23.3	22.6	22.6	20.1	20.0
27	22.4	23.2	23.2	22.4	22.4	19.6 19.5	19.5 19.4
28	107.2			107.2	107.2	24.1 24.0	24.2 24.0
29						12.6 12.7	12.4 12.7
MeGal$_f$ I							
1′	107.6	107.7	107.7	107.8	107.6	107.6	107.7
2′	83.8	83.8	83.7	83.9	83.8	83.7	83.6
3′	82.1	82.2	82.2	82.1	82.1	82.1	78.3
4′	84.4	84.4	84.4	84.5	84.4	84.3	84.5
5′	70.0	69.9	69.8	70.0	69.9	69.9	70.2
6′	75.9	75.8	75.8	75.9	75.9	75.8	75.9
6′-OCH_3	59.4	59.4	59.3	59.4	59.4	59.4	59.3
MeGal$_f$ II							
1″	109.4	109.4	109.3	109.5	109.4	109.3	108.5
2″	83.6	83.6	83.6	83.6	83.6	83.6	83.3
3″	78.3	78.3	78.3	78.5	78.3	78.3	78.8
4″	84.1	84.1	84.1	84.4	84.1	84.1	84.3
5″	69.9	70.0	70.0	70.2	70.0	70.0	70.8
6″	75.4	75.4	75.3	75.4	75.4	75.3	75.6
6″-OCH_3	59.4	59.5	59.4	59.4	59.4	59.4	59.3

[a] Assignments aided by $^1H-^1H$ COSY, TOCSY, DEPT, HSQC, and HMBC experiments.

Positive HRESITOFMS analysis of anthenoside F (**6**) exhibited a quasimolecular ion peak at *m*/*z* 821.4667 $[M + Na]^+$, corresponding to the molecular formula $C_{42}H_{70}O_{14}Na$. The ^1H and ^{13}C NMR signals were completely assigned by extensive 2D NMR studies. The NMR data indicated that it shared the same steroidal aglycone with **4** and the same side chain with **3** (Tables 2 and 3). Therefore, the structure of **6** was confirmed as (20*R*)-16-*O*-[6-*O*-methyl-β-D-galactofuranosyl-(1→3)-(6-*O*-methyl-β-D-galactofuranosyl)]-24-methyl-5α-cholest-8(14),24(28)-diene-3β,6β,7β,16α-tetrol.

Anthenoside G (**7**), a white crystalline powder, gave an $[M + Na]^+$ pseudomolecular ion at *m*/*z* 821.4664 by HRESITOFMS, which was consistent with the molecular formula $C_{42}H_{70}O_{14}Na$. The NMR data (Tables 2 and 4) showed that **6** and **7** had a similar structure except for the configuration for the 3-OH. Comparing the NMR data for the A ring of **7** with **5** showed that they shared the same A ring. Accordingly, the structure of **7** was concluded to be (20*R*)-24-methyl-16-*O*-[6-*O*-methyl-β-D-galactofuranosyl-(1→3)-(6-*O*-methyl-β-D-galactofuranosyl)]-5α-cholest-8(14),24(28)-diene-3α,6β,7β,16α-tetrol.

Compounds **8** and **9** could not be separated by repeated reversed-phase HPLC. They have the same molecular formula, $C_{43}H_{74}O_{14}$, deduced by a HRESITOFMS spectrum on the pseudomolecular ion peak at *m*/*z* 837.4979 $[M + Na]^+$. The ^1H and ^{13}C NMR spectra of the mixture clearly indicated that they had the same steroidal aglycone nucleus as **5**, with the only difference being in the side chain. The assignments of the NMR signals associated with the side chains of **8** and **9** were derived from $^1H-^1H$ COSY, TOCSY, HSQC, and HMBC experiments. The location of the ethyl at C-24 was further confirmed by HMBC correlations from Me-29 to C-25 and C-28. The complexity of the methyl region of ^{13}C NMR data clearly showed the presence of an epimeric mixture with 24*R* ethyl and 24*S* ethyl groups. The chemical shifts of the side chain carbons for both epimers were compared with the corresponding ^{13}C NMR signals of authentic commercial sitosterol and clionasterol to establish the configuration at C-24. It was known that the ^1H NMR data of the side chain of the epimeric mixture of related steroids in CD_3OD were very similar, but in $CDCl_3$ or pyridine-d_5 the ^{13}C NMR data had greater variation, such as the chemical shift at C-29 of the 24*S* epimer being more deshielded than that in the 24*R* epimer, and the chemical shifts at C-25, C-26, and C-28 being more shielded than those in the 24*R* epimer.[8] Diagnostic differences of the epimeric mixture of **8** and **9** were noted for the chemical shift values of the C-21, C-22, C-23, C-24, C-25, C-27, C-28, and C-29 signals. Therefore, the structures of the epimeric mixture of **8** and **9** were defined as (20*R*,24*R*)-16-*O*-[6-*O*-methyl-β-D-galactofura-

