

Constituents of the Sea Cucumber *Cucumaria okhotensis*. Structures of Okhotosides B₁–B₃ and Cytotoxic Activities of Some Glycosides from this Species¹

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Three new triterpene oligoglycosides, okhotosides B₁ (**1**), B₂ (**2**), and B₃ (**3**), have been isolated from the sea cucumber *Cucumaria okhotensis* along with the known compounds frondoside A (**4**), frondoside A₁, cucumarioside A₂-5, and koreoside A. The structures of **1**–**3** were elucidated on the basis of their spectroscopic data (2D NMR and MS). Compounds **1**–**3** were moderately toxic against HeLa tumor cells. Frondoside A (**4**) showed more potent cytotoxicity against THP-1 and HeLa tumor cell lines (with IC₅₀ values of 4.5 and 2.1 $\mu\text{g/mL}$, respectively) and decreased both the AP-1-dependent transcriptional activities induced by UVB, EGF, or TPA in JB6-LucAP-1 cells and the EGF-induced NF- κ B-dependent transcriptional activity in JB6-LucNF- κ B cells at doses of about 1 $\mu\text{g/mL}$. At the same doses, it increased the p53-dependent transcriptional activity in nonactivated JB6-Lucp53 cells and inhibited the colony formation of JB6 P⁺ Cl 41 cells activated with EGF (INCC₅₀ = 0.8 $\mu\text{g/mL}$).

Professor G. Robert Pettit is noted for his outstanding work on anticancer pharmaceutical leads from new highly cytotoxic natural compounds. His group has collected marine organisms in many geographical areas of the world's oceans, including participation in two cruises on Russian research vessels together with Russian scientists. Among many other marine natural products, sea cucumber cytotoxic saponins have attracted the attention of Prof. Pettit in terms of their potential anticancer properties as early as 1976.¹

As a continuation of studies on triterpene saponins from sea cucumbers (holothurians), including those belonging to the genus *Cucumaria*,^{2–5} we report herein the structures of three new (**1**–**3**) and the identification of four previously known cytotoxic triterpene glycosides from the Sea of Okhotsk holothurian *Cucumaria okhotensis* (Cucumariidae, Dendrochirotrida, Holothurioidea) and the activity of these isolated compounds against human tumor and some other cell lines.

Results and Discussion

Sea cucumbers were collected in the Sea of Okhotsk near the Western shore of the Kamchatka Peninsula. The ethanolic extract of *C. okhotensis* was submitted sequentially to column chromatography on Polychrom-1 (powdered Teflon) and silica gel to give fractions containing mono-, di-, and trisulfated glycosides. The fractions obtained were submitted to HPLC and yielded the corresponding glycosides. As result, four compounds, namely, okhotoside B₁ (**1**), frondoside A (**4**), frondoside A₁, and cucumarioside A₂-5, from the fraction containing monosulfated glycosides, two glycosides, okhotosides B₂ (**2**) and B₃ (**3**), from the fraction with disulfated glycosides, and koreoside A from the trisulfated glycoside fraction were isolated in addition to compounds previously reported from this species.² The structures of compounds **1**–**3** were elucidated mainly by spectroscopic methods (¹H and ¹³C NMR, DEPT, HSQC, HMBC, COSY, NOESY, TOCSY, LSI-MS, and LSI- and MALDITOFMS).

Frondosides A and A₁ are previously known from *C. frondosa*,^{6,7} cucumarioside A₂-5 is known from *C. conicospermium*,⁸ and koreoside A is known from *C. koraiensis*,⁹ respectively.

The ¹³C NMR spectrometric data of the aglycon parts of new glycosides **1**–**3** were shown to be identical to one another (Table 1) and coincident with those of cucumarioside A₀-2 isolated first from the sea cucumber *Cucumaria japonica*¹⁰ and found later in the glycosides of many other species of sea cucumbers.^{2,11–13} The common aglycon of **1**–**3** belongs to the holostane type [from the signals of the 18(20)-lactone at δ 179.3 (C-18) and 84.8 ppm (C-20) in the ¹³C NMR spectrum] and contains the 7(8)-double bond [the signals of a tertiary carbon (C-7) at 120.1 ppm and a quaternary carbon (C-8) at 145.5 ppm in the ¹³C NMR and DEPT spectra] as well as the 16 β -acetoxy group [the signals of the carbonyl group (C-16) at 74.9 ppm, quaternary carbon (OCOCH₃) at 169.9 ppm, and the methyl carbon (OCOCH₃) at 21.2 ppm in the ¹³C NMR and DEPT spectra]. In the side chain, a 25(26)-double bond [from signals of C-25 at 145.3 ppm and C-26 at 110.6 ppm] was indicated (Table 1). The structure of the aglycon of okhotosides B₁–B₃ (**1**–**3**) as 16 β -acetoxyholosta-7,25-dien-3 β -ol was also confirmed by the 2D NMR spectroscopic data.

