Constituents of the Sea Cucumber *Cucumaria okhotensis.* Structures of Okhotosides B_1–B_3 and Cytotoxic Activities of Some Glycosides from this Species[⊥]

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Three new triterpene oligoglycosides, okhotosides B_1 (**1**), B_2 (**2**), and B_3 (**3**), have been isolated from the sea cucumber *Cucumaria okhotensis* along with the known compounds frondoside A (**4**), frondoside A_1, cucumarioside A_2-5, 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 IC_{50} values of 4.5 and 2.1 μg/mL, respectively) and decreased both the AP-1-dependent trascriptional activities induced by UVB, EGF, or TPA in JB6-LucAP-1 cells and the EGF-induced NF-κB-dependent transcriptional activity in JB6-LucNF-kB cells at doses of about 1 μg/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 ($INCC_{50}$ = 0.8 μg/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, Dendrochirotida, 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 B_1 (**1**), frondoside A (**4**), frondoside A_1, and cucumarioside A_2-5, from the fraction containing monosulfated glycosides, two glycosides, okhotosides B_2 (**2**) and B_3 (**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 (^{1}H and ^{13}C NMR, DEPT, HSQC, HMBC, COSY, NOESY, TOCSY, LSIMS, and LSI- and MALDITOFMS).

Frondosides A and A_1 are previously known from *C. frondosa*,[6,7] cucumarioside A_2-5 is known from *C. conicospermium*,[8] and koreoside A is known from *C. koraiensis*,[9] respectively.

The ^{13}C 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 A_0-2 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 ^{13}C 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 ^{13}C NMR and DEPT spectra] as well as the 16β-acetoxy group [the signals of the carbonyl group (C-16) at 74.9 ppm, quaternary carbon ($O\underline{C}OCH_3$) at 169.9 ppm, and the methyl carbon ($OCO\underline{C}H_3$) at 21.2 ppm in the ^{13}C 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 B_1–B_3 (**1**−**3**) as 16β-acetoxyholosta-7,25-dien-3β-ol was also confirmed by the 2D NMR spectroscopic data.

The HRMALDITOFMS (positive-ion mode) of okhotoside B_1 (**1**) exhibited a pseudomolecular ion peak $[M_{Na} + Na]^+$ at *m/z* 1269.4817, which along with the ^{13}C NMR spectoscopic data allowed the determination of the molecular formula of okhotoside B_1 (**1**) as $C_{56}H_{87}O_{27}SNa$. The carbohydrate chain of **1** consisted of four monosaccharide residues as deduced from the ^{13}C 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 ^{13}C NMR and DEPT spectra of the carbohydrate moiety of **1** (Table 2) were similar to those of okhotoside A_2-1 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

[⊥] 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 ^{1}H NMR Chemical Shifts and HMBC and NOESY Correlations of Aglycon Moieties of the Glycosides **1–3**

position	δ_C mult.[a]	δ_H mult. (*J* 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 C_5D_5N/D_2O (4:1). Multiplicity by DEPT. [b] Recorded at 500 MHz in C_5D_5N/D_2O (4:1).

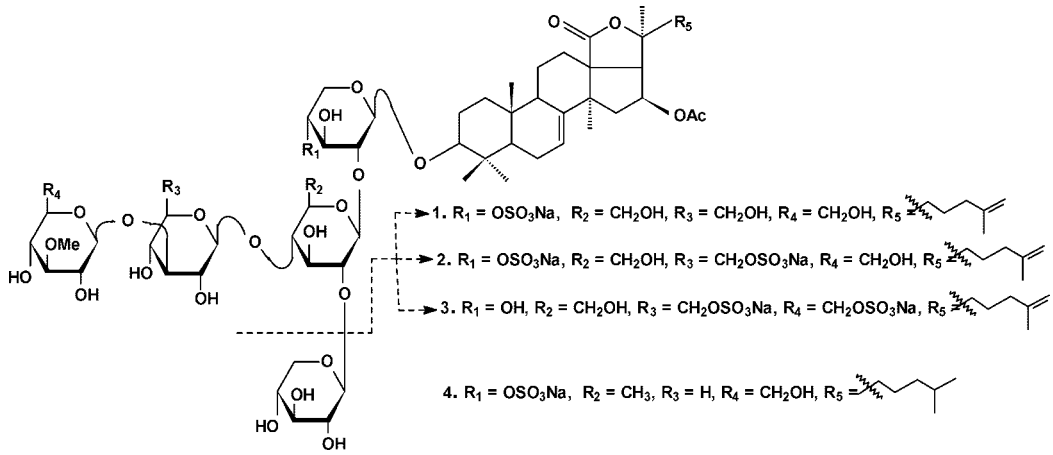
Table 2. ^{13}C and ^{1}H NMR Data of Carbohydrate Chains of Okhotosides (**1–3**)

