Triterpene Glycosides from the Far Eastern Sea Cucumber *Pentamera calcigera* II: Disulfated Glycosides

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Three new triterpene glycosides, calcigerosides D_1 (**1**), D_2 (**2**), and E (**3**), have been isolated from the sea cucumber *Pentamera calcigera*. Their structures have been deduced from extensive spectral analysis (NMR and MS) and chemical evidence. All the compounds are disulfated pentaosides differing in aglycon structure and position of sulfate group, which were determined by the measurement of NT_1 values in the cases of glycosides **1** and **2**. Glycoside **1** is a nonholostane derivative, that is, it lacks an 18(20)-lactone, which is very rare among the sea cucumber glycosides.

As a continuation of our studies on the triterpene glycosides from the Far Eastern sea cucumber *Pentamera calcigera* Stimpson (Thyoninae, Phyllophoridae),[1] collected by trawling during the 18th scientific cruise of the r/v (research vessel) *Akademik Oparin* in the southwestern area of the Peter-the-Great Gulf (the Sea of Japan), we have investigated the fraction of disulfated glycosides. In this paper we report the isolation of three new disulfated glycosides: calcigerosides D_1 (**1**), D_2 (**2**), and E (**3**) (Chart 1).

Results and Discussion

The ethanolic extract of *P. calcigera* (425 g dry wt) was sequentially submitted to column chromatography on Polychrom-1 (powdered Teflon) and Si gel. Final separation of the polar glycosides and isolation of individual compounds was achived by reversed-phase HPLC on Silasorb C_{18} to give calcigeroside D_1 (**1**), calcigeroside D_2 (**2**), and calcigeroside E (**3**).

The molecular formula of calcigeroside D_1 (**1**) was determined as $C_{54}H_{82}O_{31}S_2Na_2$ by the pseudomolecular ions at *m/z* 1335 $[M_{Na,Na} - H]^-$ and 1313 $[M_{Na,Na} - Na]^-$ in its API−ESMS$^{(-)}$ (Figure 1) and by the pseudomolecular ion at *m/z* 1359 $[M_{Na,Na} + Na]^+$ in its API−ESMS$^{(+)}$ (Figure 2), which also showed the fragment ions at *m/z* 1257 $[M_{Na,Na} - SO_3Na + H + Na]^+$ and 1155 $[M_{Na,Na} - 2SO_3Na + 2H + Na]^+$, indicating the presence of two sulfate groups in this glycoside.

The presence of 3-*O*-methylxylose, xylose, quinovose, and glucose in a 1:1:1:2 ratio in the glycoside **1** was established by acid hydrolysis with aqueous 1 N trifluoroacetic acid followed by GC−MS analysis of the corresponding aldononitrile peracetates. Solvolysis of the glycoside **1** gave the desulfated derivative, compound **4**, whose ^{13}C and ^1H NMR spectra and physical constants were found to be identical with those of the known desulfated derivative of calcigeroside C_1 described in our previous communication.[1] Thus, calcigeroside D_1 (**1**) has the same sugar sequence and aglycon structure as the known calcigeroside C_1 and differs only in the number of sulfate groups in the carbohydrate chain.

Comparison of ^{13}C NMR spectroscopic data of calcigeroside D_1 (**1**) (Tables 1 and 2) with those of its desulfated derivative (**4**)[1] confirmed the presence of two sulfate groups and allowed us to locate these at C-4 of the first xylose residue and at C-6 of a glucose residue. Indeed, the downfield shift (by 6.06 ppm) of the C-4 signal and the upfield shift (by 2.02 ppm) of the C-5 signal of the xylose residue in the spectrum of **1**, in comparison with those of **4**, indicated the attachment of one sulfate to C-4 of xylose.[2] In a similar way, the downfield shift (by 5.33 ppm) of the C-6 signal and the upfield shift (by 3.79 ppm) of the C-5 signal of a glucose residue in the spectrum of **1**, in comparison with those of **4**, indicated the attachment of the second sulfate to C-6 of a glucose residue.[2] However, due to the presence of two glucose units (inner and terminal glucose units) in the molecule, the problem was to determine in which unit the second sulfate group was located. Careful analysis of the API−ESMS and, especially, of the NT_1 values (^{13}C spin lattice relaxation time) for the carbon atoms of the carbohydrate chain, enabled us to locate the second sulfate group on the C-6 of the inner glucose unit. In fact, the presence of the terminal sulfated bioside residue linked to the quinovose unit was deduced by the fragment ions at *m/z* 387 [3-*O*-Me-Xyl-*O*-Glc-OSO_3Na − H − Na]$^-$ and 903 $[M_{Na,Na}$ − 3-*O*-Me-Xyl-*O*-Glc-OSO_3Na − H − Na]$^-$ in the API−ESMS$^{(-)}$ of **1** (Figure 1) and by the fragment at *m/z* 949 $[M_{Na,Na}$ − 3-*O*-Me-Xyl-*O*-Glc-OSO_3Na + H + Na]$^+$ in the API−ESMS$^{(+)}$ of **1** (Figure 2).

Moreover, HMQC, HMBC, and $^1H-^1H$ COSY experiments allowed us to assign all the protons to their corresponding carbons and to correlate most of the proton signals in both terminal and inner glucose units. As a result, we were able to identify a glucose unit (the inner one) sulfated at C-6 (67.26 ppm) that has H_2-6 proton signals at 4.95 and 4.57 ppm. These signals correlate by TOCSY to the proton signals of H-5 at 4.08 ppm (74.82 ppm) and H-4 at 3.78 ppm (68.91 ppm). In contrast, a second glucose (the terminal one) was identified with C-6 (61.38 ppm) and H_2-6 (4.38 and 4.15 ppm), and these signals were shifted upfield in relation to the former (inner) glucose. These data indicate the absence of a sulfate group in this residue. The NT_1 values obtained for the C-6 (67.26 ppm) and C-5 (74.82 ppm) carbons of the sulfated glucose

