Triterpene Glycosides from Antarctic Sea Cucumbers. 1. Structure of Liouvillosides A_1, A_2, A_3, B_1, and B_2 from the Sea Cucumber *Staurocucumis liouvillei*: New Procedure for Separation of Highly Polar Glycoside Fractions and Taxonomic Revision

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Five new triterpene glycosides, liouvillosides A_1 (**1**), A_2 (**2**), A_3 (**3**), B_1 (**4**), and B_2 (**5**), have been isolated from the Antarctic sea cucumber *Staurocucumis liouviellei* along with the known liouvilloside A (**6**), isolated earlier from the same species, and hemoiedemosides A (**7**) and B (**8**), isolated earlier from the Patagonian sea cucumber *Hemioedema spectabilis*. The isolation was carried out using a new chromatographic procedure including application of ion-pair reversed-phase chromatography followed by chiral chromatography on a cyclodextrin ChiraDex column. The structures of the new glycosides were elucidated using extensive NMR spectroscopy (^{1}H and ^{13}C NMR spectrometry, DEPT, ^{1}H−^{1}H COSY, HMBC, HMQC, and NOESY), ESI-FTMS, and CID MS/MS, and chemical transformations. Glycosides **1**−**3** are disulfated tetraosides and glycosides **4** and **5** are trisulfated tetraosides. Glycosides **2** and **3** contain 3-*O*-methylquinovose, found for the first time as a natural monosaccharide in sea cucumber glycosides. On the basis of analyses of glycoside structures a taxonomic revision is proposed.

Holothurians contain a great diversity of triterpene glycosides belonging mainly to the so-called holostane series. Their carbohydrate chains are comprised of two to six monosaccharide units.[1−4] Holothurian triterpene glycosides are good taxonomic markers and have been used successfully to improve sea cucumber taxonomy.[2] These natural products have been found in many sea cucumber species collected from the tropical Pacific and Indian Oceans,[1] Mediterranean Sea,[5] North Atlantic,[6] and North Pacific.[7] Nevertheless only one paper has focused on the study of triterpene glycosides from an Antarctic sea cucumber: *Staurocucumis liouvillei* (Vaney) (family Cucumariidae, order Dendrochirotida).[8] Two trisulfated tetraosides, liouvillosides A and B, were isolated from this holothurian. It is generally known that sea cucumbers belonging to the Cucumariidae have very complicated and often completely nonseparable mixtures of mono-, di-, and trisulfated triterpene glycosides.[2,9] This explains why it is often so difficult to isolate any glycoside constituents from glycoside fractions. As part of our search for new triterpene glycosides from sea cucumbers belonging to the Cucumariidae,[6,7,10,11] we report the reinvestigation of the glycoside fraction of *S. liouvillei*. A new procedure for separation of complicated polysulfated glycoside fractions was developed, and the taxonomic positions of two sea cucumber species from the Antarctic were investigated.

Results and Discussion

Twenty-three specimens of the sea cucumber *S. liouvillei* were collected near the sub-Antarctic Island of Buovet (South Atlantic Ocean) during the Antarctic expedition ANT XXI/2. Collections were made in November 2003 from the research vessel *Polarstern* (Alfred Wegener Institute for Polar and Marine Research) at 134 m depth using an Agassiz trawl and were extracted with ethanol. The concentrated ethanolic extract was chromatographed on a column with Teflon powder Polychrom-1 for desalting and elimination of polar substances and a Si gel column with $CHCl_3$/EtOH/H_2O (100:100:17 and 100:125:25) to yield two glycoside subfractions, A and B. Further separation of these subfractions by HPLC using various direct and reversed-phased columns in different chromatographic systems was unsuccessful because of high polarities of the glycosides and their small structural differences, sometimes in the position of a double bond in polycyclic moieties of the aglycones only (for example, between **1** and **7** or between **4** and **8**). We succeeded in separating the subfractions into eight individual glycosides by applications of direct-phase and reversed-phase ion-pair chromatography followed by ion-pair chiral chromatography on a cyclodextrin Aligrent ChiraDex column. Subfraction A was separated by HPLC on Supelco Sil [$CHCl_3$/EtOH/NH_4OAc (1 M solution), 65:45:2], Supelco C-18 [EtOH/H_2O/NH_4OAc (1 M solution), 55:45:2], and cyclodextrin Agilent ChiraDex [CH_3CN/H_2O/NH_4OAc (1 M solution), 60:40:1] columns, yielding liouvillosides A_1 (**1**), A_2 (**2**), and A_3 (**3**) and the known glycoside identified by ^{13}C NMR spectrum as hemoiedemoside A (**7**), previously reported from *Hemioedema spectabilis*.[12] Subfraction B was subsequently separated by HPLC on Supelco C-18 [EtOH/H_2O/NH_4OAc (1 M solution), 55:45:2] and Agilent ChiraDex BETA [CH_3CN/H_2O/NH_4OAc (1 M solution), 65:35:1, 55:45:1, 50:50:1] columns to give liouvellosides B_1 (**4**) and B_2 (**5**) and the previously known liouvilloside A (**6**) from *Staurocucumis liouvillei*[8] and hemoiedemoside B (**8**) from *H. spectabilis*.[12] This is the first use of chiral chromatography to separate these types of glycoside mixtures.

The ^{13}C NMR spectra of aglycone parts of the glycosides **1**, **2**, and **4** were identical and coincident with the aglycone parts of known cucumarioside A_2-2 from *Cucumaria japonica*[13] (Table 1). The common aglycone of **1**, **2**, and **4** belongs to the holostane type [from the signals of a 18(20)-lactone at δ 179.3 (C-18) and 83.9 ppm (C-20) in the ^{13}C NMR spectrum] and contains a 7(8)-double bond [the signals of a tertiary carbon (C-7) at 121.8 ppm and a quaternary carbon (C-8) at 143.9 ppm in the ^{13}C NMR spectrum] and a 16-keto group [the signals of a quaternary carbon at 214.3

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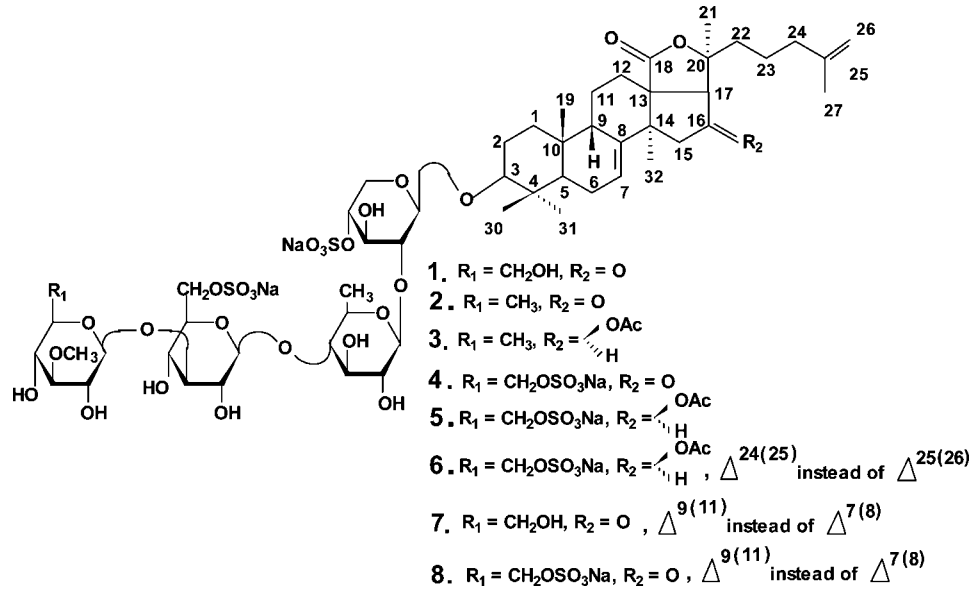
Table 1. ^{13}C and ^{1}H NMR Chemical Shifts and HMBC and NOESY Correlations of Aglycone Moieties of the Glycosides **1**, **2**, and **4**