The HRMALDITOFMS (positive-ion mode) of okhotoside B₁ (**1**) exhibited a pseudomolecular ion peak [M_{Na} + Na]⁺ at m/z 1269.4817, which along with the ¹³C NMR spectroscopic data allowed the determination of the molecular formula of okhotoside B₁ (**1**) as C₅₆H₈₇O₂₇Sn. The carbohydrate chain of **1** consisted of four monosaccharide residues as deduced from the ¹³C NMR spectrum, which showed the signals of four anomeric carbons at 104.2–105.5 ppm, correlated by the HSQC spectrum with the corresponding signals of anomeric protons at 4.73 (d, J = 7.2 Hz), 5.17 (d, J = 8.9 Hz), 5.19 (d, J = 8.2 Hz), and 5.26 (d, J = 7.9 Hz) ppm (Table 2). The coupling constants of the anomeric protons were indicative of a β -configuration of the glycosidic bonds in all cases.¹⁴

The ¹³C NMR and DEPT spectra of the carbohydrate moiety of **1** (Table 2) were similar to those of okhotoside A₂-1 isolated from *C. okhotensis* earlier.² In the NMR spectra of the carbohydrate chain of **1**, the signals of a xylose, two glucoses, and a 3-O-methylglucose residue were identified. All sugars of glycosides from *C. okhotensis* belong to the D-series, as established earlier.² The positions of interglycosidic linkages were deduced from NOESY and HMBC spectra, where cross-peaks between H-1 of the xylose residue and H-3

¹ Dedicated to Dr. G. Robert Pettit of Arizona State University for his pioneering work on bioactive natural products.

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Table 1. ^{13}C and ^1H NMR Chemical Shifts and HMBC and NOESY Correlations of Aglycon Moieties of the Glycosides **1–3**

position	δ_{C} mult. ^a	δ_{H} mult. (<i>J</i> in Hz) ^b	HMBC	NOESY
1	35.9 t	1.42 m		H-3
2	26.9 t	2.08 m, 1.87 m		
3	88.7 d	3.24 dd (3.9, 11.7)		H-1, H-5, H-31, H1-Xyl1
4	39.3 s			
5	47.8 d	0.97 dd (5.5, 10.0)	C: 4, 6, 10, 30	H-3, H-31
6	23.1 t	1.95 m		H-30, H-31
7	120.1 d	5.63 m		H-15, H-32
8	145.5 s			
9	46.9 d	3.46 brd (14.1)		H-19
10	35.4 s			
11	22.4 t	1.78 m 1.54 m		H-32
12	31.2 t	2.15 m 1.97 m		H-21
13	59.1 s			
14	47.3 s			
15	44.0 t	2.58 dd (7.5, 12.0) 1.74 m	C: 13, 14, 17, 32	H-7
16	74.9 d	5.93 brq (8.3)		H-32
17	54.6 d	2.77 d (9.4)	C: 12, 13, 18, 21	H-21, H-32
18	179.3 s			
19	23.8 q	1.19 s	C: 1, 5, 9, 10	H-9
20	84.8 s			
21	28.0 q	1.49 s	C: 17, 20, 22	H-12, H-17
22	38.4 t	2.38 m, 1.90 m		
23	23.1 t	1.45 m, 1.57 m		
24	38.2 t	2.00 m		
25	145.3 s			
26	110.6 t	4.81 brs	C: 24, 27	
27	22.0 q	1.70 s	C: 24, 25, 26	
30	17.2 q	1.07 s	C: 3, 4, 5, 31	H-6
31	28.6 q	1.18 s	C: 3, 4, 5, 30	H-6
32	32.0 q	1.24 s	C: 8, 13, 14, 15	H-7, H-11, H-16, H-17
OCOCH ₃	169.9 s, 21.1 q	2.04 s		

^a Recorded at 125.77 MHz in C₅D₅N/D₂O (4:1). Multiplicity by DEPT. ^b Recorded at 500 MHz in C₅D₅N/D₂O (4:1).**Table 2.** ^{13}C and ^1H NMR Data of Carbohydrate Chains of Okhotosides (**1–3**)