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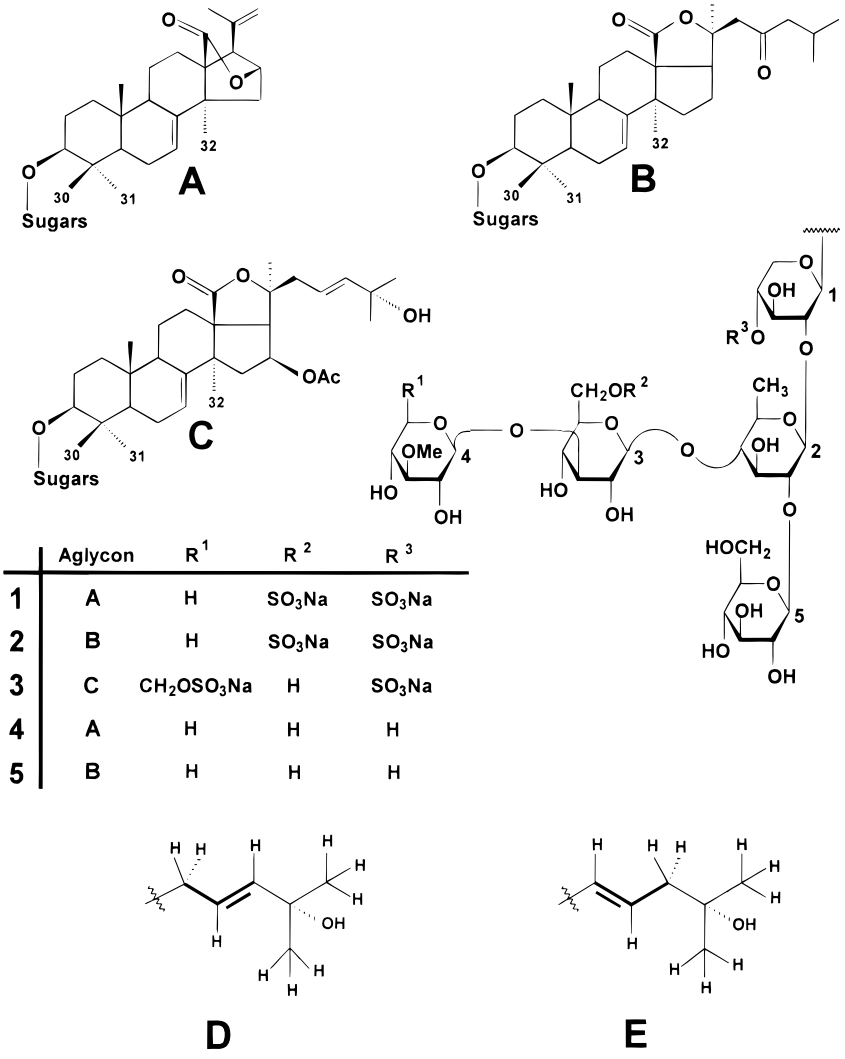
Chart 1



	Aglycon	R^1	R^2	R^3
1	A	H	SO_3Na	SO_3Na
2	B	H	SO_3Na	SO_3Na
3	C	CH_2OSO_3Na	H	SO_3Na
4	A	H	H	H
5	B	H	H	H

residue were smaller than those for the corresponding C-6 (61.38 ppm) and C-5 (77.92 ppm) signals of nonsulfated glucose (see Figure 7). In addition, taking into account that the terminal monosaccharide residues have larger NT_1 values than the inner ones,[3−5] these results confirmed the presence of the second sulfate group on the inner glucose unit.[6]

Thus, the structure of calcigeroside D_1 (**1**) was determined as 18(16)-lactone-23,24,25,26,27-pentanor-3-*O*-{3-*O*-methyl-*β*-D-xylopyranosyl-(1→3)-6-*O*-sodium sulfate-*β*-D-glucopyranosyl-(1→4)-[*β*-D-glucopyranosyl-(1→2)]-*β*-D-quinovopyranosyl-(1→2)-4-*O*-sodium sulfate-*β*-D-xylopyranosyl}-lanosta-7,20(22)-dien-3*β*-ol. Glycosides with such a carbohydrate chain were previously unknown.

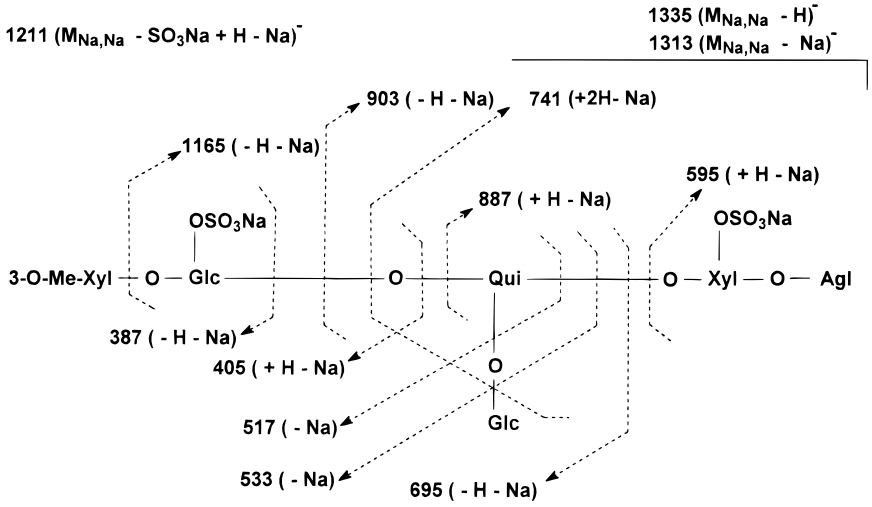


Figure 1. Fragmentation of calcigeroside D_1 (**1**) in the API−ESMS$^{(-)}$.

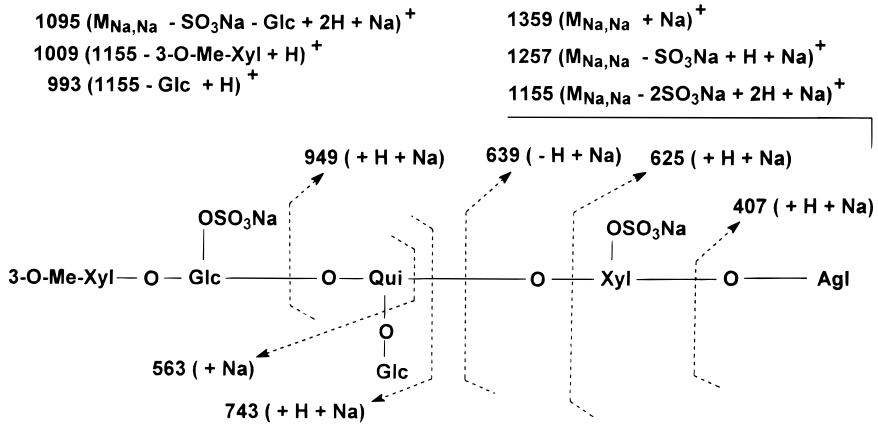


Figure 2. Fragmentation of calcigeroside D_1 (**1**) in the API−ESMS$^{(+)}$.

Table 1. ^{13}C and ^1H NMR Chemical Shifts and HMBC Correlations for the Aglycon Moiety of Calcigeroside D_1 (**1**)

position	δ_C mult[a]	δ_H mult[b] (*J* in Hz)	HMBC
1	35.61 t	1.29 m	
2	26.59 t	1.88 m, 1.70 m	
3	88.92 d	3.14 dd (11.4, 3.4)	C: 1 Xyl
4	39.18 s		
5	47.17 d	0.81 m	
6	23.06 t	1.94 m	
7	122.46 d	5.56 m	
8	147.22 s		
9	46.25 d	2.80 br d (13.9)	
10	35.24 s		
11	21.59 t	1.97 m, 1.39 m	
12	19.87 t	2.25 m, 1.84 m	
13	56.72 s		
14	46.00 s		
15	43.56 t	2.09 br s	
16	80.96 d	4.78 s	
17	59.05 d	2.95 s	C: 13, 21
18	181.87 s		
19	23.70 q	0.84 s	C: 9, 10
20	139.90 s		
21	23.01 q	1.71 s	C: 17, 20, 22
22	113.76 t	4.94 s, 4.88 s	
30	17.16 q	0.97 s	C: 3, 4, 5, 31
31	28.54 q	1.17 s	C: 3, 4, 5, 30
32	33.94 q	1.34 s	C: 8, 13, 14, 15

[a] Recorded at 125 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).