position	δ_C mult.[a]	δ_H mult. (J in Hz)[b]	HMBC	NOESY
1	35.7 CH	1.44 m		H-19
2	26.8 CH_2	2.08 m, 1.89 m		H-19, H-30
3	80.3 CH	3.28 dd (4.0, 11.5)		H-5, H1-Xyl
4	39.3 C			
5	48.2 CH	1.02 dd (4.0, 11.0)		H-3
6	23.1 CH_2	2.04 m		H-19, H-30, H-31
7	121.8 CH	5.76 m		H-15, H-32
8	143.9 C			
9	47.1 CH	3.63 brd (14.5)		H-19
10	35.4 C			
11	22.3 CH_2	1.92 m 1.65 m		H-32
12	29.5 CH_2	2.33 m		H-17, H-21
13	56.8 C			
14	45.6 C			
15	51.9 CH_2	2.77 d (15.8) 2.42 d (15.8)	C: 13	H-7, H-32
16	214.3 C			
17	63.4 CH	2.99 s	C: 12, 13, 16, 18, 21	H-12, H-21, H-32
18	179.3 C			
19	23.9 CH_3	1.22 s	C: 1, 5, 9, 10	H-1, H-2, H-6, H-9
20	83.9 C			
21	26.1 CH_3	1.58 s	C: 17, 20, 22	H-12, H-17
22	38.2 CH_2	1.81 m, 1.66 m		
23	22.1 CH_2	1.83 m, 1.52 m		
24	37.8 CH_2	2.00 m		
25	145.5 C			
26	110.5 CH_2	4.79 brd (6.7)	C: 24, 27	
27	22.1 CH_3	1.73 s	C: 24, 25,26	
30	17.2 CH_3	1.11 s	C: 3, 4, 5, 31	H-2, H-6, H6-Qui
31	28.6 CH_3	1.28 s	C: 3, 4, 5, 30	H1-Xyl, H6
32	31.9 CH_3	1.28 s	C: 8, 13, 14, 15	H-7, H-11, H-15, H-17

[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).



ppm in the ^{13}C NMR spectrum]. Signals of C-25 at 145.5 ppm and C-26 at 110.5 ppm indicate a 25(26)-double bond in the side chain (Table 1). The structure of the aglycone of liouvillosides A_1 (**1**), A_2 (**2**), and B_1 (**4**) as 16-keto-holosta-7,25-dien-3β-ol was also confirmed by 2D NMR spectroscopic data.

The ^{13}C NMR spectrometric data of the aglycone parts of new glycosides **3** and **5** were shown to be identical to one another (Table 2) and coincident with those of cucumarioside A_0-2 found in the sea cucumber *Cucumaria japonica*[14] and in the glycosides of many other species of sea cucumbers.[1,7,15,16] The common aglycone of **3** and **5** belongs to the holostane type [from 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 a 7(8)-double bond [signals of a tertiary carbon (C-7) at 120.2 ppm and a quaternary carbon (C-8) at 145.5 ppm in the ^{13}C NMR spectrum] and a 16β-acetoxy group [signals at 74.9 ppm (C-16), quaternary carbon (O$\underline{C}$OCH$_3$) at 169.6 ppm, and the methyl carbon (OCO$\underline{C}$H$_3$) at 21.1 ppm in the ^{13}C NMR spectrum]. 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 2). The structure of the aglycone of liouvillosides A_3 (**3**) and B_2 (**5**) as 16β-acetoxyholosta-7,25-dien-3β-ol was confirmed by 2D NMR spectroscopic data (Table 2).

The ESI-FTMS (negative ion mode) of liouvilloside A_1 (**1**) exhibited pseudomolecular ion peaks at *m/z* 1265.4310 (calc 1265.4337) $[M_{2Na} - Na]^-$, 1243.4460 (calc 1243.4518) $[M_{H,Na} - Na]^-$, and 621.2210 (calc 621.2223) $[M_{2Na} - 2Na]^{2-}$. This and ^{13}C NMR spectroscopic data allowed the determination of the molecular formula of **1** as $C_{54}H_{82}O_{28}S_2Na_2$.

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 104.2−104.8 ppm, correlated by the HSQC spectrum with the corresponding signals of anomeric

Table 2. ^{13}C and ^{1}H NMR Chemical Shifts and HMBC and NOESY Correlations of Aglycone Moieties of the Glycosides **3** and **5**

position	δ_C mult.[a]	δ_H mult. (*J* in Hz)[b]	HMBC	NOESY
1	35.9 CH_2	1.45 m		H-3, H-5, H-11
2	27.0 CH_2	2.11 m, 1.90 m		
3	88.7 CH	3.26 dd (4.0, 11.6)		H-1, H-5, H1-Xyl
4	39.3 C			
5	47.9 CH	1.03 t (7.0)	C: 4, 10, 19, 30	H-1, H-3, H-31
6	23.1 CH_2	2.04 m		H-19, H-30
7	120.2 CH	5.67 m		H-15, H-32
8	145.5 C			
9	47.0 CH	3.48 m		H-19
10	35.4 C			
11	22.4 CH_2	1.81 m 1.53 m		H-1 H-32
12	31.2 CH_2	2.16 m 1.98 m		H-21 H-17
13	59.1 C			
14	47.3 C			
15	43.5 CH_2	2.60 dd (7.5, 12.5) 1.75 m	C: 17	H-7, H-32
16	74.9 CH	5.93 brq (8.7)		H-32
17	54.6 CH	2.62 d (9.0)	C: 13, 18	H-21, H-12
18	179.3 C			
19	23.8 CH_3	1.23 s	C: 1, 5, 9, 10	H-9, H-6
20	84.8 C			
21	28.0 CH_3	1.49 s	C: 17, 20, 22	H-12, H-17
22	38.3 CH_2	2.38 m, 1.91 m		
23	23.1 CH_2	1.46 m		
24	38.2 CH_2	2.01 m		
25	145.3 C			
26	110.6 CH_2	4.81 brs	C: 24, 27	
27	22.0 CH_3	1.70 s	C: 24, 25,26	
30	17.0 CH_3	1.07 s	C: 3, 4, 5, 31	H-6 MeQui, H-6
31	28.4 CH_3	1.26 s	C: 3, 4, 5, 30	H-5, H-1 Xyl
32	32.0 CH_3	1.16 s	C: 8, 13, 14, 15	H-7, H-11, H-15, H-16
$OCOCH_3$	169.9 C, 21.1 CH_3	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 3. ^{13}C and ^{1}H NMR Chemical Shifts and HMBC and NOESY Correlations of the Carbohydrate Moiety of Glycoside **1**