1			2			3		
δ_{C} mult. ^{a,b,c}		δ_{H} mult. (<i>J</i> in Hz) ^d	δ_{C} mult. ^{a,b,c}		δ_{H} mult. (<i>J</i> in Hz) ^d	δ_{C} mult. ^{a,b,c}		δ_{H} mult. (<i>J</i> in Hz) ^d
Xyl (1→C-3)								
1	105.0 d	4.73 d (7.2)	104.9 d	4.80 d (7.6)		105.0 d	4.83 d (7.8)	
2	82.7 d	4.12 m	81.1 d	4.23 dd (7.1, 8.9)		81.8 d	4.20 t (8.3)	
3	75.4 d	4.38 t (8.9)	74.9 d	4.39 t (8.8)		77.2 d	4.29 t (8.7)	
4	75.6 d	5.20 m	76.1 d	5.11 m		70.2 d	4.22 m	
5	64.2 t	4.80 m 3.77 brt (10.6)	64.0 t	4.92 dd (6.0, 12.0) 3.85 t (11.8)		66.1 t	4.36 dd (4.8, 11.0) 3.75 t (11.0)	
Glc 1 (1→2Xyl)								
1	105.2 d	5.17 d (8.9)	104.2 d	5.25 d (7.4)		104.3 d	5.29 d (7.8)	
2	76.2 d	4.01 t (10.0)	75.2 d	4.01 t (8.4)		75.2 d	4.06 t (9.3)	
3	75.5 d	4.21 t (9.1)	75.2 d	4.13 t (9.0)		75.5*d	4.13 t (9.0)	
4	80.4 d	4.40 t (9.7)	81.9 d	4.06 t (8.2)		82.0 d	4.07 t (8.5)	
5	76.5 d	3.81 m	76.0 d	3.81 m		76.1 d	3.85 m	
6	61.1 t	4.59 dd (3.1, 12.2) 4.39 m	61.2 t	4.41 brs		61.3 t	4.46 brd (9.7) 4.41 dd (4.3, 11.9)	
Glc 2 (1→4Glc 1)								
1	104.2 d	5.19 d (8.2)	104.0 d	4.99 d (8.2)		103.9 d	5.00 d (7.9)	
2	73.4 d	4.09 t (9.9)	73.5 d	3.96 t (8.5)		73.5 d	3.95 t (8.5)	
3	87.6 d	4.19 t (9.2)	85.9 d	4.27 t (9.1)		86.3 d	4.24 t (9.1)	
4	69.4 d	4.06 t (8.9)	69.2 d	3.94 t (9.0)		69.2 d	3.91 t (9.4)	
5	77.6 d	3.94 m	74.9 d	4.16 m		74.9 d	4.18 m	
6	61.7 t	4.44 dd (2.4, 11.7) 4.20 m	67.2 t	5.08 brd (10.2) 4.73 dd (5.4, 9.9)		67.3 t	5.12 brd (9.0) 4.73 dd (7.3, 11.2)	
MeGlc (1→3Glc 2)								
1	105.5 d	5.26 d (7.9)	104.4 d	5.31 d (7.8)		104.6 d	5.25 d (7.9)	
2	74.9 d	4.00 t (8.5)	74.6 d	3.97 t (9.0)		74.3 d	3.90 t (8.6)	
3	87.9 d	3.73 t (9.0)	87.0 d	3.79 m		86.3 d	3.76 t (8.9)	
4	70.4 d	4.14 t (8.9)	70.3 d	4.02 m		69.7 d	4.16 t (9.5)	
5	78.1 d	3.98 m	77.5 d	4.02 m		75.6* d	4.12 m	
6	62.0 t	4.49 dd (3.3, 11.6) 4.28 dd (5.6, 11.8)	61.7 t	4.47 brd (12.1) 4.17 m		66.9 t	5.05 brd (9.8) 4.90 dd (5.4, 11.8)	
OMe	60.6 q	3.87 s	60.7 q	3.92 s		60.5 q	3.87 s	

^a Recorded at 125 MHz in C₅D₅N/D₂O (4:1). Multiplicity by DEPT. ^b Bold = interglycosidic positions. ^c Italic = sulfate position. ^d Recorded at 500 MHz in C₅D₅N/D₂O (4:1).