Solvolysis of calcigeroside D_2 (**2**) gave compound **5**, which was found to be identical to the known desulfated derivative obtained from calcigeroside C_2 described in our previous communication.[1] For this reason we were able to establish the aglycon part and the sugar sequence of **2**. On the other hand, analysis of the NMR spectra (^{13}C, DEPT, HMQC, HMBC, $^1H-^1H$ COSY) of the carbohydrate chains of calcigerosides D_1 (**1**) and D_2 (**2**) (Tables 3 and 4) allowed us to confirm its sugar chain structure and to locate the positions of the sulfate group in glycoside **2**. Indeed, the API−ESMS$^-$ of **2** (Figure 3) showed a pseudomolecular ion at *m/z* 1399 [$M_{Na,Na}$ − Na]$^-$, corresponding to the molecular formula $C_{59}H_{92}O_{32}S_2Na_2$, and fragment ions at *m/z* 387 [3-*O*-Me-Xyl-*O*-Glc-OSO_3Na − H − Na]$^-$ and 989 [$M_{Na,Na}$ − 3-*O*-Me-Xyl-*O*-Glc-OSO_3Na − H − Na]$^-$. This latter peak was due to cleavage between the terminal sulfated bioside residue, composed of 3-*O*-methylxylose linked to a sulfated glucose unit, and the quinovose residue. In a similar way, the positive-ion FABMS (Figure 4) showed the pseudomolecular ion at *m/z* 1445 [$M_{Na,Na}$ + Na]$^+$ and a fragment ion at *m/z* 1035 [$M_{Na,Na}$ − 3-*O*-Me-Xyl-*O*-Glc-OSO_3Na + H + Na]$^+$.

Table 2. ^{13}C and ^1H NMR Chemical Shifts and HMBC Correlations for the Sugar Moiety of Calcigeroside D_1 (**1**)

position	δ_C mult[a,b]	δ_H mult[c] (*J* in Hz)	HMBC
Xyl (1→C-3)			
1	104.33 d	4.77 d (6.9)	C: C-3 & 5 Xyl
2	*82.09* d	*3.91* m	C: 1 Qui
3	74.56 d	4.42 t (9.1)	C: 2 Xyl
4	**75.80** d	4.96 t (10.2)	
5	63.84 t	4.72 m, 3.88 m	
Qui (1→2Xyl)			
1	102.47 d	5.13 d (7.6)	C: 2 Xyl
2	*82.99* d	*3.89* m	C: 1 Glu_2
3	74.61 d	3.98 m	C: 2 Qui & 4 Qui
4	*86.35* d	*3.38* t (8.7)	C: 1 Glu_1
5	70.67 d	3.60 m	
6	17.67 q	1.54 d (7.0)	C: 4 Qui & 5 Qui
Glu_1 (1→4Qui)			
1	103.95 d	4.76 d (7.8)	C: 4 Qui
2	73.36 d	3.81 m	C: 1 Glu_1
3	*85.78* d	*4.10* m	
4	68.91 d	3.78 m	
5	74.82 d	4.08 m	
6	**67.26** t	4.95 m, 4.57 dd (17.4, 10.4)	
MeXyl (1→3Glu_1)			
1	105.07 d	5.10 d (7.6)	C: 3 Glu_1 & 2 MeXyl
2	74.10 d	3.79 m	
3	86.51 d	3.57 t (8.8)	C: O-Me & 2 MeXyl
4	69.58 d	3.95 m	C: 3 MeXyl & 5 MeXyl
5	66.24 t	4.14 m, 3.60 m	C: 1 MeXyl & 3 MeXyl
OMe	60.51 q	3.78 s	C: 3 MeXyl & 4 MeXyl
Glu_2 (1→2Qui)			
1	104.85 d	5.16 d (7.7)	C: 2 Qui
2	75.37 d	3.92 m	C: 1 Glu_2
3	76.36 d	4.11 m	
4	70.20 d	3.95 m	
5	77.92 d	3.85 m	C: 6 Glu_2
6	61.38 t	4.38 br d (11.3), 4.15 m	

[a] Recorded at 125 MHz in C_5D_5N/D_2O (4:1); multiplicity by DEPT. [b] Italic entries = interglycosidic positions; boldface entries = sulfate positions. [c] Recorded at 500 MHz in C_5D_5N/D_2O (4:1).

On the basis of all above-mentioned data, the structure of calcigeroside D_2 (**2**) was elucidated as 3-*O*-{3-*O*-methyl-*β*-D-xylopyranosyl-(1→3)-6-*O*-sodium sulfate-*β*-D-glucopyranosyl-(1→4)-[*β*-D-glucopyranosyl-(1→2)]-*β*-D-quinovopyranosyl-(1→2)-4-*O*-sodium sulfate-*β*-D-xylopyranosyl}-holost-7-en-23-on-3*β*-ol.

The molecular formula of calcigeroside E (**3**) was determined as $C_{62}H_{96}O_{35}S_2Na_2$ by the pseudomolecular ions at *m/z* 1549 [$M_{Na,Na}$ + K]$^+$ and 1527 [$M_{Na,K}$ + H]$^+$ in the positive-ion FABMS and at *m/z* 1487 [M_{Na}]$^-$ in the API−MS$^{(-)}$ (Figures 5 and 6).

The aglycon structure of calcigeroside E (**3**), that is, 16*β*-acetoxyholosta-7,23*E*-diene-3*β*,25-diol, was shown to be

Table 3. ^{13}C and ^{1}H NMR Chemical Shifts and HMBC Correlations for the Aglycon Moiety of Calcigeroside D_2 (**2**)

position	δ_C mult[a]	δ_H mult[b] (J in Hz)	HMBC
1	35.89 t	1.29 m	
2	26.68 t	1.91 m, 1.71 m	
3	89.04 d	3.18 br dd	C: 1 Xyl
4	39.27 s		
5	47.75 d	0.89 m	
6	23.01 t	1.90 m	
7	119.84 d	5.66 m	
8	146.40 s		
9	47.17 d	3.27 br d (14.0)	
10	35.26 s		
11	23.02 t	1.65 m	
12	29.90 t	1.92 m, 1.45 m	
13	57.59 s		
14	51.15 s		
15	33.97 t	1.33 s	
16	25.38 t	1.91 m, 1.60 m	
17	53.33 d	2.66 br dd	C: 13, 20, 21
18	180.64 s		
19	23.80 q	1.07 s	C: 1, 9, 10
20	83.04 s		
21	26.91 q	1.53 s	C: 17, 20, 22
22	51.84 t	3.06 d (18.8), 2.98 d (18.8)	
23	208.98 s		
24	52.06 t	2.30 d (6.7), 2.06 m	C: 25, 26, 27
25	24.40 d	0.80 m	
26	22.21 q	0.83 d (6.5)	C: 24, 25, 27
27	22.21 q	0.83 d (6.5)	C: 24, 25, 26
30	17.33 q	1.01 s	C: 3, 4, 5, 31
31	28.61 q	1.20 s	C: 3, 4, 5, 30
32	30.56 q	1.01 s	C: 13, 14, 16