	1		**2**		**3**	
	δ_C mult.[a,b,c]	δ_H mult. (*J* in Hz)[d]	δ_C mult.[a,b,c]	δ_H mult. (*J* in Hz)[d]	δ_C mult.[a,b,c]	δ_H mult. (*J* 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 C_5D_5N/D_2O (4:1). Multiplicity by DEPT. [b] Bold = interglycosidic positions. [c] Italic = sulfate position. [d] Recorded at 500 MHz in C_5D_5N/D_2O (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 $[M_{Na} - Na - MeGlc - Glc - Glc]^-$.

Chart 1



The differences between the spectra of okhotoside B_1 (**1**) and okhotoside A_2-1 resulted from the lack of signals of a fifth terminal xylose residue in the spectra of **1** and in the corresponding shifts of some glucose residue signals (the second monosaccharide in the carbohydrate chain) due to the absence of a xylosyl substituent in this residue. Therefore, okhotoside B_1 (**1**) has a linear tetraoside sugar chain with the glucose residue as the second monosaccharide unit and a sulfate group at C-4 of the xylose residue. All these data indicated that okhotoside B_1 (**1**) is 3*β*-*O*-[3-*O*-methyl-*β*-D-glucopyranosyl-(1→3)-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→2)-4-*O*-sodium sulfate-*β*-D-xylopyranosyl]-16*β*-acetoxyholosta-7,25-diene.

The molecular formula of okhotoside B_2 (**2**) was established as $C_{56}H_{86}O_{30}S_2Na_2$ by the pseudomolecular ion $[M_{2Na} + Na]^+$ at *m*/*z* 1371.4428 in the HRMALDITOFMS (positive-ion mode) and the ^{13}C NMR spectroscopic data. The ^{13}C NMR spectrum of the carbohydrate chain of **2** showed the signals of four anomeric carbons at 104.0–104.9 ppm, which were correlated with the corresponding signals of anomeric protons at 4.80 (d, $J = 7.6$ Hz), 5.25 (d, $J = 7.4$ Hz), 4.99 (d, $J = 8.2$ Hz), and 5.31 (d, $J = 7.8$ Hz) ppm by the HSQC spectrum (Table 2). Coupling constants of the anomeric protons were indicative of *β*-configurations of glycosidic bonds in all cases.[13] These data indicated that the carbohydrate chain of **2** consists of four monosaccharide residues.