(C-3) of the aglycon, H-1 of the glucose and H-2 (C-2) of the xylose residue, H-1 of the second glucose (the third monosaccharide residue) and H-4 (C-4) of the first glucose (the second monosaccharide residue) unit, and H-1 of 3-*O*-methylglucose and H-3 (C-3) of the second glucose unit, correspondingly, were observed. The sequence of

monosaccharide units in the carbohydrate chain was confirmed by (–) LSIMS data. Indeed, the spectrum indicated, besides the peak of the [M_{Na} – Na][–] ion at *m/z* 1223, peaks for fragment ions at *m/z* 1047 [M_{Na} – Na – MeGlc][–], 885 [M_{Na} – Na – MeGlc – Glc][–], and 723 [M_{Na} – Na – MeGlc – Glc – Glc][–].

sulfated at the C-6 (the third sugar unit) in the ^{13}C and ^1H NMR spectra of **3** were very close to the related signals in the spectra of koreoside A, a trisulfated glycoside isolated from the sea cucumber *Cucumaria koraiensis*.⁹ The positions of the interglycosidic linkages were corroborated by the NOESY spectrum of **3**, where the cross-peaks between H-1 of the xylose residue and H-3 of the aglycon, H-2 of the xylose and H-1 of the glucose (the second monosaccharide unit), H-4 of the same glucose and H-1 of the third monosaccharide residue (glucose), and H-1 of the terminal 3-*O*-methylglucose and H-3 of the second glucose (the third monosaccharide residue) were observed. Analogous cross-peaks were also observed in the HMBC spectrum of okhotoside B₃ (**3**). Therefore, the carbohydrate chain of okhotoside B₃ (**3**) is new and consists of four monosaccharide units, with the third and fourth ones being sulfated. Mass spectra confirmed the presence of two sulfate groups and the end position of the sulfated 3-*O*-methylglucose in the sugar part of **3**. There were peaks of fragment ions at m/z 1223 [$\text{M}_{2\text{Na}} - \text{Na} - \text{SO}_3\text{Na} + \text{H}$]⁺ and 1047 [$\text{M}_{2\text{Na}} - \text{Na} - \text{SO}_3\text{Na} - \text{MeGlc} + \text{H}$]⁺ in the (−) MALDI and (−) LSIMS. All these data indicated that okhotoside B₃ (**3**) is 3 β -*O*-[6-*O*-sodium sulfate-3-*O*-methyl- β -D-glucopyranosyl-(1→3)-6-*O*-sodium sulfate- β -D-glucopyranosyl-(1→4)- β -D-glucopyranosyl-(1→2)- β -D-xylopyranosyl]-16 β -acetylholosta-7,25-diene.

The triterpene glycosides of the sea cucumbers belonging to the genus *Cucumaria* are characterized by a species-specific set of aglycons on one hand and by a similar set of carbohydrate chains for all the studied species of the genus on the other hand.³ The pentaoside branched oligosaccharide moieties containing from one to three sulfate groups are specific for the glycosides of all *Cucumaria* spp. The sea cucumbers *Aslia* (= *Cucumaria*) *lefevrei* and *Pseudocnus* (= *Cucumaria*) *echinata*, containing glycosides quite different (having other carbohydrate chains) from the glycosides of the other representatives of genus *Cucumaria*, have been excluded from this genus.³

The situation with the glycosides of *Cucumaria okhotensis* seems to be uncommon. This sea cucumber also contained some glycosides typical for the sea cucumber belonging to the genus *Cucumaria* such as cucumarioside A₀-1,² isolated earlier from *C. japonica*, frondoside A₁ and frondoside A (**4**) previously isolated from *C. frondosa*,^{6,7} cucumarioside A₂-5 isolated from *C. conicospermium*,⁸ and koreoside A isolated from *C. koraiensis*.⁹ Glycosides having a trisulfated branched pentaoside carbohydrate chain, like koreoside A, have been isolated from all species of the genus *Cucumaria* investigated. Therefore, the presence of this glycoside undoubtedly confirms the inclusion of *C. okhotensis* to the genus *Cucumaria*.³ Although okhotoside A₂-1 isolated earlier from *C. okhotensis* and having glucose as the second monosaccharide residue is uncommon for sea cucumbers belonging to the genus *Cucumaria*, its carbohydrate chain has the same branched pentasaccharide “architecture” as in other glycosides isolated from representatives of this genus.² However, the remaining five glycosides isolated from *C. okhotensis* contained tetraoside linear carbohydrate chains instead of characteristic pentaoside branched ones. The carbohydrate chain of okhotoside A₁-1² was identical to that of frondoside A₁, the only tetraoside isolated so far from the representatives of the genus *Cucumaria*.³