[a] Recorded at 125 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).

identical to that of the known cucumarioside G_4, isolated from the sea cucumber *Eupentacta fraudatrix*[8] and eximisoside A from the sea cucumber *Psolus eximius*,[9] by comparison of the NMR spectra of their corresponding aglycon parts. This fact was confirmed by extensive analysis of the NMR spectra of **3** (^{1}H, ^{13}C, DEPT, COSY, HMBC, HMQC) (Table 5). Thus, the ^{1}H NMR spectrum of the side chain in the aglycon moiety showed a singlet at 1.45 ppm corresponding to two methyl groups (H_3-26 and H_3-27), two olefinic protons at 5.76 (1H, m) and 5.90 ppm (1H, d, J = 15.4 Hz) corresponding to a trans double bond, and a methylene group at 2.57 and 3.20 ppm (1H, m each). Given these data, only two side chains (D and E) were possible. Assignment of the side chain structure of **3** as D was made on the basis of HMBC cross-peaks displayed by H_3-21 protons at 1.52 ppm and a methylene carbon at 41.70 ppm (t) (locating them at C-22), as well as by the HMBC correlations between the methyl protons H_3-26 and H_3-27 at 1.45 ppm and the quaternary carbon C-25 (69.95 ppm) and olefinic carbons C-24 and C-23 (143.24 and 120.46 ppm).

The presence of five monosaccharide units in the carbohydrate chain of **3** was deduced from the ^{13}C NMR and DEPT spectra, which exhibited five signals of anomeric carbons at 104.36, 102.41, 103.76, 104.65, and 105.09 ppm, and from the ^{1}H NMR spectrum, which displayed five doublet signals corresponding to the anomeric protons at 4.81 (d, J = 6.7 Hz), 5.12 (d, J = 7.7 Hz), 4.83 (d, J = 7.8 Hz), 5.12 (d, J = 7.7 Hz), and 5.15 (d, J = 7.7 Hz) ppm. The J values (6.7−7.8 Hz) agree with a β configuration for the glycosidic bonds (Table 6).[2]

Comparison of the NMR (^{1}H and ^{13}C NMR, $^{1}H-^{1}H$ COSY, HMQC, and HMBC) and MS data [positive-ion FABMS and API−ESMS$^{(-)}$] of the carbohydrate chain of **3** with those of the known cucumarioside A_6-2, isolated from *Cucumaria japonica*,[7] showed a very similar monosaccharide content and a sugar sequence that differs only in the presence of a terminal glucose unit linked to C-2 of quinovose in **3** rather than a terminal xylose unit at that position in cucumarioside A_6-2. The positions of the two sulfate groups in **3** at C-4 of the xylose unit and at C-6 of the 3-*O*-methyl-glucose terminal residue were deduced by careful analysis of the NMR spectra (Table 6) and MS data [positive FABMS and API−ESMS$^{(-)}$]. Indeed, the carbon and proton chemical shifts arising from position **4** of the xylose unit [C-4 (75.95 ppm, d) and H-4 (4.96 ppm, 1H, m)] and the fragment ions at m/z 769 [Agl-*O*-Xyl-OSO_3Na + H + Na]$^+$ and at m/z 897 [Agl-*O*-Xyl-OSO_3Na-*O*-Qui + Na]$^+$ in the positive FABMS, are in agreement with a sulfate group at that position. On the other hand, the carbon and proton chemical shifts arising from position 6 of the terminal 3-*O*-methylglucose unit [66.99 t/4.95 and 4.70 (each 1H, m)] and the fragment ions at m/z 255 [3-*O*-Me-Glc-OSO_3Na − H − Na]$^-$, 1209 [$M_{Na,Na}$ − 3-*O*-Me-Glc-OSO_3-Na + H − Na]$^-$, and 1193 [$M_{Na,Na}$ − 3-*O*-Me-Glc-OSO_3Na-O + H − Na]$^-$ in the API−ESMS$^{(-)}$ allowed us to locate the second sulfate group at that position. Thus, the structure of calcigeroside E (**3**) was determined as 16β-acetoxy-3-*O*-{6-*O*-sodium sulfate-3-*O*-methyl-β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→2)]-β-D-quinovopyranosyl-(1→2)-4-*O*-sodium sulfate-β-D-xylopyranosyl}-holosta-7,23*E*-diene-3β,25-diol.

Calcigerosides D_1 (**1**), D_2 (**2**), and E (**3**) are new triterpene disulfated glycosides isolated from the sea cucumber *P.*

Table 4. ^{13}C and ^{1}H NMR Chemical Shifts and HMBC Correlations for the Sugar Moiety of Calcigeroside D_2 (**2**)

position	δ_C mult[a,b]	δ_H mult[c] (J in Hz)	HMBC
Xyl (1→C-3)			
1	104.38 d	4.77 d (6.7)	C: C-3
2	*82.13* d	*3.94* m	
3	74.58 d	4.44 t (9.1)	
4	**75.83** d	4.98 t (10.2)	
5	63.88 t	4.76 m, 3.90 m	
Qui (1→2Xyl)			
1	102.43 d	5.14 br d	C: 2 Xyl
2	*82.99* d	*3.89* m	
3	74.63 d	3.97 m	
4	*86.35* d	*3.38* t (8.7)	
5	70.68 d	3.59 m	
6	17.80 q	1.56 d (7.6)	
Glu_1 (1→4Qui)			
1	103.97 d	4.76 d (8.1)	C: 4 Qui
2	73.37 d	3.80 m	
3	*85.79* d	*4.10* t (9.3)	
4	68.90 d	3.77 m	
5	74.81 d	4.08 m	
6	**67.26** t	4.96 m, 4.58 br t	
MeXyl (1→3Glu_1)			
1	105.08 d	5.11 d (7.7)	C: 3 Glu_1
2	74.11 d	3.78 m	
3	86.52 d	3.58 t (8.7)	
4	69.59 d	3.96 m	
5	66.25 t	4.14 m, 3.60 m	
OMe	60.51 q	3.78 s	C: 4 MeXyl
Glu_2 (1→2Qui)			
1	104.87 d	5.16 br d	C: 2 Qui
2	75.39 d	3.93 m	
3	76.36 d	4.11 m	
4	70.21 d	3.95 m	
5	77.94 d	3.85 m	
6	61.41 t	4.38 br d (11.5), 4.14 m	

[a] Recorded at 125 MHz in C_5D_5N/D_2O (4:1); multiplicity by DEPT. [b] Italic entries = interglycosidic positions; boldface entries = sulfate positions. [c] Recorded at 500 MHz in C_5D_5N/D_2O (4:1).