atom	δ_C mult.[a,b,c]	δ_H mult. (*J* in Hz)[d]	HMBC	NOESY
		Xyl (1→C-3)		
1	104.8 CH	4.77 d (7.2)	C: 3	H-3, H-3,5 Xyl, H-31
2	**82.3** CH	4.08 dd (7.2, 8.9)	C: 3 Xyl	H-1 Qui
3	75.0 CH	4.35 t (8.9)	C: 2, 4 Xyl	H-1,5 Xyl
4	*76.2* CH	5.10 m	C: 3 Xyl	
5	64.0 CH_2	4.91 dd (5.0, 11.4) 3.85 dd (9.6, 11.8)	C: 3 Xyl	H-1,3 Xyl
		Qui (1→2Xyl)		
1	104.6 CH	5.09 d (7.4)	C: 2 Xyl	H-2 Xyl, H-3,5 Qui
2	75.4 CH	3.98 t (9.0)		
3	75.2 CH	4.02 t (9.2)	C: 2 Qui	H-1,5 Qui
4	**86.9** CH	3.54 t (8.7)	C: 1 Glc; 3 Qui	H-1 Glc, H-6 Qui
5	71.3 CH	3.72 m		H-1,3 Qui
6	17.8 CH_3	1.70 d (6.0)	C: 4,5 Qui	H-4 Qui, H-1 Glc, H-30
		Glc (1→4Qui)		
1	104.2 CH	4.87 d (8.0)	C: 4-Qui	H-4,6 Qui, H-3,5 Glc
2	73.5 CH	3.95 t (7.9)		
3	**85.9** CH	4.29 t (9.1)	C: 1 MeGlc, 2,4 Glc	H-1 MeGlc, H-1,5 Glc
4	69.2 CH	3.94 t (8.7)		
5	75.0 CH	4.20 m		H-1,3 Glc
6	*67.2* CH_2	5.11 m4.72 dd (6.6, 11.3)		
		MeGlc (1→3Glc)		
1	104.4 CH	5.32 d (7.8)	C: 3 Glc	H-3 Glc, H-3 MeGlc
2	74.5 CH	3.96 dd (7.5, 9.2)		
3	86.9 CH	3.79 t (9.0)	C: 4 MeGlc, OMe	H-1 MeGlc
4	70.3 CH	4.01 m		
5	77.5 CH	4.01 m		
6	61.7 CH_2	4.45 brd (11.2) 4.16 m	C: 5 MeGlc	
Ome	60.7 CH_3	3.91 s	C: 3 MeGlc	

[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).

protons at 4.77 (d, $J = 7.2$ Hz), 4.87 (d, $J = 8.0$ Hz), 5.09 (d, $J = 7.4$ Hz), and 5.32 (d, $J = 7.8$ Hz) ppm (Table 3). The coupling constants of the anomeric protons were indicative of β-configuration of the glycosidic bonds in all cases.[17]

The ^{13}C NMR and DEPT spectra of the carbohydrate part of **1** were similar to those of the known holothurin A_2 from *Holothuria edulis*[18] and other glycosides belonging to holothurin A type[19,1,4] with a linear tetrasaccharide carbohydrate chain consisting of D-xylose (sulfated at C-4), D-quinovose, D-glucose, and 3-*O*-methyl-D-glucose. The differences were in the third monosaccharide residue (glucose), where the signal of C-6 was shifted downfield 4.8 ppm and C-5 was shifted upfield 2.7 ppm, corresponding to α- and β-shifted effects of sulfate groups.[17] Hence, the liouvilloside A_1 (**1**) carbohydrate chain contains an additional sulfate at C-6 of the

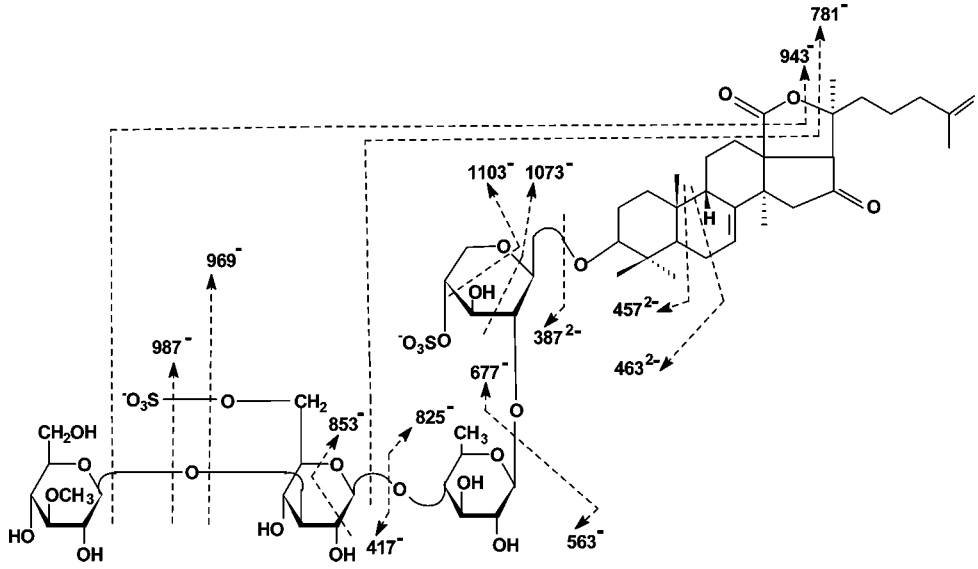


Figure 1. Main CID-type fragmentations in (−) ESI-FTMS/MS of the ion $[M_{2Na} - 2Na]^{2-}$ at *m/z* 621.22 of liouvilloside A_1 (**1**).

glucose residue. The position of the sulfate group in the glucose residue was confirmed by TOCSY experiment ($t_m = 100$ ms), where cross-peaks between all carbohydrate protons (CHO) of sulfated glucose including H-1 at 4.87 ppm and 2 H-6 at 5.11 and 4.72 ppm were observed.

The positions of interglycosidic linkages were deduced from NOESY and HMBC spectra (Table 3), where cross-peaks between H-1 of the xylose residue and H-3 (C-3) of the aglycone, H-1 of the quinovose and H-2 (C-2) of the xylose residue, H-1 of the glucose and H-4 (C-4) of the quinovose unit, and H-1 of 3-*O*-methylglucose and H-3 (C-3) of glucose unit, respectively, were observed.

The sequence of monosaccharide units in the carbohydrate chain was confirmed by (−) ESI-FTMS/MS data. The MS/MS spectrum of the ion of $[M_{2Na} - 2Na]^{2-}$ at *m/z* 621.22 of **1** indicated the peaks for fragment ions at *m/z* 1145.48 $[M_{2Na} - NaSO^-_4 - Na - H]^-$, 987.42 $[M_{2Na} - MeGlc - NaSO_3 + 2H - Na]^-$, 969.41 $[M_{2Na} - OMeGlc - NaSO_3 - Na]^-$, 943.44 $[M_{2Na} - MeGlc - NaSO_3 + 2H - Na - CO_2]^-$, 925.42 $[M_{2Na} - OMeGlc - NaSO_3 - Na - CO_2]^-$, 825.37 $[M_{2Na} - MeGlc - GlcNaSO_3 + H - Na]^-$, 781.38 $[M_{2Na} - MeGlc - Glc - NaSO_3 + H - Na - CO_2]^-$, 679.31 $[M_{2Na} - MeGlu - Glc - NaSO_3 - Qui + H - Na]^-$, 563.13 $[M_{2Na} - Xyl - NaSO_3 - OAgl - H - Na]^-$, 417.07 $[M_{2Na} - Qui - Xyl - NaSO_3 - OAgl - H - Na]^-$, and 387.06 $[M_{2Na} - OAgl - 2Na - H]^{2-}$ (Figure 1).

Acid hydrolysis of liouvilloside A_1 (**1**) with TFA was carried out to ascertain its monosaccharide composition. The mixture of sugars obtained was submitted to HPLC to give individual monosaccharides. Subsequent alcoholysis of each monosaccharide by (*R*)-(−)-2-octanol followed by acetylation, GLC analysis, and comparison with standard monosaccharides allowed us to determine the absolute D-configuration of all monosaccharide residues comprising the carbohydrate moiety of **1** (xylose, quinovose, glucose, and 3-*O*-methylglucose).