The new tetraosides **1–3** contain a new carbohydrate chain, not only atypical for the glycosides from the sea cucumber belonging to the genus *Cucumaria* but also not found in other triterpene glycosides earlier. The glycosides having a glucose residue as the second monosaccharide unit in the carbohydrate chain are very rare in sea cucumber glycosides, because the majority of these contain a quinovose residue in this position.^{11,16} Moreover, okhotoside B₃ (**3**) contains sulfate groups attached to C-6 of the glucose residue (the third monosaccharide unit) and to C-6 of the 3-*O*-methylglucose residue (the terminal monosaccharide unit), while the first monosaccharide residue (xylose) is not sulfated or glycosylated at C-4.

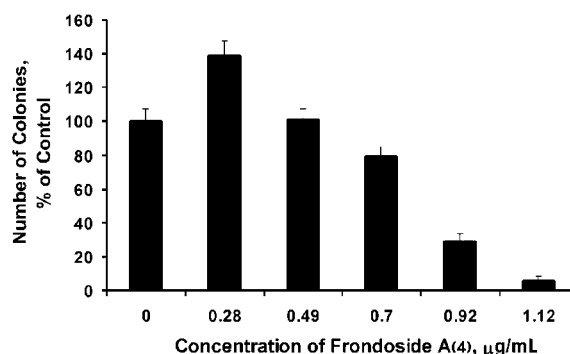


Figure 1. Frondoside A (**4**) prevents EGF-induced malignant transformation of JB6 P⁺ Cl 41 cells.

Glycoside **3** represents the second case of a sea cucumber triterpene glycoside (another example is psolusoside A from *Psolus fabricii*)¹⁷ with this structural peculiarity. The present study shows a more significant structural diversity of triterpene glycosides in sea cucumbers belonging to the genus *Cucumaria* than considered earlier.³

Okhotosides B₁–B₃ (**1–3**) and frondoside A (**4**) were studied as potential cytotoxic agents using human tumor cell lines. The following results were obtained: okhotoside B₁, IC₅₀ 34.0 µg/mL against HeLa; okhotoside B₂, IC₅₀ 13.0 µg/mL against HeLa; okhotoside B₃, IC₅₀ 17.8 µg/mL against HeLa; frondoside A, IC₅₀ of 4.5, 2.1, and 5.9 µg/mL against the THP-1, HeLa, and JB6 Cl 41 cell lines, respectively.

On a cellular level the biological action of frondoside A (**4**) was studied in more detail using JB6 Cl 41 cells and their modifications as models. The ability of this compound to influence AP-1- and NF-κB-dependent transcriptional activities was evaluated using viable JB6-LucAP-1 or JB6-LucNF-κB cells, as previously described.¹⁸ Frondoside A (**4**) decreased by 41% the UVB-induced AP-1-dependent transcriptional activity at a concentration of 0.7 µg/mL as well as at a concentration of 1.1 µg/mL by 20% and 51% the TPA-induced and EGF-induced AP-1-dependent transcriptional activities, respectively, in JB6-LucAP-1 cells. At a concentration of 0.7 µg/mL it decreased by 37% the EGF-induced NF-κB-dependent transcriptional activity in JB6-LucNF-κB cells. At a concentration of 1.8 µg/mL, it increased the p53-dependent transcriptional activity by 39% in nonactivated JB6-Lucp53 cells.

Therefore, frondoside A (**4**) influences signal transduction in living cells in the same manner as some anticancer and cancer chemopreventive agents. Moreover, when this glycoside was evaluated using JB6 P⁺ Cl 41 cells, activated with EGF (10 ng/mL) in a soft agar test, it inhibited formation of cell colonies with INCC₅₀ = 0.8 µg/mL (Figure 1). It is of special interest that frondoside inhibits JB6 Cl41 cell transformation induced by the action of EGF even at concentrations about 10-fold less than cytotoxic ones. However, even at as small a concentration as 280 ng/mL it seems to enhance colony stimulatory action of EGF.

Experimental Section

General Experimental Procedures. All melting points were determined with a Kofler-Thermogenerate apparatus. Optical rotations were measured with a Perkin-Elmer 343 polarimeter. ^1H and ^{13}C NMR spectra were recorded using a Bruker AMX 500 spectrometer at 500.12 MHz for proton and 125.67 MHz for carbon in $\text{C}_5\text{D}_5\text{N}/\text{D}_2\text{O}$ (4:1) with TMS as an internal reference ($\delta = 0$). The MALDITOFMS (positive- and negative-ion modes) were recorded using a Bruker mass spectrometer, model BIFLEX III, with impulse extraction of ions, on a 2,5-dihydroxybenzoic acid matrix. Negative LSI mass spectra were recorded on an AMD-604S double focusing mass spectrometer using glycerol as a matrix. HPLC was performed using an Agilent 1100 chromatograph equipped with a differential refractometer on Discovery C₈ (4.6 × 250) and Discovery C₁₈ (4.6 × 250) columns.