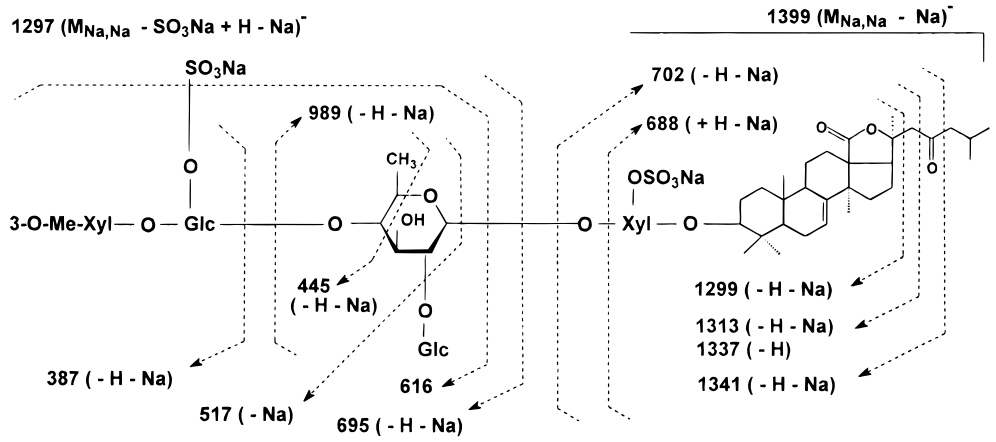


Figure 3. Fragmentation of calcigeroside D_2 (**2**) in the API−ESMS$^{(-)}$.

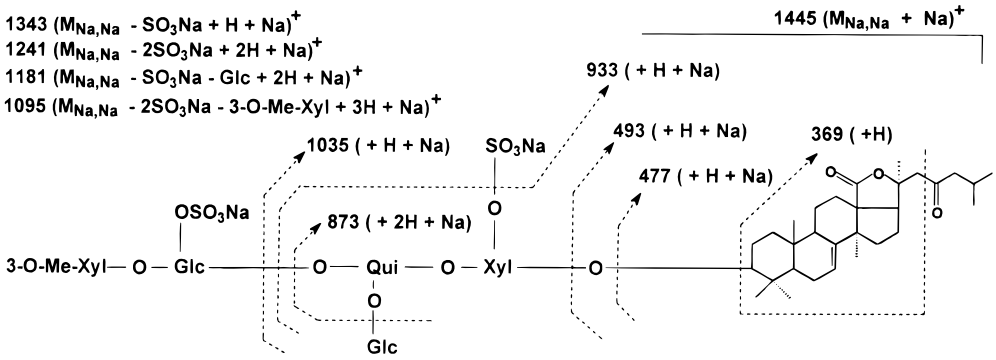


Figure 4. Fragmentation of calcigeroside D_2 (**2**) in the FABMS$^{(+)}$.

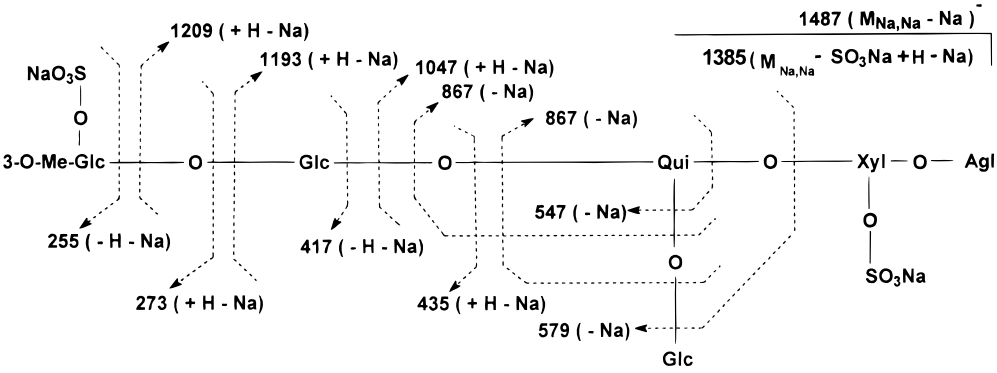


Figure 5. Fragmentation of calcigeroside E (**3**) in the API−ESMS$^{(-)}$.

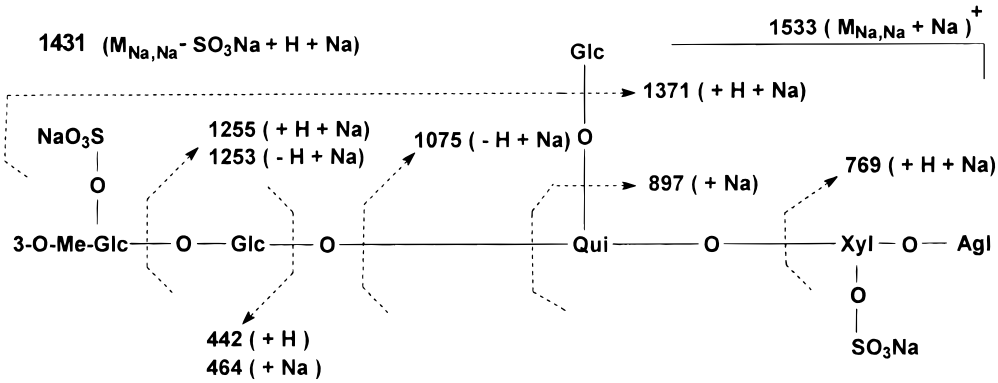


Figure 6. Fragmentation of calcigeroside E (**3**) in the FABMS$^{(+)}$.

calcigera. These glycosides are characterized by a fifth terminal glucose residue in the carbohydrate chain, which is a very uncommon situation.[9] Glycoside **1** is a "nonholostane" glycoside with an aglycon having an 18(16)-lactone and shortened side chain, which is, again, not commonly found.[9]

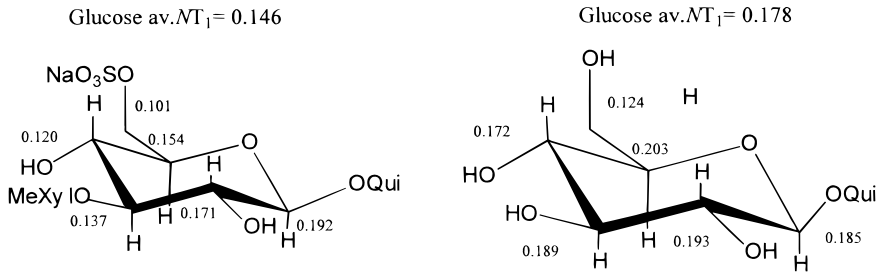


Figure 7. NT_1 values measured for the glucose moieties and average NT_1 calculated for the glucose moieties of calcigeroside D_1 (**1**).