All these data indicated that liouvilloside A_1 (**1**) is 3*β*-*O*-[3-*O*-methyl-*β*-D-glucopyranosyl-(1→3)-6-*O*-sodium sulfate-*β*-D-glucopyranosyl-(1→4)-*β*-D-quinovopyranosyl-(1→2)-4-*O*-sodium sulfate-*β*-D-xylopyranosyl]-16-keto-holosta-7,25-diene.

The ESI-FTMS (negative ion mode) of liouvilloside A_2 (**2**) exhibited pseudomolecular ion peaks at *m/z* 1249.4420 (calc 1249.4393) $[M_{2Na} - Na]^-$, 1227.4537 (calc 1227.4568) $[M_{H,Na} - Na]^-$, 1147.5017 (calc 1147.5000) $[M_{H,Na} - NaSO_3]^-$, and 613.2201 (calc 613.2248) $[M_{2Na} - 2Na]^{2-}$, which along with the ^{13}C NMR spectroscopic data allowed the determination of the molecular formula of **2** as $C_{54}H_{82}O_{27}S_2Na_2$.

The carbohydrate chain of **2** consisted of four monosaccharide residues as deduced from the ^{13}C NMR spectrum, which showed the signals of four anomeric carbons at 104.9−105.2 ppm, correlated by the HSQC spectrum with the corresponding signals of anomeric protons at 4.71 (d, $J = 7.4$ Hz), 4.94 (d, $J = 7.6$ Hz), 4.84 (d, $J = 7.9$ Hz), and 5.16 (d, $J = 7.9$ Hz) ppm (Table 4). The coupling constants of the anomeric protons were indicative of a *β*-configuration of the glycosidic bonds in all cases.[17]

The ^{13}C NMR and DEPT spectra of **2** were similar to those of liouvilloside A_1 (**1**). The differences were in the terminal monosaccharide residue, where the signal of C-6 characteristic for 3-*O*-methylglucose was absent, but the signal of CH_3 at 18.0 ppm characteristic for an additional quinovose residue was present. Hence, the liouvilloside A_2 (**2**) carbohydrate chain contains 3-*O*-methylquinovose instead of 3-*O*-methylglucose as a terminal monosaccharide residue. Indeed, the signals of the terminal monosaccharide residue of **2** in the ^{13}C NMR spectra were similar to those of model methyl-3-*O*-methyl-*β*-D-quinovopyranoside. The signals of the model compound were recorded on the mixture of methyl-3-*O*-methyl-*β*-D-quinovopyranoside and methyl-3-*O*-methyl-α-D-quinovopyranoside (1:2) after a 2D NMR study of the mixture. The mixture was obtained by methanolysis of methyl-3-*O*-methyl-α-D-quinovopyranoside with methanol saturated by HCl. The synthesis of methyl-3-*O*-methyl-α-D-quinovopyranoside was carried out as described earlier.[20]

The positions of interglycosidic linkages were deduced from NOESY and HMBC spectra (Table 4), where cross-peaks between H-1 of the xylose residue and H-3 (C-3) of the aglycone, H-1 of the quinovose and H-2 (C-2) of the xylose residue, H-1 of the glucose and H-4 (C-4) of the quinovose unit, and H-1 of 3-*O*-methylquinovose and H-3 (C-3) of glucose unit, respectively, were observed.

The sequence of monosaccharide units in the carbohydrate chain was confirmed by (−) ESI-FTMS/MS data. The MS/MS spectrum of the ion of $[M_{2Na} - 2Na]^{2-}$ at *m/z* 613.22 of **2** indicated the peaks for fragment ions at *m/z* 1129.49 $[M_{2Na} - NaSO_4 - Na - H]^-$, 987.42 $[M_{2Na} - MeQui - NaSO_3 + 2H - Na]^-$, 969.41 $[M_{2Na} - OMeQui - NaSO_3 - Na]^-$, 943.44 $[M_{2Na} - MeQui - NaSO_3 + 2H - Na - CO_2]^-$, 925.43 $[M_{2Na} - OMeQui - NaSO_3 - Na - CO_2]^-$, 825.37 $[M_{2Na} - MeQui - Glc - NaSO_3 + H - Na]^-$, 781.38 $[M_{2Na} - MeQui - Glc - NaSO_3 + H - Na - CO_2]^-$, 679.32 $[M_{2Na} - MeQui - Glc - NaSO_3 - Qui + H - Na]^-$, 379.06 $[M_{2Na} - OAgl - 2Na - H]^{2-}$, 547.13 $[M_{2Na} - Xyl - NaSO_3 - OAgl - H - Na]^-$, and 401.08 $[M_{2Na} - Qui - Xyl - NaSO_3 - OAgl - H - Na]^-$ (Figure 2).

Table 4. ^{13}C and ^{1}H NMR Chemical Shifts and HMBC and NOESY Correlations of the Carbohydrate Moiety of Glycosides **2** and **3**

atom	δ_C mult.[a,b,c]	δ_H mult. (*J* in Hz)[d]	HMBC	NOESY
		Xyl (1→C-3)		
1	105.1 CH	4.71 d (7.4)	C: 3	H-3, H-3,5 Xyl, H-31
2	**83.1** CH	4.01 dd (7.3, 9.3)	C: 1,4 Xyl	H-1 Qui
3	75.8 CH	4.35 t (9.5)	C: 2, 4 Xyl	H-1,5 Xyl
4	*75.6* CH	5.20 m		
5	64.1 CH_2	4.63 dd (5.6, 11.6) 3.71 m	C: 4 Xyl	H-1,3 Xyl
		Qui (1→2Xyl)		
1	105.0 CH	4.94 d (7.6)	C: 2 Xyl	H-2 Xyl, H-3,5 Qui
2	76.0 CH	3.85 t (8.4)	C: 1,3 Qui	H-4 Qui
3	75.0[f] CH	3.96 m	C: 1 Qui	H-1,5 Qui
4	**88.1** CH	3.49 t (8.6)	C: 1 Glc; 3,5 Qui	H-1 Glc, H-2,6 Qui
5	71.4 CH	3.72 m		H-1,3 Qui
6	17.9 CH_3	1.66 d (6.0)	C: 4,5 Qui	H-4 Qui, H-1 Glc, H-30
		Glc (1→4Qui)		
1	104.9 CH	4.84 d (7.9)	C: 4 Qui	H-4,6 Qui, H-3,5 Glc
2	73.4 CH	3.98 m		H-4 Glc
3	**86.8** CH	4.14 t (9.0)	2,4 Glc	H-1 MeQui, H-1,5 Glc
4	70.1 CH	3.81 t (9.2)		H-2,6 Glc
5	75.2 CH	4.29 td (9.5, 2.6)		H-1,3 Glc
6	*67.5* CH_2	5.29 dd (2.5, 10.9) 4.83 dd (8.9, 10.8)	C: 5 Glc	H-4 Glu
		MeQui (1→3Glc)		
1	105.2 CH/105.1[e]	5.16 d (7.9)	C: 3 Glc	H-3 Glc, H-3,5 MeQui
2	74.8[f] CH/74.7[e]	3.94 m		
3	87.4 CH/87.8[e]	3.58 m	C: 4 MeQui	H-1 MeQui
4	75.4 CH/75.7[e]	3.58 m	C: 3 MeQui	
5	73.0 CH/72.6[e]	3.73 m		H-1 MeQui
6	18.0 CH_3/18.0[e]	1.48 d (6.0)	C: 4,5 MeQui	
OMe	60.5 CH_3/60.6[e]	3.85 s	C: 3 MeQui	

[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). [e] Signals of model 3-*O*-methyl-β-D-methylquinovopyranoside. [f] Signals may be interchanged.