Comparison of ^{13}C and DEPT NMR spectra of the sugar chain of **2** with that of okhotoside B_1 (**1**) showed that signals of the first, second, and terminal monosaccharide units are similar, suggesting xylose as the first monosaccharide unit, glucose as the second residue, and 3-*O*-methylglucose as the fourth monosaccharide residue in the carbohydrate chain of glycoside **2**. In order to assign the carbon signals of the third monosaccharide residue, in the carbohydrate chain of **2** the corresponding proton signals were deduced from the NOESY and TOCSY spectra and then correlated by the HSQC spectrum with the corresponding carbons. These ^{13}C NMR spectroscopic data indicated that the third monosaccharide unit in the sugar chain of **2** is glucose sulfated at C-6. Indeed, the signal of C-6 of the glucose residue in the ^{13}C NMR spectrum of okhotoside B_2 (**2**) was shifted downfield by 5.5 ppm, and the signal of C-5 of the same monosaccharide residue was shifted upfield by 2.7 ppm, in comparison with the spectrum of okhotoside B_1 (**1**), due to the α- and *β*-effects of the sulfate group.[13] The sequence of monosaccharides in the carbohydrate chain of okhotoside B_2 (**2**) was corroborated by the NOESY spectrum, where cross-peaks between H-1 of the xylose and H-3 of the aglycon, H-1 of the first glucose and H-2 of the xylose, H-1 of the sulfated glucose and H-4 of the glucose, and H-1 of the terminal 3-*O*-methylglucose and H-3 of the second (sulfated) glucose were observed. The analogous cross-peaks were also identified in the HMBC spectrum of **2**. This sequence was confirmed by (−) MALDITOFMS data. The spectrum indicated, besides the peak of the $[M_{2Na} - Na]^-$ ion at *m*/*z* 1325, peaks of fragment ions at *m*/*z* 1223 $[M_{2Na} - Na - SO_3Na + H]^-$, 1047 $[M_{2Na} - Na - SO_3Na - MeGlc + H]^-$, 885 $[M - Na - SO_3Na - MeGlc - Glc + H]^-$, and 723 $[M_{2Na} - Na - SO_3Na - MeGlc - Glc - Glc + H]^-$.

Therefore, okhotoside B_2 (**2**) differs from **1** in the presence of an additional sulfate group in the carbohydrate moiety and was assigned as 3*β*-*O*-[3-*O*-methyl-*β*-D-glucopyranosyl-(1→3)-6-*O*-sodium sulfate-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→2)-4-*O*-sodium sulfate-*β*-D-xylopyranosyl]-16*β*-acetoxyholosta-7,25-diene.

The molecular formula of okhotoside B_3 (**3**) was established as $C_{56}H_{86}O_{30}S_2Na_2$ by the pseudomolecular ion $[M_{2Na} + Na]^+$ at *m*/*z* 1371.4157 in the HRMALDITOFMS (positive-ion mode) and NMR spectroscopic data. The ^{13}C NMR spectrum of the carbohydrate chain of **3** showed signals of four anomeric carbons at 103.9–105.0 ppm, which were correlated with the corresponding signals of the anomeric protons at 4.83 (d, $J = 7.8$ Hz), 5.29 (d, $J = 7.8$ Hz), 5.00 (d, $J = 7.9$ Hz), and 5.25 (d, $J = 7.9$ Hz) ppm by the HSQC spectrum (Table 2). The coupling constants of the anomeric protons were indicative of a *β*-configuration of the glycosidic bonds in all cases.[14] These data indicated that the carbohydrate chain of **3** consists of four monosaccharide residues, like those of okhotosides B_1 (**1**) and B_2 (**2**).

Comparison of the ^{13}C NMR spectra of okhotosides B_3 (**3**) and B_2 (**2**) showed the signals of the second and the third monosaccharide residues to be very close, indicating a glucose residue as the second sugar unit and a glucose residue sulfated at C-6 as the third sugar unit in the carbohydrate chain. The signals of a xylose unit were also present in the NMR spectra of **3**, but it was revealed that the signal of C-4 of this monosaccharide residue was shifted upfield by 5.9 ppm, with the signals of C-3 and C-5 shifted downfield by 2.3 and 2.1 ppm, correspondingly, due to the absence of a sulfate group at C-4 of this xylose residue. The chemical shifts of the xylose residue in the ^{13}C and ^1H NMR spectra of okhotoside B_3 (**3**) were similar to those of the unsulfated xylose residue in the spectra of synallactoside A_1 from the sea cucumber *Synallactes nozawai*.[15] In order to assign the carbon signals of the terminal monosaccharide residue (3-*O*-methylglucose) in the carbohydrate moiety of okhotoside B_3 (**3**), the corresponding protons were first deduced from the TOCSY and NOESY spectra and then correlated with the corresponding carbon signals by the HSQC spectrum. The signal of C-6 in the 3-*O*-methylglucose residue was shifted downfield by 5.2 ppm and the signal at C-5 was shifted upfield to 1.9 ppm in ^{13}C NMR spectrum of **3** in comparison with the same signals in the spectrum of **2**, due to the α- and *β*-effects of a sulfate group attached to C-6 of the 3-*O*-methylglucose residue in the sugar chain of okhotoside B_3 (**3**) (Table 2). Indeed, the signals of the terminal sulfated 3-*O*-methylglucose residue and glucose residue