Table 3. ^{1}H NMR Spectroscopic Data (500 MHz, CD_3OD) for Anthenosides C−F (**3**−**6**)[a] (δ in ppm, J in Hz)

position	3	4	5	6
1	α 1.33, m;	α 1.15, m;	α 1.57, m;	α 1.13, m;
	β 1.55, m	β 1.63, m	β 1.33, m	β 1.61, m
2	α 2.02, m;	α 1.72, m;	1.63, m	α 1.78, m;
	β 1.55, m	β 1.38, m		β 1.36, m
3	3.75, m	3.60, m	4.10, m	3.60, m
4	3.89, m	α 1.73, m;	α 1.41, m; β 1.98, td (13.5, 2.5)	α 1.72, m;
		β 1.57, m		β 1.57, m
5	2.02, br s	1.70, m	2.17, br d (13.5)	1.69, m
6	3.99, m	3.60, m	3.55, br t (2.0)	3.60, m
7	4.23, d (2.0)	4.27, d (2.5)	4.29, d (2.0)	4.27, d (2.5)
9	2.23, m	2.15, br t (8.0)	2.26, br t (8.0)	2.15, br t (8.0)
11	α 1.61, m;	α 1.58, m;	α 1.67, m;	α 1.58, m;
	β 1.55, m	β 1.66, m	β 1.57, m	β 1.65, m
12	α 1.24, m;	α 1.83, br d (12.0);	α 1.26, m;	α 1.83, br d (12.0);
	β 1.83, br d (12.0)	β 1.24, m	β 1.82, br d (12.5)	β 1.25, m
15	α 2.45, dd (17.0, 3.0)	α 2.43, dd (17.0, 3.0)	α 2.44, dd (17.0, 3.0)	α 2.44, dd (17.0, 3.0)
	β 2.95, ddd (17.0, 9.0, 3.0)	β 2.95, ddd (17.0, 9.0, 3.0)	β 2.94, ddd (17.0, 9.0, 3.0)	β 2.96, ddd (17.0, 9.0, 3.0)
16	4.47, td (9.0, 5.0)	4.45, td (9.0, 5.0)	4.45, td (9.0, 5.0)	4.47, td (9.0, 5.0)
17	1.50, dd (9.0, 5.0)	1.45, dd (9.0, 5.0)	1.48, dd (9.0, 5.0)	1.49, dd (9.0, 5.0)
18	0.92, s	0.92, s	0.93, s	0.92, s
19	1.16, s	0.86, s	0.85, s	0.86, s
20	1.68, m	1.65, m	1.66, m	1.68, m
21	1.07, d (7.0)	1.04, d (7.0)	1.04, d (7.0)	1.07, d (7.0)
22	1.46, m; 1.81, m	1.35, m; 1.61, m	1.35, m; 1.61, m	1.46, m; 1.78, m
23	2.26, m; 1.98, m	1.52, m; 1.23, m	1.22, m; 1.52, m	2.25, m; 1.96, m
24		1.22, m	1.23, m	
25	2.28, m	1.57, m	1.57, m	2.27, m
26	1.06, d (6.5)	0.90, d (6.5)	0.92, d (7.0)	1.05, d (7.0)
27	1.06, d (6.5)	0.91, d (6.5)	0.92, d (7.0)	1.05, d (7.0)
28	4.77, s; 4.74, s			4.76, s; 4.74, s
MeGal$_f$ I				
1′	4.96, d (1.5)	4.95, d (1.0)	4.95, d (1.0)	4.96, d (1.0)
2′	4.01, m	4.02, m	4.03, m	4.02, m
3′	4.03, m	4.04, m	4.04, m	4.03, m
4′	4.14, dd (6.5, 3.5)	4.15, dd (6.5, 3.5)	4.15, dd (6.5, 3.5)	4.14, dd (6.0, 3.5)
5′	3.99, m	4.01, m	4.01, m	3.98, m
6′	3.50, m	3.50, m	3.51, d (7.0)	3.48, m
6′-OCH_3	3.37, s	3.38, s	3.38, s	3.37, s
MeGal$_f$ II				
1″	5.03, d (2.5)	5.04, d (2.0)	5.05, d (2.0)	5.03, d (1.5)
2″	3.96, m	3.96, m	3.97, m	3.96, m
3″	4.01, m	4.03, m	4.02, m	4.01, m
4″	3.86, dd (7.0, 3.0)	3.87, dd (7.0, 3.0)	3.88, dd (7.0, 3.0)	3.86, dd (7.0, 2.5)
5″	3.81, m	3.82, m	3.83, m	3.82, m
6″	3.48, m	3.48, m	3.49, d (7.0)	3.48, m
6″-OCH_3	3.38, s	3.39, s	3.39, s	3.38, s

[a] Assignments aided by ^{1}H−^{1}H COSY, TOCSY, DEPT, HSQC, and HMBC experiments.