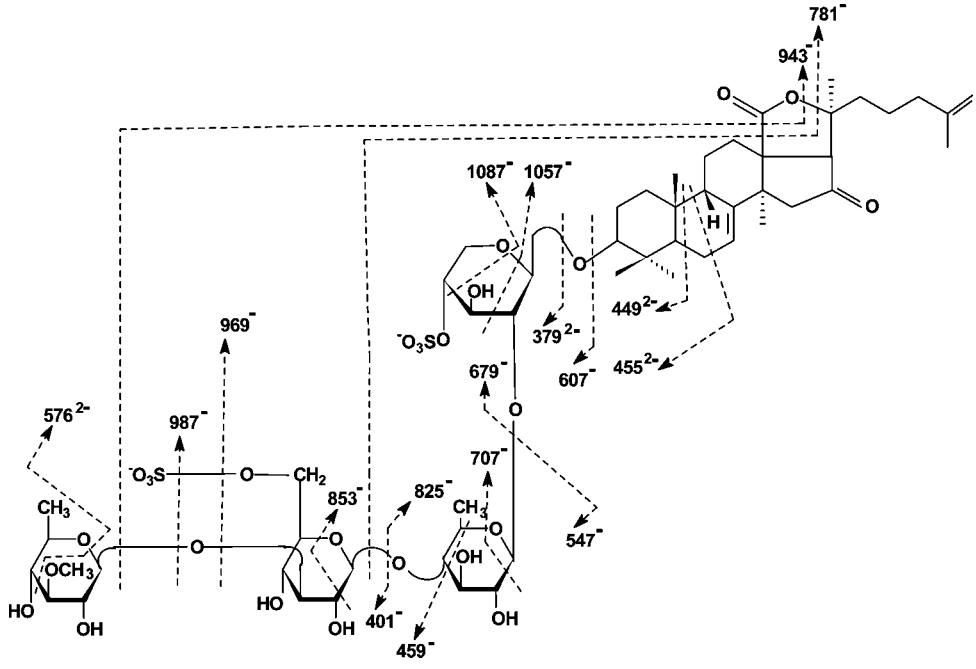


Figure 2. Main CID-type fragmentations in (−) ESI-FTMS/MS of the ion $[M_{2Na} - 2Na]^{2-}$ at *m/z* 613.22 of liouvilloside A_2 (**2**).

Liouvilloside A_2 (**2**) was hydrolyzed with TFA and treated as liouvilloside A_1 (**1**) to ascertain its monosaccharide composition. The absolute D-configurations of all monosaccharide residues (xylose, quinovose, glucose, and 3-*O*-methylquinovose) were determined. The reference sample of 3-*O*-methylquionovose was obtained by acid hydrolysis with TFA of α-methyl-3-*O*-methyl-D-quinovopyranoside.[20]

All these data indicated that liouvilloside A_2 (**2**) is 3β-*O*-[3-*O*-methyl-β-D-quinovopyranosyl-(1→3)-6-*O*-sodium sulfate-β-D-glucopyranosyl-(1→4)-β-D-quinovopyranosyl-(1→2)-4-*O*-sodium sulfate-β-D-xylopyranosyl]-16-keto-holosta-7,25-diene.

The ESI-FTMS (negative ion mode) of liouvilloside A_3 (**3**) exhibited a pseudomolecular ion peaks at *m/z* 1293.4678 (calc 1293.4650) $[M_{2Na} - Na]^-$, 1271.4810 (calc 1271.4831) $[M_{H,Na} - Na]^-$, 1191.5251 (calc 1191.5263) $[M_{H,Na} - NaSO_3]^-$, and 635.2339 (calc 635.2379) $[M_{2Na} - 2Na]^{2-}$, which along with the ^{13}C NMR spectroscopic data allowed the determination of the molecular formula of **3** as $C_{56}H_{86}O_{28}S_2Na_2$.

All the signals of the carbohydrate part of glycoside **3** in the NMR spectra including ^{13}C and ^{1}H NMR, NOESY, and HMBC were very close to those of liouvilloside A_2 (**2**) (Table 4).

The sequence of monosaccharide units in the carbohydrate chain was confirmed by (−) ESI-FTMS/MS data. The MS/MS spectrum of the ion of $[M_{2Na} - 2Na]^{2-}$ at *m/z* 635.24 of **3** indicated the peaks for fragment ions at *m/z* 1173.51 $[M_{2Na} - NaSO_4 - Na - H]^-$, 1031.45 $[M_{2Na} - MeQui - NaSO_3 + 2H - Na]^-$, 1013.44 $[M_{2Na} - OMeQui - NaSO_3 - Na]^-$, 869.40 $[M_{2Na} - MeQui - Glc - NaSO_3 + H - Na]^-$, 809.38 $[M_{2Na} - MeQui - Glc - NaSO_3 +$

Table 5. ^{13}C and ^{1}H NMR Chemical Shifts and Selected HMBC and NOESY Correlations of the Carbohydrate Moiety of Glycosides **4** and **5**

atom	δ_C mult.[a,b,c]	δ_H mult. (*J* in Hz)[d]	HMBC	NOESY
		Xyl (1→C-3)		
1	104.8 CH	4.79 d (6.8)	C: 3	H-3, H-3,5 Xyl, H-31
2	**82.4** CH	4.07 dd (7.3, 8.7)	C: 1 Qui; 3 Xyl	H-1 Qui
3	74.8 CH	4.35 t (8.8)	C: 2,4 Xyl	H-1,5 Xyl
4	*76.1* CH	5.10 m		
5	63.9 CH_2	4.91 dd (5.4, 11.8) 3.86 dd (9.2, 12.2)		H-1,3 Xyl
		Qui (1→2Xyl)		
1	104.6 CH	5.07 d (7.6)	C: 2 Xyl	H-2 Xyl, H-3,5 Qui
2	75.4 CH	3.97 t (8.0)	C: 1 Qui	H-4 Qui
3	75.1 CH	4.02 t (9.0)	C: 2 Qui	H-1,5 Qui
4	**87.0** CH	3.55 t (8.9)	C: 1 Glc; 3 Qui	H-1 Glc, H-2 Qui
5	71.3 CH	3.72 m		H-1,3 Qui
6	17.9 CH_3	1.70 d (6.0)		H-4 Qui, H-1 Glc, H-30
		Glc (1→4Qui)		
1	104.1 CH	4.89 d (7.8)	C: 4 Qui	H-4,6 Qui, H-3,5 Glc
2	73.5 CH	3.95 t (8.5)	C: 1 Glc	
3	**86.5** CH	4.26 t (9.0)	C: 2,4 Glc	H-1 MeGlc, H-1 Glc
4	69.3 CH	3.88 m	C: 3 Glc	
5	74.8 CH	4.23 m		H-1 Glc
6	*67.5* CH_3	5.13 brd (11.4) 4.70 dd (7.0, 11.2)		
		MeGlc (1→3Glc)		
1	104.8 CH	5.28 d (7.8)	C: 3 Glc	H-3 Glc, H-3,5 MeClc
2	74.4 CH	3.90 t (9.0)	C: 3 MeGlc	
3	86.3 CH	3.76 t (8.7)	C: 2,4 MeGlc	H-1 MeGlc
4	69.8 CH	4.15 t (9.9)		
5	75.5 CH	4.12 m		H-1 MeGlc
6	*67.0* CH_2	5.04 brd (10.4) 4.88 m		
OMe	60.5 CH_3	3.87 s	C: 3 MeGlc	

[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).