Table 5. ^{13}C and ^{1}H NMR Chemical Shifts and HMBC Correlations for the Aglycon Moiety of Calcigeroside E (**3**)

positions	δ_C mult[a]	δ_H mult[b] (*J* in Hz)	HMBC
1	35.79 t	1.27 m	
2	26.61 t	1.91 m, 1.72 m	
3	88.87 d	3.18 m	
4	39.21 s		
5	47.71 d	0.86 m	C: 6
6	23.02 t	1.91 m	
7	120.21 d	5.73 m	
8	143.24 s		
9	47.26 d	3.27 br d (14.0)	
10	35.24 s		
11	22.33 t	1.41 m	
12	30.97 t	1.66 m, 2.01 m	
13	59.08 s		
14	46.90 s		
15	43.40 t	2.54 m, 1.59 m	
16	74.94 d	5.86 q (8.2)	
17	54.18 d	2.72 d (9.1)	
18	180.15 s		
19	23.75 q	1.06 s	C: 9, 10
20	84.94 s		
21	28.58 q	1.52 s	C: 17, 20, 22
22	41.70 t	2.57 m, 3.20 m	
23	143.24 d	5.76 m	
24	120.46 d	5.90 d (15.4)	
25	69.95 s		
26	29.60[c] q	1.45 s	C: 23, 24, 25, 27
27	29.82[c] q	1.45 s	C: 23, 24, 25, 26
30	17.24 q	0.99 s	C: 3, 4, 5, 31
31	28.05 q	1.19 s	C: 3, 4, 5, 30
32	32.03 q	1.06 s	C: 13, 14
OAc	171.00 s		C: O-*C*O-CH_3
	21.13 q	1.94 s	

[a] Recorded at 125 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). [c] The signals may be changed.

Table 6. ^{13}C and ^{1}H NMR Chemical Shifts and HMBC Correlations for the Sugar Moiety of Calcigeroside E (**3**)

position	δ_C mult[a,b]	δ_H mult[c] (*J* in Hz)	HMBC
Xyl (1→C-3)			
1	104.36 d	4.81 d (6.7)	C: C-3
2	*82.22* d	*3.94* m	C: 1 Qui
3	74.65 d	4.45 d (8.7)	C: 2 Xyl
4	**75.95** d	4.96 m	
5	63.94t	4.77 m, 3.89 m	
Qui (1→2Xyl)			
1	102.41 d	5.12 d (7.7)	C: 2 Xyl
2	*83.05* d	*3.93* m	C: 1 Glu_2
3	75.03 d	3.97 m	
4	*85.59* d	*3.51* m	C: 1 Glu_1
5	70.92 d	3.57 m	
6	17.80 q	1.57 d (5.5)	C: 4 Qui, 5 Qui
Glu_1 (1→4Qui)			
1	103.76 d	4.83 d (7.8)	C: 4 Qui
2	73.37 d	3.89 m	
3	*87.29* d	*4.12* m	C: 1 MeGlu
4	69.76 d	3.98 m	
5	77.99 d	3.82 m	
6	61.61[d] t	4.38 d (11.0), 4.16 m	
MeGlu (1→3Glu_1)			
C-1	104.65 d	5.12 d (7.7)	C: 3 Glu_1
C-2	74.20 d	3.76 m	C: 3 MeGlu
C-3	86.27 d	3.64 t (8.9)	C: 2 MeGlu & 4 MeGlu & OMe
C-4	70.31 d	3.93 m	C: OMe
C-5	75.32 d	4.04 m	C: 4 MeGlu
C-6	**66.99** t	4.95 m, 4.70 m	
O-Me	60.51 q	3.75 s	C: 3 MeGlu
Glu_2 (1→2Qui)			
C-1	105.09 d	5.15 d (7.7)	C: 2 Qui
C-2	76.88 d	3.89 m	C: 3 Glu_2
C-3	76.64 d	4.09 m	C: 4 Glu_2
C-4	75.53 d	4.05 m	
C-5	69.24 d	3.98 m	
C-6	61.46[d] t	4.29 d (11.0), 3.99 m	

[a] Recorded at 125 MHz in C_5D_5N/D_2O (4:1); multiplicity by DEPT. [b] Italic entries = interglycosidic positions; boldface entries = sulfate positions. [c] Recorded at 500 MHz in C_5D_5N/D_2O (4:1). [d] The signals may be changed.

Experimental Section

General Experimental Procedures. All melting points were determined using a Kofler-Thermogenerate apparatus. Optical rotations were measured with a Perkin-Elmer 141 polarimeter. ^{1}H and ^{13}C NMR spectra were obtained using a Bruker AMX 500 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 ($\delta = 0$). The FABMS (positive- and negative-ion modes) were recorded using a Micromass apparatus, model Autospec-M, on a glycerol/thioglycerol matrix. API−ESMS spectra were obtained on a Hewlett-Packard 1100 MSD apparatus by direct injection. HPLC was performed using an Yanako L-2000L chromatograph equipped with a differential refractometer on a Silasorb C_{18} column (10 × 150 mm). GC analysis was carried out using a Tsvet-110 apparatus, with a glass column (0.3 × 150 cm) and 1.5% QF-1 as the stationary liquid phase and Chromatrone N−HMDS as the stationary solid phase. The following experimental conditions were used: carrier gas, Ar at 60 mL/min; column temperature, from 150 to 225 °C at 5 °C/min. GLC−MS was performed using an LKB 9000s apparatus with a glass column (0.3 × 300 cm) with 1.5% OF-1 as the stationary liquid phase and Chromatrone N−HMDS as the stationary solid phase and He at 50 mL/min as the carrier gas. The following conditions were selected: injection port 275 °C, molecular separator 265 °C, ion source 255 °C, column 110−210 °C at 4 °C/min, ionizing voltage 70 ev. The atomic absorption spectroscopy data were obtained using a Jarrell Ash AA-780 spectrometer. The cations in all the glycosides were determined to be Na^+.

Animal Material. Specimens of *P. calcigera* were collected at a depth of 70−80 m by Sigsby trawl in the southwestern area of the Peter-the-Great Gulf (Sea of Japan) between Furugelm Island and Gamov Cape in September 1995, during the 18th scientific cruise of r/v *Akademik Oparin* and were stored in ethanol at room temperature. The sea cucumber was

identified by Dr. V. S. Levin (Kamchatka Institute of Fishery and Oceanography, Petropavlovsk-Kamchatsky, Russia). A voucher specimen [no. 018-68(13)] is on a deposit at the marine specimen collection of the Pacific Institute of Bioorganic Chemistry, Vladivostok, Russia.

Extraction and Isolation. The sea cucumbers (425 g, dried residue) were cut into pieces and extracted twice with refluxing ethanol. The combined extracts were concentrated, and the extract residue was dissolved in water. Desalting was carried out by passing this fraction through a Polychrom-1 column (powdered Teflon; Biolar, Latvia), first eluting the inorganic salts and polar impurities with H_2O, and then eluting the crude glycoside fraction (3.5 g) with 65% aqueous methanol. The glycoside fraction was further chromatographed on Si gel, eluting with $CHCl_3$/MeOH/H_2O (65:25:4) and $CHCl_3$/MeOH/H_2O (650:300:54) to give fractions A (450 mg), B (450 mg), C (600 mg), D (365 mg), and E (100 mg).