sulfated at the C-6 (the third sugar unit) in the ^{13}C and ^{1}H 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 B_3 (**3**). Therefore, the carbohydrate chain of okhotoside B_3 (**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 $[M_{2Na} - Na - SO_3Na + H]^-$ and 1047 $[M_{2Na} - Na - SO_3Na - MeGlc + H]^-$ in the (−) MALDI and (−) LSIMS. All these data indicated that okhotoside B_3 (**3**) is 3β-*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]-16β-acetoxyholosta-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 *Pseudoocnus* (=*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 A_0-1,[2] isolated earlier from *C. japonica*, frondoside A_1 and frondoside A (**4**) previously isolated from *C. frondosa*,[6,7] cucumarioside A_2-5 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 A_2-1 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 A_1-1[2] was identical to that of frondoside A_1, 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 B_3 (**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.

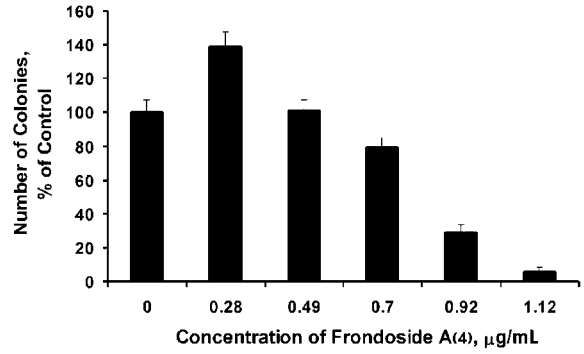


Figure 1. Frondoside A (**4**) prevents EGF-induced malignant transformation of JB6 P^+ Cl 41 cells.

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 B_1−B_3 (**1**–**3**) and frondoside A (**4**) were studied as potential cytotoxic agents using human tumor cell lines. The following result were obtained: okhotoside B_1, IC_{50} 34.0 μg/mL against HeLa; okhotodside B_2, IC_{50} 13.0 μg/mL against HeLa; okhitosude B_3, IC_{50} 17.8 μg/mL against HeLa; frondoside A, IC_{50} 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 $INCC_{50}$ = 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. ^{1}H 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 C_5D_5N/D_2O (4:1) with TMS as an internal reference (δ = 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 C_8 (4.6 × 250) and Discovery C_{18} (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 H_2O 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 $CHCl_3$/EtOH/H_2O, 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 $CHCl_3$/EtOH/H_2O (100:75:10) to give the glycosides belonging to the groups A (130 mg), A_1 (85 mg), and A_2 (94 mg).[1] The individual glycosides were isolated by HPLC using different proportions of solvents MeOH/H_2O/($Na_2HPO_4 \cdot 12H_2O$ (358 mg) + $NaH_2PO_4 \cdot 2H_2O$ (156 mg) in 100 mL of water) as the mobile phase followed by desalting on a Polychrom-1 column, as described above. Besides okhotosides A_1-2 and A_2-1 and cucumarioside A_0-1,[2] reported earlier, the following compounds were obtained.