H − Na − AcOH]$^-$, 723.34 [M_{2Na} − MeQui − Glc − $NaSO_3$ − Qui + H − Na]$^-$, 605.23 [M_{2H} − AcOH − 2H]$^{2-}$, 583.23 [M_{2H} − AcOH − CO_2 − 2H]$^{2-}$, 547.13 [M_{2Na} − Xyl − $NaSO_3$ − OAgl − H − Na]$^-$, 401.08 [M_{2Na} − Qui − Xyl − $NaSO_3$ − OAgl − H − Na]$^-$, and 379.06 [M_{2Na} − OAgl − 2Na − H]$^{2-}$ (Figure 1, Supporting Information).

All these data indicated that liouvilloside A_3 (**3**) is 3*β*-*O*-[3-*O*-methyl-*β*-D-quinovopyranosyl-(1→3)-6-*O*-sodium sulfate-*β*-D-glucopyranosyl-(1→4)-*β*-D-quinovopyranosyl-(1→2)-4-*O*-sodium sulfate-*β*-D-xylopyranosyl]-16*β*-*O*-acetoxyholosta-7,25-diene.

The ESI-FTMS (negative ion mode) of liouvilloside B_1 (**4**) exhibited a pseudomolecular ion peaks at *m/z* 1367.3729 (calc 1367.3725) [M_{3Na} − Na]$^-$, 1345.3900 (calc 1345.3905) [$M_{H,2Na}$ − Na]$^-$, 1323.4063 (calc 1323.4086) [$M_{2H,Na}$ − Na]$^-$, 1243.4464 (calc 1243.4518) [$M_{H,2Na}$ − Na − $NaSO_3$]$^-$, 672.1898 (calc 672.1917) [M_{3Na} − 2Na]$^{2-}$, 661.1983 (calc 661.2007) [$M_{H,2Na}$ − 2Na]$^{2-}$, 621.2201 (calc 621.2223) [M_{3Na} − $NaSO_3$ − 2Na + H]$^{2-}$, 440.4627 (calc 440.4647) [M_{3Na} − 3Na]$^{3-}$, which along with the ^{13}C NMR spectroscopic data allowed the determination of the molecular formula of **4** as $C_{54}H_{81}O_{31}S_3Na_3$.

The ^{13}C NMR and DEPT spectra of the carbohydrate part of **4** were similar to those of liouvilloside A_1 (**1**) (Tables 1, 3, and 5). The differences were in the terminal monosaccharide residue (3-*O*-methylglucose), where the signal of C-6 was shifted downfield by 5.3 ppm and C-5 was shifted upfield by 2.0 ppm, which corresponded to α- and *β*-shifted effects of sulfate groups.[17] Hence, the liouvilloside B_1 (**4**) carbohydrate chain contains an additional sulfate at C-6 of the 3-*O*-methylglucose residue.

The positions of interglycosidic linkages were deduced from NOESY and HMBC spectra (Table 3), where cross-peaks between H-1 of the xylose residue and H-3 (C-3) of the aglycone, H-1 of the quinovose and H-2 (C-2) of the xylose residue, H-1 of the glucose and H-4 (C-4) of the quinovose unit, and H-1 of 3-*O*-methylglucose and H-3 (C-3) of glucose unit, respectively, were observed.

The sequence of monosaccharide units in the carbohydrate chain was confirmed by (−) ESI-FTMS/MS data. The spectrum of the ion [M_{3Na} − 3Na]$^{3-}$ at *m/z* 440.46 of **4** indicated the peaks for fragment ions at *m/z* 1127.49 [M_{3Na} − 2$NaSO_4$ − Na − 2H]$^-$, 969.42 [M_{3Na} − MeGlc − $NaSO_3$ − $NaSO_3$]$^{2-}$, 825.38 [M_{3Na} − MeGlc − $NaSO_3$ − Glc − $NaSO_3$ − Na + H]$^-$, 781.38 [M_{3Na} − MeGlc − $NaSO_3$ − GlcNaSO_3 − Na − CO_2 + H]$^-$, 679.32 [M_{3Na} − MeGlc − $NaSO_3$ − Glc − $NaSO_3$ − Qui − Na + H]$^-$, 612.22 [M_{3Na} − $NaSO_4$ − 2Na − H]$^{2-}$, 533.19 [M_{3Na} − MeGlc − $NaSO_3$]$^{2-}$, 417.07 [M_{3Na} − Qui − Xyl − $NaSO_3$ − OAgl − $NaSO_3$ − Na]$^-$, 321.04 [M_{3Na} − Xyl − $NaSO_3$ − OAgl − H − 2Na]$^{2-}$, 284.36 [M_{3Na} − OAgl − 3Na−H]$^{3-}$, 273.03 [M_{3Na} − Glc − $NaSO_3$ − Qui − Xyl − $NaSO_3$−OAgl − Na]$^-$, and 255.02 [M_{3Na} − GlcNaSO_3 − Qui − Xyl − $NaSO_3$ − OAgl − H − Na]$^-$ (Figure 3).

All these data indicated that liouvilloside B_1 (**4**) is 3*β*-*O*-[3-*O*-methyl-6-*O*-sodium sulfate-*β*-D-glucopyranosyl-(1→3)-6-*O*-sodium sulfate-*β*-D-glucopyranosyl-(1→4)-*β*-D-quinovopyranosyl-(1→2)-4-*O*-sodium sulfate-*β*-D-xylopyranosyl]-16-keto-holosta-7,25-diene.

The ESI-FTMS (negative ion mode) of liouvilloside B_2 (**5**) exhibited pseudomolecular ion peaks at *m/z* 1411.3992 (calc 1411.3987) [M_{3Na} − Na]$^-$, 1389.4145 (calc 1389.4168) [$M_{H,2Na}$ − Na]$^-$, 1367.4356 (calc 1367.4349) [$M_{2H,Na}$ − Na]$^-$, 694.2048 (calc 694.2048) [M_{3Na} − 2Na]$^{2-}$, 683.2134 (calc 683.2138) [$M_{H,2Na}$ − 2Na]$^{2-}$, and 455.1396 (calc 455.1401) [M_{3Na} − 3Na]$^{3-}$, which along with the ^{13}C NMR spectroscopic data allowed the determination of the molecular formula of **5** as $C_{56}H_{85}O_{32}S_3Na_3$.

The NMR spectra of the carbohydrate part of **5** were identical to those of the carbohydrate part of **4** (Table 5). The positions of interglycosidic linkages were deduced from NOESY and HMBC spectra (Table 5), where cross-peaks between H-1 of the xylose residue and H-3 (C-3) of the aglycone, H-1 of the quinovose and H-2 (C-2) of the xylose residue, H-1 of the glucose and H-4 (C-4) of the quinovose unit, and H-1 of 3-*O*-methylglucose and H-3 (C-3) of glucose unit, respectively, were observed.