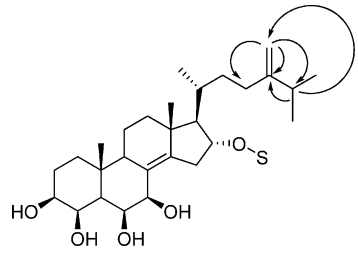


Figure 2. Key HMBC correlations of the side chain of anthenoside C (**3**).

nosyl-(1→3)-(6-*O*-methyl-β-D-galactofuranosyl)]-24-ethyl-5α-cholest-8(14)-en-3α,6β,7β,16α-tetrol (**8**) and (20*R*,24*S*)-16-*O*-[6-*O*-methyl-β-D-galactofuranosyl-(1→3)-(6-*O*-methyl-β-D-galactofuranosyl)]-24-ethyl-5α-cholest-8(14)-en-3α,6β,7β,16α-tetrol (**9**). Evaluation of the intensities of the C-29 resonances (**8**/**9** =1:8) revealed a greater proportion of the 24*S* epimer (**9**) relative to the 24*R* epimer (**8**).

Compounds **10** and **11** also could not be separated by repeated reversed-phase HPLC. Their molecular formulas were established as the same, $C_{43}H_{74}O_{14}$, on the basis of the HRESITOFMS and ^{13}C NMR spectroscopic data. The exact mass of the $[M + Na]^+$ ion (*m*/*z* 837.4973) corresponded to the molecular formula of $C_{43}H_{74}O_{14}Na$. Methanolysis of **10** and **11** and GC-MS analysis of the corresponding methylated hydrolysate compared with authentic samples afforded galactose or its derivative. The D-configuration for the galactofuranose was confirmed by analogy with the sugar configuration of **2**. Inspection of the ^{1}H and ^{13}C NMR signals and comparison of the chemical shifts with those for the mixture of **8** and **9** (Tables 2 and 4) showed that the mixture of **10** and **11** had the same sugar residues (6-*O*-methyl-β-D-galactofuranose) and the same steroidal aglycone, but the differences were at C-6, C-7, and C-8. This deduction was further supported by the HSQC, HMBC, and NOESY spectra. In the NOESY spectrum, the correlation of H-5 with H-9 indicated the A/B-*trans* ring fusion. The α configuration for H-7 was deduced from the cross-peaks H-7/H-9 and H-5/H-7, and the coupling constant $J_{H\text{-}6/H\text{-}7}$ (2.5 Hz) suggested that the configuration for H-6 was α. An HMBC experiment established the connection between the steroidal aglycone and the saccharide residues at δ_C 78.4 (C-7) and 77.5 (C-16). The linkage of MeGal$_f$ at C-7 of the aglycone was indicated by the cross-peak MeGal$_f$ II H-1/aglycone C-7 in the HMBC spectrum, and the cross-peak between C-16 of the aglycone and H-1 of MeGal$_f$ (MeGal$_f$ I) indicated that MeGal$_f$ I was connected to C-16 of the aglycone. Furthermore comparing the NMR data with those of **8** and **9**, the

Table 4. ^{1}H NMR Spectroscopic Data (500 MHz, CD_3OD) for Anthenosides G−K (**7**−**11**)[a] (δ in ppm, J in Hz)