Final purification of the polar fractions D and E was achieved by HPLC. Fraction D afforded 120 mg of pure calcigeroside D_1 (**1**) and 60 mg calcigeroside D_2 (**2**) using MeOH/H_2O (75:80) as the mobile phase and a flow of 2.5 mL/min. Fraction E gave 27 mg of pure calcigeroside E (**3**) using MeOH/H_2O (75:80).

Calcigeroside D_1 (1): mp 218−220 °C, $[\alpha]^{20}_D$ −49.0° (*c* 0.1, pyridine/water, 1:1); ^{13}C and ^1H NMR, see Tables 1 and 2; API−ESMS$^{(-)}$ *m/z* (rel int, %) 1335 ($[M_{Na,Na} - H]^-$, 5), 1113 ($[M_{Na,Na} - Na]^-$, 28), 1211 ($[M_{Na,Na} - SO_3Na + H - Na]^-$, 100), 1165 ($[M_{Na,Na}$ − 3-*O*-MeXyl − H − Na$]^-$, 4), 903 ($[M_{Na,Na}$ − 3-*O*-MeXyl-*O*-Glc-OSO_3Na − H − Na$]^-$, 11), 887 ($[M_{Na,Na}$ − 3-*O*-MeXyl-*O*-Glc-OSO_3Na − O + H − Na$]^-$, 3), 741 ($[M_{Na,Na}$ − 3-*O*-MeXyl-*O*-Glc-OSO_3Na − Glc + 2H − Na$]^-$, 8), 695 ([3-*O*-MeXyl-*O*-Glc-OSO_3Na-*O*-Qui-*O*-Glc − H − Na$]^-$, 9), 595 ($[M_{Na,Na}$ − 3-*O*-MeXyl-*O*-Glc-OSO_3Na-*O*-Qui-*O*-Glc + H − Na$]^-$, 24), 533 ([3-*O*-MeXyl-*O*-Glc-OSO_3Na-*O*-Qui-O − Na$]^-$, 7), 517 ([3-*O*-MeXyl-*O*-Glc-OSO_3Na-*O*-Qui − Na$]^-$, 64), 405 ([3-*O*-MeXyl-Glc-OSO_3Na − O + H − Na$]^-$, 5), 387 ([3-*O*-MeXyl-Glc-OSO_3Na − H − Na$]^-$, 43); API−ESMS$^{(+)}$ *m/z* (rel int, %) 1359 ($[M_{Na,Na} + Na]^+$, 72), 1257 ($[M_{Na,Na} - SO_3Na + H + Na]^+$, 48), 1155 ($[M_{Na,Na} - 2SO_3Na + 2H + Na]^+$, 100), 1095 ($[M_{Na,Na} - SO_3Na - Glc + 2H + Na]^+$, 11), 1009 ([1155 − 3-*O*-MeXyl + H$]^+$, 29), 993 ([1155 − Glc + H$]^+$, 14), 949 ($[M_{Na,Na}$ − 3-*O*-MeXyl-*O*-Glc-$OSO_3Na + H + Na]^+$, 17), 743 ([3-*O*-MeXyl-*O*-Glc-OSO_3Na-*O*-Qui-*O*-Glc + H + Na$]^+$, 98), 639 ($[M_{Na,Na}$ − 3-*O*-MeXyl-*O*-Glc-OSO_3Na-*O*-Qui-*O*-Glc − H +Na$]^+$, 33), 625 ({$M_{Na,Na}$ − 3-*O*-MeXyl-*O*-Glc-OSO_3Na-*O*-Qui-[*O*-Glc]-*O* − H + Na}$^+$, 27), 563 ([3-*O*-MeXyl-*O*-Glc-OSO_3Na-*O*-Qui + Na$]^+$, 13), 407 ({$M_{Na,Na}$ − 3-*O*-MeXyl-*O*-Glc-OSO_3Na-*O*-Qui-[*O*-Glc]-*O*-Xyl-OSO_3Na}$^+$, 12).

Calcigeroside D_2 (2): mp 242−244 °C, $[\alpha]^{20}_D$ −22.0° (*c* 0.1, pyridine/water, 1:1); ^{13}C and ^1H NMR, see Tables 3 and 4; API−ESMS$^{(-)}$ *m/z* (rel int, %) 1399 ($[M_{Na,Na} - Na]^-$, 57), 1341 (8), 1337 (4), 1313 (7), 1297 ($[M_{Na,Na} - SO_3Na + H - Na]^-$, 81), 989 ($[M_{Na,Na}$ − 3-*O*-MeXyl-*O*-Glc-OSO_3Na − H − Na$]^-$, 18), 702 ($[M_{Na,Na}$ − 3-*O*-MeXyl-*O*-Glc-OSO_3Na-*O*-Qui-*O*-Glc − H − Na$]^-$, 8), 695 ([3-*O*-MeXyl-*O*-Glc-OSO_3Na-*O*-Qui-*O*-Glc − H − Na$]^-$, 18), 688 ({$M_{Na,Na}$ − 3-*O*-MeXyl-*O*-Glc-OSO_3Na-*O*-Qui-[*O*-Glc]-*O* + H − Na}$^-$, 100), 616 ([3-*O*-MeXyl-*O*-Glc-OSO_3Na-*O*-Qui-*O*-Glc − SO_3Na$]^-$, 48), 517 ([3-*O*-MeXyl-*O*-Glc-OSO_3Na-*O*-Qui − Na$]^-$, 91), 445 (53), 387 ([3-*O*-MeXyl-*O*-Glc-OSO_3Na − H − Na$]^-$, 51); API−ESMS$^{(+)}$ *m/z* (rel int, %) 1445 ($[M_{Na,Na} + Na]^+$, 26), 1343 ($[M_{Na,Na} - SO_3Na + H + Na]^+$, 100), 1241 ($[M_{Na,Na} - 2SO_3Na + 2H + Na)]^+$, 93), 1181 ($[M_{Na,Na} - SO_3Na - Glc + 2H + Na]^+$, 19), 1095 ($[M_{Na,Na} - 2SO_3Na$ − 3-*O*-MeXyl + 3H + Na$]^+$, 38), 1035 ($[M_{Na,Na}$ − 3-*O*-MeXyl-*O*-Glc-OSO_3Na + H + Na$]^+$, 20), 933 ($[M_{Na,Na}$ − 3-*O*-MeXyl-*O*-Glc-$OSO_3Na - SO_3Na + H + Na]^+$, 27), 873 ($[M_{Na,Na}$ − 3-*O*-MeXyl-*O*-Glc-$OSO_3Na - Glc + 2H + Na]^+$, 28), 493 ({$M_{Na,Na}$ − 3-*O*-MeXyl-*O*-Glc-OSO_3Na-*O*-Qui-[*O*-Glc]-*O*-Xyl-$OSO_3Na + H + Na$}$^+$, 20), 477 ({$M_{Na,Na}$ − 3-*O*-MeXyl-*O*-Glc-OSO_3Na-*O*-Qui-[*O*-Glc]-*O*-Xyl-OSO_3Na-*O* + H + Na}$^+$, 19), 369 (24).