The sequence of monosaccharide units in the carbohydrate chain was confirmed by (−) ESI-FTMS/MS data. The spectrum of the ion [M_{3Na} − 3Na]$^{3-}$ at *m/z* 455.14 of **5** indicated the peaks for fragment ions at *m/z* 555.20 [M_{3Na} − MeGlc − $NaSO_3$]$^{2-}$, 969.42

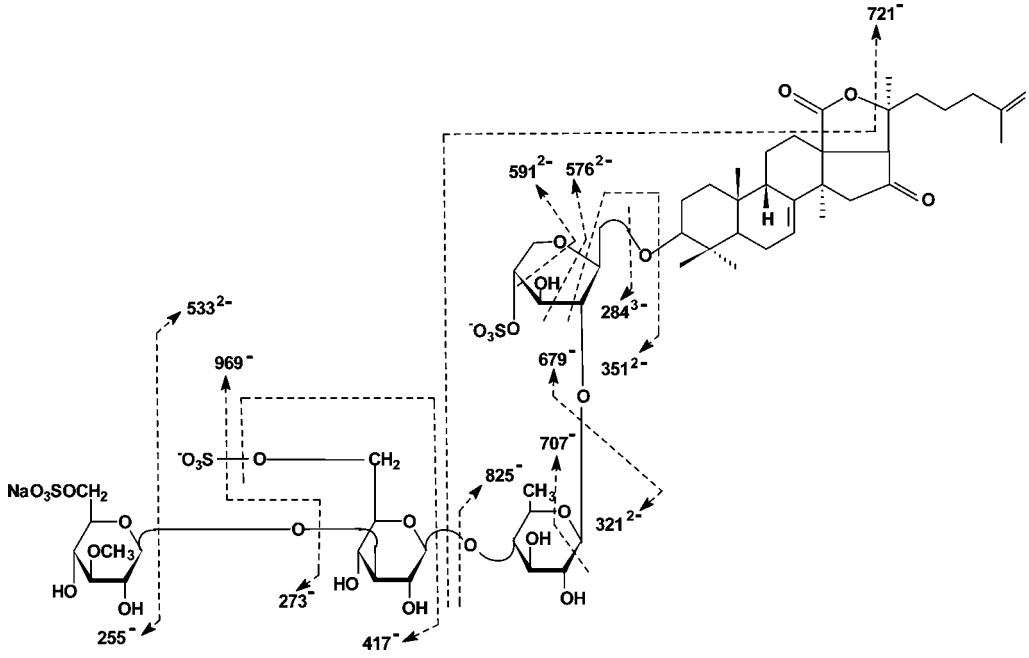


Figure 3. Main CID-type fragmentations in (−) ESI-FTMS/MS of the ion $[M_{3Na} - 3Na]^{3-}$ at *m/z* 440.46 of liouvilloside B_1 (**4**).

$[M_{3Na} - MeGlc - NaSO_3 - NaSO_3]^{2-}$, 869.40 $[M_{3Na} - MeGlc - NaSO_3 - Glc - NaSO_3 - Na + H]^-$, 809.38 $[M_{3Na} - MeGlc - NaSO_3 - Glc - NaSO_3 - Na - AcOH + H]^-$, 765.39 $[M_{3Na} - MeGlc - NaSO_3 - Glc - NaSO_3 - Na - AcOH - CO_2 + H]^-$, 723.34 $[M_{3Na} - MeGlc - NaSO_3 - Glc - NaSO_3 - Qui - Na + H]^-$, 634.23 $[M_{3Na} - NaSO_4 - H - 2Na]^{2-}$, 435.13 $[M_{3Na} - CO_2 - 3Na]^{3-}$, 420.47 $[M_{3Na} - AcOH - CO_2 - 3Na]^{3-}$, 321.04 $[M_{3Na} - Xyl - NaSO_3 - OAgl - H - 2Na]^{2-}$, 284.36 $[M_{3Na} - OAgl - H - 3Na]^{3-}$, 273.03 $[M_{3Na} - Glc - NaSO_3 - Qui - Xyl - NaSO_3 - OAgl - Na]^-$, 255.02 $[M_{3Na} - Glc - NaSO_3 - Qui - Xyl - NaSO_3 - OAgl - H - Na]^-$, and 248.01 $[M_{3Na} - Qui - Xyl - NaSO_3 - OAgl - H - 2Na]^{2-}$ (Figure 2, Supporting Information).

All these data indicated that liouvilloside B_2 (**5**) is 3*β*-*O*-[3-*O*-methyl-6-*O*-sodium sulfate-*β*-D-glucopyranosyl-(1→3)-6-*O*-sodium sulfate-*β*-D-glucopyranosyl-(1→4)-*β*-D-quinovopyranosyl-(1→2)-4-*O*-sodium sulfate-*β*-D-xylopyranosyl]-16-acetoxyholosta-7,25-diene.

Thus, we isolated five new di- and trisulfated tetraosides, namely, liouvillosides A_1 (**1**), A_2 (**2**), A_3 (**3**), B_1 (**4**), and B_2 (**5**), from *S. liouvillei*. We also found in *S. liouvillei* the previously known liouvilloside A (**6**)[8] and two other known glycosides, hemoiedemosides A (**7**) and B (**8**), isolated earlier from the Patagonian sea cucumber *Hemioedema spectabilis*.[12]

H. spectabilis (Ludwig, 1883) was originally placed in the genus *Thyone* but was later transferred by Panning first into the genus *Psolidiella* (subfamily Cucumariinae, family Cucumariidae)[21] and years later into the genus *Hemioedema*.[22] The situation of *S. liouvillei* (Vaney, 1914) is taxonomically more complicated. The species was originally described in the genus *Cucumaria*.[23] In 1927 Ekman established a new genus, *Staurocucumis*, and designated *S. liouvillei* as its type species.[24] In 1949 Panning placed the genus *Staurocucumis* in the subfamily Ypsilothuriinae previously established by Heding in 1942 as a family.[21] In 1965 Pawson and Fell placed the family Ypsilothuriidae in a new order, Dactylochirotida, but removed the genus *Staurocucumis* from Ypsilothuriidae.[25] Then, Pawson placed it in the order Dendrochirotida as *incertae sedis* without placing it in any particular family.[26] Recently, O'Loughlin definitively placed *S. liouvillei* in the family Cucumariidae but did not provide any arguments supporting his decision.[27]

Our results demonstrate significant similarities in the glycoside structures in *S. liouvillei* and *H. spectabilis*. On the basis of these data, we suggest that both *Staurocucumis* and *Hemioedema* should be considered as members of the family Cucumariidae and, most likely, the subfamily Cucumariinae. The genus *Cucumaria* is characterized by having triterpene glycosides with pentasaccharide carbohydrate chains.[28] The absence of triterpene pentaosides in *S. liouvillei* and *H. spectabilis* indicates that *Staurocucumis* and *Hemioedema* genera are not closely related to the genus *Cucumaria*, although all these genera are considered members of the subfamily Cucumariinae.

Liouvillosides A_2 (**2**) and A_3 (**3**) contain 3-*O*-methyl-D-quinovose as terminal monosaccharine residues. The presence of this sugar in carbohydrate chains has never been found before in sea cucumber triterpene glycosides. The membranolytic activities of sea cucumber glycosides could be the basis of their main adaptive role, defense against predatory fish.[29] The presence of a sulfate group at C-6 of the common terminal 3-*O*-methyl-D-glucose significantly decreases the membranolytic activities of sea cucumber glycosides.[30] The absence of a hydroxyl group at C-6 of the terminal monosaccharide unit (as in 3-*O*-methyl-D-quinovose) blocks the possibility of sulfation of this position, and this may have adaptive significance. 3-*O*-Methyl-D-quinovose was unknown as a glycoside constituent until De Marino and collaborators found it in asterosaponins from the starfish *Goniopecten demonstrans* in 2000.[31]

In most cases, sea cucumbers have been reported to accumulate glycosides with one type of polycyclic system for the aglycones that contain either the 7(8)- or 9(11)-double bond. Only five species of sea cucumbers are known to contain both glycosides with the 7(8)- and glycosides with 9(11)-double bond simultaneously. These are *Cucumaria conicospermium*,[9] *Pentacta australis*,[32] *Neothyonidium magnum*,[33,34] *Psolus fabricii*,[35,36] and *Cucumaria frondosa*.[6] However, only two pairs of glycosides from *C. conicospermium* and three pairs of glycosides from *C. frondosa* were isomeric in their double-bond positions. Thus, the fact that *S. liouvillei* contains two pairs of similar isomers, i.e., hemoiedesmoside B (**7**) and liouvilloside B_1 (**4**), and hemoiedesmoside A (**8**) and liouvilloside A_1 (**1**), differing only in the position of the double bonds in the polycyclic systems of their aglycones, is of particular interest.