Animal Material. The specimens of *Cucumaria okhotensis* were collected at a depth of 28 m by an industrial creep from the small seine-net fishing vessel *MRS-268* in the Sea of Okhotsk near the western shore of Kamchatka (52°51'00" N, 155°56'40" E) in September 2001 and kept in ethanol at room temperature. The sea cucumber was identified by Dr. V. S. Levin, Pacific Institute Biorganic Chemistry of the Far East Division of the Russian Academy of Sciences, Vladivostok, Russia.

Extraction and Isolation. The sea cucumbers (1385 g dried residue) were cut and extracted twice with refluxing ethanol. The combined extracts were concentrated to dryness, and the residue was dissolved in water. Desalting was carried out by passing the fraction through a Polychrom-1 column (powdered Teflon, Biolar, Latvia), eluting first inorganic salts and polar impurities with H₂O and then the fraction containing glycosides with 50% ethanol. The latter fraction was submitted to sequential chromatography on silica gel columns in the solvent systems CHCl₃/EtOH/H₂O, 100:75:10, 100:100:17, and 100:125:25, to give fractions of mono-, di-, and trisulfated glycosides (673, 700, and 750 mg). Separation of the fraction containing monosulfated glycosides was carried out using column chromatography at silica gel in the solvent system CHCl₃/EtOH/H₂O (100:75:10) to give the glycosides belonging to the groups A (130 mg), A₁ (85 mg), and A₂ (94 mg).¹ The individual glycosides were isolated by HPLC using different proportions of solvents MeOH/H₂O/(Na₂HPO₄·12H₂O (358 mg) + NaH₂PO₄·2H₂O (156 mg) in 100 mL of water) as the mobile phase followed by desalting on a Polychrom-1 column, as described above. Besides okhotosides A₁-2 and A₂-1 and cucumarioside A₀-1,² reported earlier, the following compounds were obtained.

Okhotoside B₁ (1): (5.3 mg) isolated from group A of monosulfated glycosides on a C₁₈ Discovery column with the mobile phase described above in the ratio 62:36:2; mp 273–275 °C, [α]_D²⁰ –5 (c 0.1 pyridine); see Tables 1 and 2 for NMR data; (+) HRMALDITOFMS, *m/z* 1269.4817 [M_{Na} + Na]⁺, C₅₆H₈₇O₂₇SN₂, calcd 1269.4951; (+) MALDIMS, *m/z* 1269 [M_{Na} + Na]⁺, 1239 [M_{Na} – Na – CH₂O]⁺, 1167 [M_{Na} + Na – SO₃Na + H]⁺, 1149 [M_{Na} + Na – SO₃Na – CO + H]⁺; (–) MALDI MS, *m/z* 1223 [M_{Na} – Na][–]; (+) LSIMS, *m/z* 1269 [M_{Na} + Na]⁺, 1239 [M_{Na} + Na – CH₂O]⁺, 1167 [M_{Na} + Na – SO₃Na + H]⁺; (–) LSI MS, *m/z* 1223 [M_{Na} – Na][–], 1046 [M_{Na} – Na – MeGlc][–], 885 [M_{Na} – Na – MeGlc – Glc][–], 867 [M_{Na} – Na – MeGlc – Glc – H₂O][–], 723 [M_{Na} – Na – MeGlc – Glc – Glc][–].