Calcigeroside E (3): mp 239−241 °C, $[\alpha]^{20}_D$ −20.0° (*c* 0.1, pyridine/water, 1:1); ^{13}C and ^1H NMR, see Tables 5 and 6; API−ESMS$^{(-)}$ *m/z* (rel int, %) 1487 ($[M_{Na,Na}$ −Na$]^-$, 13), 1385 ($[M_{Na,Na} - SO_3Na + H - Na]^-$, 27), 1209 ($[M_{Na,Na}$ − 3-*O*-MeGlc-OSO_3Na + H − Na$]^-$, 7), 1193 ($[M_{Na,Na}$ − 3-*O*-MeGlc-OSO_3-Na-*O* + H − Na$]^-$, 4), 1047 ($[M_{Na,Na}$ − 3-*O*-MeGlc-OSO_3Na-*O*-Glc + H − Na$]^-$, 4), 867 ($[M_{Na,Na}$ − 3-*O*-MeGlc-OSO_3Na-*O*-Glc-*O*-Glc − Na$]^-$, 3), 732 (100), 579 ({3-*O*-MeGlc-OSO_3Na-*O*-Glc-*O*-Qui-[*O*-Glc]-*O* − Na}$^-$, 5), 547 ([3-*O*-MeGlc-OSO_3Na-*O*-Glc-*O*-Qui − Na$]^-$, 55), 435 ([3-*O*-MeGlc-OSO_3Na-*O*-Glc-*O* + H − Na$]^-$, 4), 417 ([3-*O*-MeGlc-OSO_3Na-*O*-Glc$]^-$, 10), 273 ([3-*O*-MeGlc-OSO_3Na-*O* + H − Na$]^-$, 14), 255 ([3-*O*-MeGlc-OSO_3-Na − H − Na$]^-$, 77); FABMS (positive-ion mode) *m/z* (rel int, %) 1549 ($[M_{Na,Na} + K]^+$, 38), 1533 ($[M_{Na,Na} + Na]^+$, 3), 1527 ($[M_{Na,K} + H]^+$, 83), 1431 ($[M_{Na,Na} - SO_3Na + H + Na]^+$, 100), 1371 ($[M_{Na,Na} - Glc + H + Na]^+$, 35), 1255 ($[M_{Na,Na}$ − 3-*O*-MeGlc-$OSO_3Na + H + Na]^+$, 35), 1253 ($[M_{Na,Na}$ − 3-*O*-MeGlc-$OSO_3Na - H + Na]^+$, 30), 1075 ($[M_{Na,Na}$ − 3-*O*-MeGlc-OSO_3Na-*O*-Glc-*O* − H + Na$]^+$, 29), 897 ($[M_{Na,Na}$ − 3-*O*-MeGlc-OSO_3Na-*O*-Glc-*O* − Glc-*O* + Na$]^+$, 76), 769 ($[M_{Na,Na}$ − 3-*O*-MeGlc-OSO_3Na-*O*-Glc-*O*-Qui-[*O*-Glc]-*O* + H + Na$]^+$, 5), 464 ([3-*O*-MeGlc-OSO_3Na-*O*-Glc + Na$]^+$, 9), 442 ([3-*O*-MeGlc-OSO_3Na-*O*-Glc + H$]^+$, 16).

Hydrolysis of Calcigerosides D_1 (1) and D_2 (2). The glycoside (5 mg) was dissolved in 5 mL of 1 N trifluoroacetic acid and heated in an ampule at 100 °C for 1 h. The solution was decanted from an aglycon precipitate and concentrated. Pyridine 1 mL and NH_2OH·HCl 2 mg were added to the dry residue, and the mixture was heated at 100 °C for 1 h. After that time, 1 mL of Ac_2O was added, and heating at 100 °C was continued for a further 1 h. After removing the solvents, the resulting aldononitrile peracetates were analyzed by GC−MS, using standard aldononitrile peracetates as reference samples. Xylose, quinovose, glucose, and 3-*O*-Me-xylose were identified for the glycosides **1** and **2** in a ratio (1:1:2:1).

Desulfation of Calcigeroside D_1 (1). The glycoside **1** (45 mg) was dissolved in a mixture of pyridine/dioxane (1:1), heated under reflux for 1 h, and the mixture then concentrated under reduced pressure. The residue was chromatographed on a Si gel column with $CHCl_3$/MeOH/H_2O (700:125:7) to give the desulfated derivative **4** (23 mg), mp 194−196 °C, $[\alpha]^{20}_D$ −50.0° (*c* 0.1, pyridine).

Desulfation of Calcigeroside D_2 (2). The glycoside **2** (30 mg) was desulfated as described above to give the desulfated derivative **5** (19 mg), mp 188−190 °C, $[\alpha]^{20}_D$ −27.0° (*c* 0.1, pyridine).

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References and Notes

(1) Avilov, S. A.; Antonov, A. S.; Drozdova, O. A.; Kalinin, V. I.; Kalinovsky, A. I.; Stonik, V. A.; Riguera, R.; Lenis, L. A.; Jimenez, C. *J. Nat. Prod.* **2000**, *63*, 65−71.
(2) Sashkov, A. S.; Chizhov, O. S. *Bioorgan. Khim.* **1976**, *2*, 437−497.
(3) Kalinovsky, A. I. *Khim. Prir. Soedin.* **1988**, 605−606.
(4) Miyamoto, T.; Togawa, K.; Higuchi, R.; Komori, T.; Sasaki, T. *Liebigs Ann. Chem.* **1990**, 453−460.
(5) Rodriguez, J.; Castro, R.; Riguera, R. *Tetrahedron* **1991**, *47*, 4753−4762.
(6) Drozdova, O. A.; Avilov, S. A.; Kalinin, V. I.; Kalinovsky, A. I.; Stonik, V. A.; Riguera, R.; Jiménez, C. *Liebigs Ann. Chem.* **1997**, 2351−2356.
(7) Kalinin, V. I.; Avilov, S. A.; Kalinovsky, A. I.; Stonik, V. A.; Milgrom, Y. M.; Rashkes, Y. W. *Khim. Prir. Soedin.* **1992**, 691−694.
(8) Kalinin, V. I.; Avilov, S. A.; Kalinina, E. Y.; Korolkova; O. G., Kalinovsky; A. I., Stonik, V. A.; Riguera, R.; Jiménez C. *J. Nat. Prod.* **1997**, *60*, 817−819.
(9) Stonik, V. A.; Kalinin, V. I.; Avilov, S. A. *J. Nat. Toxins* **1999**, *8*, 235−247.

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