position	**7**	mixture of **8** and **9**	mixture of **10** and **11**
1	α 1.54, m;	α 1.54, m;	α 1.54, m;
	β 1.31, m	β 1.32, m	β 1.30, m
2	1.63, m	1.63, m	1.62, m
3	4.10, m	4.10, m	4.08, m
4	α 1.41, m;	α 1.38, m;	α 1.37, m;
	β 1.98, m	β 1.97, td (13.5, 2.5)	β 1.96, td (13.5, 2.5)
5	2.18, br d (13.0)	2.16, br d (13.5)	2.12, br d (13.5)
6	3.55, br t (2.5)	3.54, br t (2.5)	3.62, br t (2.5)
7	4.28, d (2.5)	4.26, d (2.5)	4.23, d (2.5)
9	2.27, m	2.26, br t (8.0)	2.26, br t (8.5)
11	α 1.67, m;	α 1.68, m;	α 1.65, m;
	β 1.55, m	β 1.55, m	β 1.56, m
12	α 1.25, m;	α 1.24, m;	α 1.24, m;
	β 1.83, br d (12.0)	β 1.81, br d (12.5)	β 1.81, br d (12.0)
15	α 2.44, dd (17.0, 3.0)	α 2.43, dd (17.0, 3.5)	α 2.58, dd (17.0, 3.0)
	β 2.95, ddd (17.0, 8.5, 3.0)	β 2.96, ddd (17.0, 9.0, 3.5)	β 2.88, ddd (17.0, 9.0, 3.0)
16	4.47, td (8.5, 5.5)	4.48, td (9.0, 5.5)	4.48, td (9.0, 5.5)
17	1.49, dd (9.0, 4.0)	1.49, m	1.48, m
18	0.92, s	0.92, s	0.93, s
19	0.85, s	0.85, s	0.86, s
20	1.69, m	1.62, m	1.62, m
21	1.08, d (7.0)	1.05, d (7.0)	1.05, d (7.5)
22	1.46, m; 1.80, m	1.28, m; 1.64, m	1.27, m; 1.66, m
23	2.27, m; 1.96, m	1.10, m; 1.49, m	1.10, m; 1.49, m
24		1.05, m	1.04, m
25	2.29, m	1.75, m	1.75, m
26	1.05, d (7.0)	0.88, d (7.5)	0.87, d (7.0)
27	1.06, d (7.0)	0.88, d (7.5)	0.87, d (7.0)
28	4.77, s; 4.74, s	1.23, m; 1.38, m	1.22, m; 1.38, m
29		0.90, t (7.5)	0.90, t (7.5)
MeGal$_f$ I			
1′	4.96, d (1.5)	4.96, d (1.5)	4.95, d (2.0)
2′	4.02, m	4.02, m	3.96, m
3′	4.04, m	4.04, m	4.05, m
4′	4.15, dd (6.0, 3.5)	4.15, dd (6.0, 3.0)	3.88, m
5′	3.99, m	4.00, m	3.85, m
6′	3.48, m	3.51, d (6.0)	3.50, d (6.5)
6′-OCH_3	3.37, s	3.39, s	3.38, s
MeGal$_f$ II			
1″	5.04, d (2.5)	5.04, d (2.0)	4.99, d (1.5)
2″	3.97, m	3.96, m	3.91, m
3″	4.01, m	4.01, m	3.99, m
4″	3.87, dd (7.0, 2.5)	3.87, dd (7.0, 3.0)	3.89, dd (7.0, 2.5)
5″	3.83, m	3.83, m	3.84, m
6″	3.48, m	3.49, d (6.0)	3.54, d (6.0)
6″-OCH_3	3.38, s	3.39, s	3.39, s

[a] Assignments aided by ^{1}H−^{1}H COSY, TOCSY, DEPT, HSQC, and HMBC experiments.

Table 5. Cytotoxicity of Glycosides **5** and **7**, a Mixture of **8** and **9**, and a Mixture of **10** and **11** against Three Cancer Cell Lines *in Vitro* (IC_{50}, μM)[a]

cell line	**5**	**7**	mixture of **8** and **9**	mixture of **10** and **11**	positive control
K-562	6.1 ± 1.4	4.6 ± 1.1	2.8 ± 0.9	0.6 ± 0.1	0.16 ± 0.03[b]
BEL-7402	8.3 ± 1.9	5.5 ± 1.0	4.9 ± 1.5	2.7 ± 0.5	0.36 ± 0.05[b]
U87MG	>25.4	>25.1	>24.5	3.6 ± 0.9	0.84 ± 0.04[c]

[a] IC_{50} values are means from three independent experiments (average ± SD). [b] 10-Hydroxycamptothecine (HCP) as positive control. [c] Nimustine (ACNU) as positive control.

chemical shift at C-7 was shifted downfield to δ_C 78.4 (δ_C 72.1 in **8** and **9**), and the signals at C-6 and C-8 were shifted upfield to δ_C 75.2 and 127.0 ($\delta_{C\text{-}6}$ 77.2 and $\delta_{C\text{-}8}$ 128.4 in **8** and **9**), respectively, which was consistent with the linkage of MeGal$_f$ II at C-7 of the aglycone. Accordingly, compounds **10** and **11** were respectively established as (20*R*,24*R*)-7-*O*-(6-*O*-methyl-β-D-galactofuranosyl)-16-*O*-(6-*O*-methyl-β-D-galactofuranosyl)-24-ethyl-5α-cholest-8(14)-en-3α,6β,7β,16α-tetrol (**10**) and (20*R*,24*S*)-7-*O*-(6-*O*-methyl-β-D-galactofuranosyl)-16-*O*-(6-*O*-methyl-β-D-galactofuranosyl)-24-ethyl-5α-cholest-8(14)-en-3α,6β,7β,16α-tetrol (**11**). Evaluation of the intensities of C-29 resonances revealed a ratio of 1:4 for **10** and **11**, respectively, again showing a greater amount of the 24*S* epimer (**11**). Separated MeGal$_f$ residues linked at C-7 and C-16 of the aglycone simultaneously have not been reported previously.