Okhotoside B₂ (2): (7 mg) isolated from the fraction of disulfated glycosides on a C₈ Discovery column with the mobile phase described above in the ratio 60:38:2; mp 244 °C, [α]_D²⁰ –9 (c 0.1 pyridine); see Tables 1 and 3 for NMR data; (+) HRMALDITOFMS, *m/z* 1371.4428 [M_{2Na} + Na]⁺, C₅₆H₈₆O₃₀S₂Na₃, calcd 1371.4428; (+) MALDITOFMS, *m/z* 1387 [M_{2Na} + K]⁺, 1371 [M_{2Na} + Na]⁺, 1285 [M_{2Na} + K – SO₃Na + H]⁺, 1269 [M_{2Na} + Na – SO₃Na + H]⁺, 1167 [M_{2Na} + Na – 2SO₃Na + 2H]⁺; (–) MALDI TOF MS, *m/z* 1341 [M_{Na,K} – Na][–], 1325 [M_{2Na} – Na][–], 1295 [M_{2Na} – Na – CH₂O][–], 1223 [M_{2Na} – Na – SO₃Na + H][–], 1193 [M_{2Na} – Na – SO₃Na – CH₂O + H][–], 1047 [M_{2Na} – Na – SO₃Na – MeGlc + H][–], 885 [M_{2Na} – Na – SO₃Na – MeGlc – Glc + H][–], 723 [M_{2Na} – Na – SO₃Na – MeGlc – 2Glc + H][–]; LSI MS (+), *m/z* 1387 [M_{2Na} + K]⁺, 1371 [M_{2Na} + Na]⁺, 1285 [M_{2Na} + K – SO₃Na + H]⁺, 1269 [M_{2Na} + Na – SO₃Na + H]⁺, 1167 [M_{2Na} + Na – 2SO₃Na + 2H]⁺, 991 [M_{2Na} + Na – 2SO₃Na – MeGlc + 2H]⁺; (–) LSIMS, *m/z* 1341 [M_{Na,K} – Na][–], 1325 [M_{2Na} – Na][–], 1303 [M_{Na,H} – Na][–], 1223 [M_{2Na} – Na – SO₃Na + H][–], 1193 [M_{2Na} – Na – SO₃Na – CH₂O + H][–], 1047 [M_{2Na} – Na – SO₃Na – MeGlc + H][–], 1031 [M_{2Na} – Na – SO₃Na – MeGlc – O + H][–], 885 [M_{2Na} – Na – SO₃Na – MeGlc – Glc + H][–], 867 [M_{2Na} – Na – SO₃Na – MeGlc – Glc – H₂O + H][–], 723 [M_{2Na} – Na – SO₃Na – MeGlc – 2Glc + H][–].

Okhotoside B₃ (3): (6 mg) isolated from the disulfated glycosides fraction on a C₈ Discovery column with the mobile phase 60:38:2; mp 260–262 °C, [α]_D²⁰ –10 (c 0.1 pyridine); see Tables 1 and 4 for NMR data; (+) HRMALDITOFMS, *m/z* 1371.4339 [M_{2Na} + Na]⁺, C₅₆H₈₆O₃₀S₂Na₃, calcd 1371.4157; (+) MALDITOFMS, *m/z* 1387 [M_{2Na} + K]⁺, 1371 [M_{2Na} + Na]⁺, 1285 [M_{2Na} + K – SO₃Na + H]⁺, 1269 [M_{2Na} + Na – SO₃Na + H]⁺, 1167 [M_{2Na} + Na – 2SO₃Na + 2H]⁺, 991 [M_{2Na} + Na – 2SO₃Na – MeGlc + 2H]⁺; (–) MALDITOFMS, *m/z* 1341 [M_{Na,K} – Na][–], 1325 [M_{2Na} – Na][–], 1295 [M_{2Na} – Na – CH₂O][–], 1223 [M_{2Na} – Na – SO₃Na + H][–], 1193 [M_{2Na} – Na – SO₃Na – CH₂O + H][–], 1047 [M_{2Na} – Na – SO₃Na – MeGlc + H][–], 885 [M_{2Na} – Na – SO₃Na – MeGlc – Glc + H][–], 723 [M_{2Na} – Na – SO₃Na – MeGlc – 2Glc + H][–]; (–) LSIMS, *m/z*: 1341 [M_{Na,K} – Na][–], 1325 [M_{2Na} – Na][–], 1303 [M_{Na,H} – Na][–], 1223 [M_{2Na} – Na –

SO₃Na + H][–], 1193 [M_{2Na} – Na – SO₃Na – CH₂O + H][–], 1047 [M_{2Na} – Na – SO₃Na – MeGlc + H][–], 1031 [M_{2Na} – Na – SO₃Na – MeGlc – O + H][–], 885 [M_{2Na} – Na – SO₃Na – MeGlc – Glc + H][–], 867 [M_{2Na} – Na – SO₃Na – MeGlc – Glc – H₂O + H][–], 723 [M_{2Na} – Na – SO₃Na – MeGlc – 2Glc + H][–].