Okhotoside B_1 (1): (5.3 mg) isolated from group A of monosulfated glycosides on a C_{18} Discovery column with the mobile phase described above in the ratio 62:36:2; mp 273–275 °C, $[\alpha]_D^{20}$ −5 (*c* 0.1 pyridine); see Tables 1 and 2 for NMR data; (+) HRMALDITOFMS, *m/z* 1269.4817 $[M_{Na} + Na]^+$, $C_{56}H_{87}O_{27}SNa_2$, calcd 1269.4951; (+) MALDIMS, *m/z* 1269 $[M_{Na} + Na]^+$, 1239 $[M_{Na} - Na - CH_2O]^+$, 1167 $[M_{Na} + Na - SO_3Na + H]^+$, 1149 $[M_{Na} + Na - SO_3Na - CO + H]^+$; (−) MALDI MS, *m/z* 1223 $[M_{Na} - Na]^-$; (+) LSIMS, *m/z* 1269 $[M_{Na} + Na]^+$, 1239 $[M_{Na} + Na - CH_2O]^+$, 1167 $[M_{Na} + Na - SO_3Na + H]^+$; (−) LSI MS, *m/z* 1223 $[M_{Na} - Na]^-$, 1046 $[M_{Na} - Na - MeGlc]^-$, 885 $[M_{Na} - Na - MeGlc - Glc]^-$, 867 $[M_{Na} - Na - MeGlc - Glc - H_2O]^-$, 723 $[M_{Na} - Na - MeGlc - Glc - Glc]^-$.

Okhotoside B_2 (2): (7 mg) isolated from the fraction of disulfated glycosides on a C_8 Discovery column with the mobile phase described above in the ratio 60:38:2; mp 244 °C, $[\alpha]_D^{20}$ −9 (*c* 0.1 pyridine); see Tables 1 and 3 for NMR data; (+) HRMALDITOFMS, *m/z* 1371.4428 $[M_{2Na} + Na]^+$, $C_{56}H_{86}O_{30}S_2Na_3$, calcd 1371.4428; (+) MALDITOFMS, *m/z* 1387 $[M_{2Na} + K]^+$, 1371 $[M_{2Na} + Na]^+$, 1285 $[M_{2Na} + K - SO_3Na + H]^+$, 1269 $[M_{2Na} + Na - SO_3Na + H]^+$, 1167 $[M_{2Na} + Na - 2SO_3Na + 2H]^+$; (−) MALDI TOF MS, *m/z* 1341 $[M_{Na,K} - Na]^-$, 1325 $[M_{2Na} - Na]^-$, 1295 $[M_{2Na} - Na - CH_2O]^-$, 1223 $[M_{2Na} - Na - SO_3Na + H]^-$, 1193 $[M_{2Na} - Na - SO_3Na - CH_2O + H]^-$, 1047 $[M_{2Na} - Na - SO_3Na - MeGlc + H]^-$, 885 $[M_{2Na} - Na - SO_3Na - MeGlc - Glc + H]^-$, 723 $[M_{2Na} - Na - SO_3Na - MeGlc - 2Glc + H]^-$; LSI MS (+), *m/z* 1387 $[M_{2Na} + K]^+$, 1371 $[M_{2Na} + Na]^+$, 1285 $[M_{2Na} + K - SO_3Na + H]^+$, 1269 $[M_{2Na} + Na - SO_3Na + H]^+$, 1167 $[M_{2Na} + Na - 2SO_3Na + 2H]^+$, 991 $[M_{2Na} + Na - 2SO_3Na - MeGlc + 2H]^+$; (−) LSIMS, *m/z* 1341 $[M_{Na,K} - Na]^-$, 1325 $[M_{2Na} - Na]^-$, 1303 $[M_{Na,H} - Na]^-$, 1223 $[M_{2Na} - Na - SO_3Na + H]^-$, 1193 $[M_{2Na} - Na - SO_3Na - CH_2O + H]^-$, 1047 $[M_{2Na} - Na - SO_3Na - MeGlc + H]^-$, 1031 $[M_{2Na} - Na - SO_3Na - MeGlc - O + H]^-$, 885 $[M_{2Na} - Na - SO_3Na - MeGlc - Glc + H]^-$, 867 $[M_{2Na} - Na - SO_3Na - MeGlc - Glc - H_2O + H]^-$, 723 $[M_{2Na} - Na - SO_3Na - MeGlc - 2Glc + H]^-$.