The cytotoxic activities of glycosides **5** and **7**, a mixture of **8** and **9**, and a mixture of **10** and **11** were evaluated against human leukemia K-562, human hepatoma BEL-7402, and human spongioblastoma U87MG cell lines with camptothecin or nimustine as the positive control. The mixture of **10** and **11** exhibited significant cytotoxicity against all human tumor cell lines tested, while compounds **5** and **7** and the mixture of **8** and **9** were active against K-562 and BEL-7402 cell lines (Table 5). Furthermore, when the glycosides were tested for promotion of tubulin polymerization *in vitro*, the mixture of **10** and **11** exhibited significant activity, with

a P_e value of 29% and a P_k value of 95% at 10 μg/mL, while glycosides **5** and **7** and the mixture of **8** and **9** were inactive in promoting tubulin polymerization. By comparing the cytotoxicity and the activity for promoting tubulin polymerization *in vitro* of the mixture of **10** and **11** with glycosides **5** and **7** and the mixture of **8** and **9**, the saccharide units and their points of attachment on the aglycone may be considered to be important for promoting polymerization of tubulin and inhibiting the proliferation of the human cancer cell line U87MG. The structure−function relationships for these steroidal glycosides are the subject of ongoing studies.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. NMR spectra were recorded on Bruker AVANCE-500 and ARX-600 spectrometers, and the 2D NMR spectra were obtained using standard pulse sequences. ESIMS and HRESITOFMS were recorded on a Micromass Quattro mass spectrometer. GC-MS were performed on an Agilent 6890 GS/5973 MS apparatus with an HP-1 column (30 m × 0.32 mm i.d.) for the analyses of the methylated hydrolysates. GC was performed on a Finnigan Voyager apparatus using a l-Chirasil-Val column (25 m × 0.32 mm i.d.) for the analyses of the trimethylsilated hydrolysates. HPLC was carried out on a Dionex P680 liquid chromatograph equipped with a UV 170 UV/vis detector using a YMC-Pack C_{18} column (25 cm × 4.6 mm i.d.) and monitored at 206 nm for the semipreparation or a Thermo ODS-2 column (25 cm × 4.6 mm i.d.) and monitored at 206 nm for the purification. Column chromatographies were performed on Si gel H (10−40 μm, Qingdao Marine Chemical Inc.), Sephadex LH-20 (Pharmacia), and reversed-phase Si gel (Lichroprep RP-18, 40−63 μm, Merck Inc.). Fractions were monitored by TLC, and spots were visualized by heating Si gel plates sprayed with 20% H_2SO_4 in EtOH (v/v).

Animal Material. Specimens of *Anthenea chinensis* Gay [*Anthenea pentagonula* (Lamarck, 1816), order Valvatida, family Oreasteridae][9] were collected at a depth of 2−20 m by hand using scuba or by a fishery bottom trawler from offshore waters of the Sanya Bay in the South China Sea in October 2007 and stored in 95% EtOH at room temperature. The organisms were identified by one of the authors (H.-W.L.) of the Second Military Medical University, China. A voucher specimen was deposited in the Department of Pharmacy, Xijing Hospital, Fourth Military Medical University, Xi'an, China, under the registration code number XJ-HNHX-07.

Extraction and Isolation. The starfish (18 kg, wet weight) were soaked in 95% EtOH for 2 weeks. The material was then cut into small pieces and refluxed with 75% EtOH three times respectively for 2, 1.5, and 1 h. The combined extracts were concentrated to leave a reddish residue, which was suspended in H_2O (4 L) and then partitioned successively using petroleum ether (4 L × 3) and *n*-BuOH (4 L × 6). The *n*-BuOH fraction (78 g) was chromatographed on Si gel (1200 g) eluting with $CHCl_3$ (15 L) and $CHCl_3$/MeOH/H_2O (10:1:0.035 to 6.5:3.5:1) gradient to give nine major fractions (A−I) based on TLC analysis. Fraction F (4.15 g) was chromatographed on Si gel (200 g) with $CHCl_3$/*n*-BuOH (saturated with H_2O) (1:4) to give five fractions (F-1 to F-5). Fraction F-3 was subjected to size exclusion chromatography on a Sephadex LH-20 column equilibrated with $CHCl_3$/MeOH (1:1) to remove the pigments and impurities, then was subjected to MPLC on a Lobar column (Lichroprep RP-18) eluting with MeOH/H_2O (80:20) to give four major fractions (F-3-1 to F-3-4). The crude glycoside fraction F-3-3 (768 mg) was further purified by repeated semipreparative HPLC (YMC-Pack C_{18} column) to afford pure glycosides **2** (35.0 mg, t_R = 45.8 min), **3** (10.6 mg, t_R = 42.1 min), **4** (3.50 mg, t_R = 39.3 min), **5** (31.3 mg, t_R = 47.9 min), **6** (19.1 mg, t_R = 36.5 min), **7** (28.1 mg, t_R = 44.1 min), and a mixture of **8** and **9** (8.3 mg, t_R = 74.7 min) using MeOH/H_2O (78:22) as the mobile phase and a flow rate of 2 mL/min. Fraction E (4.28 g) was chromatographed on Si gel (200 g) with $CHCl_3$/*n*-BuOH (saturated with H_2O) (1:2.5) to give six fractions (E-1 to E-6). Fraction E-5 (0.52 g) was subjected to MPLC on a Lobar column (Lichroprep RP-18) eluting with MeOH/H_2O (80:20) and finally purified by means of HPLC eluting with MeOH/H_2O (78:22) at a flow rate of 1 mL/min to yield the mixture of **10** and **11** (12.5 mg) in 37.4 min.