Frondoside A₁: (3.3 mg) isolated from the A₁ group of monosulfated glycosides on a C₁₈ Discovery column with 65:33:2 and 69:30:1 mobile phases; physical constants and ¹³C NMR spectrum were identical with literature data.⁷

Frondoside A (4): (4.4 mg) isolated from the A group of monosulfated glycosides on a C₁₈ Discovery column with the mobile phases 65:33:2 and 73:25:2; physical constants and ¹³C NMR spectrum were identical with literature data.⁶

Cucumarioside A₂-5: (3 mg) isolated from the A₂ group of monosulfated glycosides on a C₈ Discovery column with the mobile phase 62:36:2; physical constants and ¹³C NMR spectrum were identical with literature data.⁸

Koreoside A: (10 mg) isolated from the fraction of trisulfated glycosides on a C₈ Discovery column with the solvent system 46:53:1; physical constants and ¹³C NMR spectrum were identical with literature data.⁹

Tumor Cells Viability Assay. The effect of compounds 1–3 and frondoside A (4) on cell viability was evaluated using MTS reduction into its formazan product.¹⁹ The HeLa cells were cultured for 12 h in 96-well plates (6000 cells/well) in RPMI medium (100 μL/well) containing 10% FBS. Then the medium was replaced with 5% FBS-RPMI containing the indicated concentrations of the compounds, and the cells were incubated for 22 h. Next, 20 μL of the MTS reagent was added into each well, and MTS reduction was measured 2 h later spectrophotometrically at 492 and 690 nm as background using the μQuant microplate reader (Bio-Tek Instruments, Inc.). Data were obtained as the mean ± SD from six samples of two independent experiments. The statistical computer program Statistica 6.0 for Windows (StatSoft, Inc., 2001) was used to compute SD and IC₅₀ in corresponding experiments.

Effect of Frondoside A (4) on AP-1-, NF-κB-, and p53-Dependent Transcriptional Activities. JB6-LucAP-1 cells were activated with EGF (10 ng/mL) or TPA (20 ng/mL) or UVB (4 kJ/m²). JB6-LucNF-κB cells were activated with EGF (10 ng/mL). Viable activated JB6-LucAP-1 or JB6-LucNF-κB cells or nonactivated JB6-Lucp53 cells (6 × 10³) suspended in 100 μL of 5% FBS/MEM were added into each well of a 96-well plate. Plates were incubated for 24 h and then treated with various concentrations of frondoside A (4) in 100 μL of 0.1% FBS-MEM. After incubation with frondoside A (4) for 24 h, the cells were extracted for 1 h at room temperature with 100 μL/well of lysis buffer [0.1 M potassium phosphate buffer at pH 7.8, 1% Triton X-100, 1 mM dithiothreitol (DTT), 2 mM EDTA]. Then, 30 μL of lysate from each well was transferred in a plate used for luminescent analysis, and luciferase activity was measured using 100 μL/well of the luciferase assay buffer [1 mM d-luciferin, pH 6.1–6.5; 40 mM Tricin, 2.14 mM magnesium carbonate (MgCO₃)₄ × Mg(OH)₂ × 5H₂O, 5.34 mM MgSO₄ × 7H₂O, 66.6 mM DTT, 1.06 mM adenosine triphosphate, 0.54 mM coenzyme A, 0.2 mM EDTA, pH 7.8] and the Luminoscan ascent type 392 microplate reader (Labsystems). For each compound, two independent experiments with five samples for each concentration were performed.

Anchorage-Independent Soft Agar Assay. The effect of frondoside A (4) was evaluated in 6-well plates using JB6 P⁺ Cl 41 cells, activated with EGF (10 ng/mL). JB6 P⁺ Cl 41 cells (8 × 10³/mL) were treated with the indicated concentrations of the glycoside in 1 mL of 0.33% basal medium Eagle (BME) agar containing 10% FBS over 3.5 mL of 0.5% BME agar containing 10% FBS and indicated concentrations of the test compound. The cultures were maintained in a 37 °C, 5% CO₂ incubator for 1 week. Cell colonies were then scored using a Leica DM IRB inverted research microscope (Leica Mikroskopie und Systeme GmbH) and Image-Pro Plus software, version 3.0 for Windows (Media Cybernetics). For each compound, two independent experiments in triplicate for each concentration were performed. Results of the application of this method to frondoside A (4) are given in Figure 1.

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Supporting Information Available: NMR data concerning carbohydrate chains of **1–3**, including results of HMBC and NOESY experiments (Tables 1–3) as well as some 2D NMR spectra are provided free of charge at <http://pubs.acs.org>.

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