Okhotoside B_3 (3): (6 mg) isolated from the disulfated glycosides fraction on a C_8 Discovery column with the mobil phase 60:38:2; mp 260–262 °C, $[\alpha]_D^{20}$ −10 (*c* 0.1 pyridine); see Tables 1 and 4 for NMR data; (+) HRMALDITOFMS, *m/z* 1371.4339 $[M_{2Na} + Na]^+$, $C_{56}H_{86}O_{30}S_2Na_3$, calcd 1371.4157; (+) MALDITOFMS, *m/z* 1387 $[M_{2Na} + K]^+$, 1371 $[M_{2Na} + Na]^+$, 1285 $[M_{2Na} + K - SO_3Na + H]^+$, 1269 $[M_{2Na} + Na - SO_3Na + H]^+$, 1167 $[M_{2Na} + Na - 2SO_3Na + 2H]^+$, 991 $[M_{2Na} + Na - 2SO_3Na - MeGlc + 2H]^+$; (−) MALDITOFMS, *m/z* 1341 $[M_{Na,K} - Na]^+$, 1325 $[M_{2Na} - Na]^-$, 1295 $[M_{2Na} - Na - CH_2O]^-$, 1223 $[M_{2Na} - Na - SO_3Na + H]^-$, 1193 $[M_{2Na} - Na - SO_3Na - CH_2O + H]^-$, 1047 $[M_{2Na} - Na - SO_3Na - MeGlc + H]^-$, 885 $[M_{2Na} - Na - SO_3Na - MeGlc - Glc + H]^-$, 723 $[M_{2Na} - Na - SO_3Na - MeGlc - 2Glc + H]^-$; (−) LSIMS, *m/z*: 1341 $[M_{Na,K} - Na]+$, 1325 $[M_{2Na} - Na]^-$, 1303 $[M_{Na,H} - Na]^-$, 1223 $[M_{2Na} - Na - SO_3Na + H]^-$, 1193 $[M_{2Na} - Na - SO_3Na - CH_2O + H]^-$, 1047 $[M_{2Na} - Na - SO_3Na - MeGlc + H]^-$, 1031 $[M_{2Na} - Na - SO_3Na - MeGlc - O + H]^-$, 885 $[M_{2Na} - Na - SO_3Na - MeGlc - Glc + H]^-$, 867 $[M_{2Na} - Na - SO_3Na - MeGlc - Glc - H_2O + H]^-$, 723 $[M_{2Na} - Na - SO_3Na - MeGlc - 2Glc + H]^-$.

Frondoside A_1: (3.3 mg) isolated from the A_1 group of monosulfated glycosides on a C_{18} Discovery column with 65:33:2 and 69:30:1 mobile phases; physical constants and ^{13}C NMR spectrum were identical with literature data.[7]

Frondoside A (4): (4.4 mg) isolated from the A group of monosulfated glycosides on a C_{18} Discovery column with the mobile phases 65:33:2 and 73:25:2); physical constants and ^{13}C NMR spectrum were identical with literature data.[6]

Cucumarioside A_2-5: (3 mg) isolated from the A_2 group of monosulfated glycosides on a C_8 Discovery column with the mobile phase 62:36:2; physical constants and ^{13}C NMR spectrum were identical with literature data.[8]

Koreoside A: (10 mg) isolated from the fraction of trisulfated glycosides on a C_8 Discovery column with the solvent system 46:53:1; physical constants and ^{13}C 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 IC_{50} in corresponding experiments.

Effect of Frondoside A (4) on AP-1-, NF-kB-, 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/m^2). JB6-LucNF-kB cells were activated with EGF (10 ng/mL). Viable activated JB6-LucAP-1 or JB6-LucNF-kB cells or nonactivated JB6-Lucp53 cells (6×10^3) 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 $(MgCO_3)_4 \times Mg(OH)_2 \times 5H_2O$, 5.34 mM $MgSO_4 \times 7H_2O$, 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^+ Cl41 cells (8×10^3/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% CO_2 incubator for 1 week. Cell colonies were then scored using a Leica DM IRB inverted research microscope (Leica Mikroskopie and 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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