Anthenoside B (2): colorless, crystalline powder; $[\alpha]^{22}_D$ −75.9 (*c* 0.170, MeOH); ^{1}H and ^{13}C NMR, see Table 1; ESIMS (positive ion mode) *m/z* 1627 [2 M + Na]$^+$, 825 [M + Na]$^+$; ESIMS (negative ion mode) *m/z* 1603 [2 M − H]$^-$, 801 [M − H]$^-$; HRESIMS (positive ion mode) *m/z* 825.4611 [M + Na]$^+$ (calcd for $C_{41}H_{70}O_{15}Na$, 825.4612).

Anthenoside C (3): colorless, crystalline powder; $[\alpha]^{22}_D$ −134 (*c* 0.085, MeOH); ^{1}H and ^{13}C NMR, see Tables 2 and 3; ESIMS (positive ion mode) *m/z* 1651 [2 M + Na]$^+$, 853 [M + K]$^+$, 837 [M + Na]$^+$; ESIMS (negative ion mode) *m/z* 1627 [2 M − H]$^-$, 813[M − H]$^-$; HRESIMS (positive ion mode) *m/z* 837.4617 [M + Na]$^+$ (calcd for $C_{42}H_{70}O_{15}Na$, 837.4612).

Anthenoside D (4): colorless, crystalline powder; $[\alpha]^{22}_D$ −38.3 (*c* 0.060, MeOH); ^{1}H and ^{13}C NMR, see Tables 2 and 3; ESIMS (positive ion mode) *m/z* 1595 [2 M + Na]$^+$, 809 [M + Na]$^+$; ESIMS (negative ion mode) *m/z* 1571 [2 M − H]$^-$, 785 [M − H]$^-$; HRESIMS (positive ion mode) *m/z* 809.4661 [M + Na]$^+$ (calcd. for $C_{41}H_{70}O_{14}Na$, 809.4663).

Anthenoside E (5): colorless, crystalline powder; $[\alpha]^{22}_D$ −115.5 (*c* 0.570, MeOH); ^{1}H and ^{13}C NMR, see Tables 2 and 3; ESIMS (positive ion mode) *m/z* 1595 [2 M + Na]$^+$, 809 [M + Na]$^+$; ESIMS (negative ion mode) *m/z* 1571 [2 M − H]$^-$, 785 [M − H]$^-$; HRESIMS (positive ion mode) *m/z* 809.4660 [M + Na]$^+$ (calcd for $C_{41}H_{70}O_{14}Na$, 809.4663).

Anthenoside F (6): colorless, crystalline powder; $[\alpha]^{22}_D$ −66.0 (*c* 0.100, MeOH); ^{1}H and ^{13}C NMR, see Tables 2 and 3; ESIMS (positive ion mode) *m/z* 1619 [2 M + Na]$^+$, 821 [M + Na]$^+$; ESIMS (negative ion mode) *m/z* 1595 [2 M − H]$^-$, 797 [M − H]$^-$; HRESIMS (positive ion mode) *m/z* 821.4667 [M + Na]$^+$ (calcd for $C_{42}H_{70}O_{14}Na$, 821.4663).

Anthenoside G (7): white, crystalline powder; $[\alpha]^{22}_D$ −123 (*c* 0.145, MeOH); ^{1}H and ^{13}C NMR, see Tables 2 and 4; ESIMS (positive ion mode) *m/z* 1619 [2 M + Na]$^+$, 821 [M + Na]$^+$; ESIMS (negative ion mode) *m/z* 1595 [2 M − H]$^-$, 797 [M − H]$^-$; HRESIMS (positive ion mode) *m/z* 821.4664 [M + Na]$^+$ (calcd for $C_{42}H_{70}O_{14}Na$, 821.4663).

Mixture of Anthenoside H (8) and Anthenoside I (9): white crystalline powder; $[\alpha]^{22}_D$ −100.8 (*c* 0.125, MeOH); ^{1}H and ^{13}C NMR, see Tables 2 and 4; ESIMS (positive ion mode) *m/z* 1651 [2 M + Na]$^+$, 853 [M + K]$^+$, 837 [M + Na]$^+$; ESIMS (negative ion mode) *m/z* 1627 [2 M − H]$^-$, 813 [M − H]$^-$; HRESIMS (positive ion mode) *m/z* 837.4979 [M + Na]$^+$ (calcd for $C_{43}H_{74}O_{14}Na$, 837.4976).

Mixture of Anthenoside J (10) and Anthenoside K (11): white, crystalline powder; $[\alpha]^{22}_D$ −109.6 (*c* 0.235, MeOH); ^{1}H and ^{13}C NMR, see Tables 2 and 4; ESIMS (positive ion mode) *m/z* 837 [M + Na]$^+$; ESIMS (negative ion mode) *m/z* 813 [M − H]$^-$; HRESIMS (positive ion mode) *m/z* 837.4973 [M + Na]$^+$ (calcd for $C_{43}H_{74}O_{14}Na$, 837.4976).

Methanolysis of 2−11.[4] Each sample (1 mg) was resolved with 2 M HCl/MeOH (0.5 mL) and heated at 80 °C for 12 h. The reaction mixture was evaporated to dryness, and the residue was partitioned between EtOAc and H_2O. The aqueous phase was concentrated under reduced pressure. Then, DMSO (0.3 mL) and 50% aqueous sodium hydroxide (0.03 mL) were added to the dried residue, and the mixture was stirred vigorously. Iodomethane (0.05 mL) was immediately added dropwise, and the mixture was further stirred for 4 h. The resulting suspension was then poured into water (0.5 mL) and extracted with CH_2Cl_2 (4 mL × 3). The CH_2Cl_2 extracts were washed with water and dried over sodium sulfate, and then concentrated under reduced pressure. The resulting products and the standard sugar derivatives prepared under the same conditions were analyzed by GC-MS with an initial temperature of 100 °C for 2 min and then temperature programming to 240 °C at a rate of 8 °C/min. The derivatives of D-glucose and D-galactose were detected with t_R of 11.91 and 12.85 min, respectively. All the compounds gave peaks of the derivative of D-galactose.

Demethylation and Acid Hydrolysis of 2 and the Mixture of 10 and 11.[5] For demethylation of **2** and the mixture of **10** and **11**, respectively, each sample (1 mg) was mixed with 1 mL of dry dichloromethane and 0.01 mL of boron tribromide at −80 °C for 30 min and then stood overnight at 10 °C under anhydrous conditions. The solvent and the reagent were evaporated to dryness *in vacuo* at room temperature. The demethylated derivative of each sample was heated with 1 mL of 2 M CF_3COOH at 120 °C for 2 h. The reaction mixture was evaporated under vacuum, and the residue was partitioned between CH_2Cl_2 and H_2O. The aqueous phase was concentrated and dissolved in 1-(trimethylsilyl)imidazole and anhydrous pyridine (0.1 mL). Then, the solution was stirred at 60 °C for 5 min and dried with a stream of N_2. The residue was partitioned between CH_2Cl_2 and H_2O. The CH_2Cl_2 layer was analyzed by GC with an initial temperature of 100 °C for 1 min and then temperature programming to 180 °C at a rate of 5 °C/min. The peaks of the derivatives of the samples were detected at 13.82 and 14.79 min (**2**) and 13.81 and 14.79 min (the mixture of **10** and **11**), respectively. Retention times for authentic

samples after being treated simultaneously with 1-(trimethylsilyl)imidazole in pyridine were 13.80 and 14.78 min (D-galactose) and 13.62 and 14.59 min (L-galactose), respectively.

Bioassays. The cytotoxicity of glycosides **5** and **7**, a mixture of **8** and **9**, and a mixture of **10** and **11** against human leukemia K-562 cells and human spongioblastoma U87MG cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay described in a previous paper.[10] The cytotoxicity against human hepatoma BEL-7402 cells was evaluated by the sulforhodamine B (SRB) protein assay following a reported method.[11] Dose−response curves were plotted for the samples, and the IC_{50} values were calculated as the concentrations of the test glycosides resulting in 50% reduction of absorption compared to the control cells. The data represented the means of three independent experiments in which each compound concentration was tested in three replicate wells. The anticancer agent 10-hydroxycamptothecine (HCP) was used as the positive control with $IC_{50} = 0.16 \pm 0.03$ μM against K-562 cells and $IC_{50} = 0.36 \pm 0.05$ μM against BEL-7402 cells, and nimustine (ACNU) was used as the positive control with $IC_{50} = 0.84 \pm 0.04$ μM against U87MG cells. Glycosides **5** and **7**, a mixture of **8** and **9**, and a mixture of **10** and **11** were screened by a high-throughput model for screening antitumor agents capable of promoting polymerization of tubulin *in vitro* as previously reported.[2] The anticancer agent paclitaxel was used as positive control with $P_e = 31\%$ and $P_k = 100\%$ at 10 μg/mL.

Acknowledgment. This work was supported by the National High-Tech Research and Development Project (863 Project, No. 2006AA09Z423) and the grant of the National Natural Science Foundation of China (No. 20502035).

Supporting Information Available: Comparison of ^{13}C NMR data of the sugar in anthenoside B (**2**) with antarcticoside I (Table S1), comparison of ^{13}C NMR data of ring A in anthenoside D (**4**) with certonardosterol E (Table S2), H-3 and C-3 NMR data for several 3-OH steroidal compounds having A/B-*trans* or A/B-*cis* connection of the rings (Table S3), key NOE correlations of anthenoside D (**4**) (Figure S1), HRESITOFMS and NMR spectra of compounds **2**−**11** (Figures S2−S32), descriptions of tubulin-polymerization assay, MTT assay, and SRB assay. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Iorizzi, M.; De Marino, S.; Zollo, F. *Curr. Org. Chem.* **2001**, *5*, 951–973.
(2) Tang, H. F.; Cheng, G.; Wu, J.; Chen, X. L.; Zhang, S. Y.; Wen, A. D.; Lin, H. W. *J. Nat. Prod.* **2009**, *72*, 284–303.
(3) Ma, N.; Tang, H. F.; Qiu, F.; Lin, H. W.; Tian, X. R.; Zhang, W. *Chin. Chem. Lett.* **2009**, *20*, 1231–1234.
(4) Wang, H.; Sun, L. H.; Glazebnik, S.; Zhao, K. *Tetrahedron Lett.* **1995**, *36*, 2953–2596.
(5) (a) Araki, S.; Abe, S.; Odani, S.; Ando, S.; Fujii, N.; Satake, M. *J. Biol. Chem.* **1987**, *262*, 14141–14145. (b) De Marina, S.; Borbone, N.; Iorizzi, M.; Esposito, G.; McClintock, J. B.; Zollo, F. *J. Nat. Prod.* **2003**, *66*, 515–519.
(6) (a) Iorizzi, M.; De Marino, S.; Minale, L.; Zollo, F.; Le Bert, V.; Roussakis, C. *Tetrahedron* **1996**, *52*, 10997–11012. (b) Iorizzi, M.; Bifulco, G.; De Riccardis, F.; Minale, L.; Riccio, R.; Zollo, F. *J. Nat. Prod.* **1995**, *58*, 10–26. (c) De Marino, S.; Iorizzi, M.; Zollo, F.; Minale, L.; Amsler, C. D.; Baker, B. J.; McClintock, J. B. *J. Nat. Prod.* **1997**, *60*, 959–966. (d) Kicha, A. A.; Kalinovskii, A. I.; Stonik, V. A. *Chem. Nat. Compd.* **1989**, *25*, 569–572. (e) Riccio, R.; Minale, L.; Bano, S.; Uddin Ahmad, V. *Tetrahedron Lett.* **1987**, *28*, 2291–2294.
(7) Wang, W. H.; Li, F. M.; Park, Y. J.; Hong, J. K.; Lee, C. O.; Kong, J. Y.; Shin, S.; Im, K. S.; Jung, J. H. *J. Nat. Prod.* **2003**, *66*, 384–391.
(8) (a) Migliuolo, A.; Notaro, G.; Piccialli, V.; Sica, D. *J. Nat. Prod.* **1990**, *53*, 1414–1424. (b) Kimura, Y.; Akihisa, T.; Yasukawa, K.; Takido, M.; Tamura, T. *Chem. Pharm. Bull.* **1995**, *43*, 1813–1817.
(9) Appeltans, W.; Bouchet, P.; Boxshall, G. A.; Fauchald, K.; Gordon, D. P.; Hoeksema, B. W.; Poore, G. C. B.; van Soest R. W. M.; Stöhr, S.; Walter, T. C.; Costello, M. J., Eds. *World Register of Marine Species*; 2009, accessed at http://www.marinespecies.org.
(10) Sargent, J. M.; Taylor, C. G. *Br. J. Cancer* **1989**, *60*, 206–210.
(11) Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst.* **1990**, *82*, 1107–1112.

NP9007188