Experimental Section

General Experimental Procedures. All melting points were determined with a Kofler-Thermogenerate apparatus. Specific rotation was measured on a Perkin-Elmer 343 polarimeter. NMR spectra were recorded on a DRX-500 Bruker spectrometer at 500.13/125.75 MHz ($^1H/^{13}C$) in C_5D_5N/D_2O (4:1) with TMS as an internal reference (δ =

0). The ESI-FTMS and CID MS/MS (negative ion mode) were recorded using a Bruker ESI-FTICR mass spectrometer, model Apex Qe, sample concentration was 0.01 mg/mL, and CH_3CN/H_2O (1:1) was the solvent. HPLC was performed using an Agilent 1100 chromatograph equipped with a differential refractometer on Supelco Sil (4.6 × 250 mm, 5 μm), C-18 (10 × 250 mm, 5 μm), and cyclodextrin Agilent ChiraDex (4 × 244 mm, 5 μm) columns. GLC analysis was carried out on Aligent 6850 Series apparatus, carrier gas He (1.7 mL/min) at 100 °C (0.5 min) → 250 °C (5 °C/min, 10 min), capillary column HP-5 MS (30 m × 0.25 mm). Temperatures of injector and detector were 150 and 280 °C, respectively.

Animal Material. Twenty-three specimens of the sea cucumber *Staurocucumis liouvillei* (Vaney) (family Cucumariidae; order Dendrochirotida) were collected near the sub-Antarctic Island of Buovet (3°17,58′ E, 54°22,49′ S) by Agassiz trawl on board the research vessel *Polarstern* [Alfred Wegener Institute for Polar and Marine Research (Bremenhaven, Germany)] during the Antarctic expedition ANT XXI/2. Sampling was performed on November 25, 2003, at a depth of 134 m (collectors C. Avila and M. Ballesteros). Sea cucumbers were identified by M. Ballesteros and A. Bosch (University of Barcelona, Spain); voucher specimens are preserved in the collection of the Department of Animal Biology (invertebrates), Faculty of Biology, University of Barcelona.

Extraction and Isolation. The sea cucumbers were minced and extracted twice with refluxing 70% EtOH. The dry wt of the residue was 5.6 g. The combined extracts were concentrated to dryness *in vacuo*, dissolved in H_2O, and chromatographed on a Polychrom-1 column (powdered Teflon, Biolar, Latvia), eluting first inorganic salts and polar impurities with H_2O and then the glycosides with 60% acetone. The latter fraction was submitted to sequential chromatography on Si gel columns eluting with $CHCl_3$/EtOH/H_2O (100:100:17 and 100:125:25) solvent systems to give 47 and 95 mg of glycoside fractions A and B, respectively, as individual spots on TLC. Fraction A was subsequently separated by HPLC on a Supelco Sil column with $CHCl_3$/EtOH/NH_4OAc (1 N H_2O solution) (65:45:2) as mobile phase, Supelco C-18 column with EtOH/H_2O/NH_4OAc (1 N H_2O solution) (55:45:2) as mobile phase, and Agilent ChiraDex column with CH_3CN/H_2O/NH_4OAc (1 N H_2O solution) (60:40:1) as mobile phase to give 1.2 mg of liouvilloside A_1 (**1**), 1.6 mg of liouvilloside A_2 (**2**), 1.3 mg of liouvilloside A_3 (**3**), and 2.8 mg of the previously described hemoidemoside A.[12] Fraction B was subsequently separated by HPLC on Supelco C-18 column with EtO/H_2O/NH_4OAc (1 N H_2O solution) (55:45:2) as mobile phase and an Agilent ChiraDex column with CH_3CN/H_2O/NH_4OAc (1 N water solution) (65:35:1, 55:45:1, 50:50:1) as mobile phase to yield 2.6 mg of liouvelloside B_1 (**4**), 1.4 mg of liouvilloside B_2 (**5**), 2.9 mg of the previously described hemoidemosides B (**7**),[12] and 2.1 mg of liouvilloside A (**6**).[8]

Liouvilloside A_1 (1): mp 228−230 °C, $[\alpha]_D^{20}$ −48 (*c* 0.1 pyridine); see Tables 1 and 3 for NMR data; ESI-FTMS (−) *m/z* 1265.4310 (calc 1265.4337) $[M_{2Na} - Na]^-$, 1243.4460 (calc 1243.4518) $[M_{H,Na} - Na]^-$, and 621.2210 (calc 621.2223) $[M_{2Na} - 2Na]^{2}$; ESI-FTMS/MS (−) of the ion $[M_{2Na} - 2Na]^{2-}$ at *m/z* 621.22, *m/z* 45.48 $[M_{2Na} - NaSO_4 - Na - H]^-$, 987.42 $[M_{2Na} - MeGlc - NaSO_3 + 2H - Na]^-$, 969.41 $[M_{2Na} - OMeGlc - NaSO_3 - Na]^-$, 943.44 $[M_{2Na} - MeGlc - NaSO_3 + 2H - Na - CO_2]^-$, 925.42 $[M_{2Na} - OMeGlc - NaSO_3 - Na - CO_2]^-$, 825.37 $[M_{2Na} - MeGlc - GlcNaSO_3 + H - Na]^-$, 781.38 $[M_{2Na} - MeGlc - Glc - NaSO_3 + H - Na - CO_2]^-$, 679.31 $[M_{2Na} - MeGlu - Glc - NaSO_3 - Qui + H - Na]^-$, 563.13 $[M_{2Na} - Xyl - NaSO_3 - OAgl - H - Na]^-$, 417.07 $[M_{2Na} - Qui - Xyl - NaSO_3 - OAgl - H - Na]^-$, and 387.06 $[M_{2Na} - OAgl - 2Na - H]^{2-}$ (also see Figure 1).

Liouvilloside A_2 (2): mp 227−229 °C, $[\alpha]_D^{20}$ −53 (*c* 0.1 pyridine); see Tables 1 and 4 for NMR data; ESI-FTMS (−) *m/z* 1249.4420 (calc 1249.4393) $[M_{2Na} - Na]^-$, 1227.4537 (calc 1227.4568) $[M_{H,Na} - Na]^-$, 1147.5017 (calc 1147.5000) $[M_{H,Na} - NaSO_3]^-$, and 613.2201 (calc 613.2248) $[M_{2Na} - 2Na]^{2-}$; ESI-FTMS/MS (−) of the ion $[M_{2Na} - 2Na]^{2-}$ at *m/z* 613.22, *m/z* 1129.49 $[M_{2Na} - NaSO_4 - Na - H]^-$, 987.42 $[M_{2Na} - MeQui - NaSO_3 + 2H - Na]^-$, 969.41 $[M_{2Na} - OMeQui - NaSO_3 - Na]^-$, 943.44 $[M_{2Na} - MeQui - NaSO_3 + 2H - Na - CO_2]^-$, 925.43 $[M_{2Na} - OMeQui - NaSO_3 - Na - CO_2]^-$, 825.37 $[M_{2Na} - MeQui - Glc - NaSO_3 + H - Na]^-$, 781.38 $[M_{2Na} - MeQui - Glc - NaSO_3 + H - Na - CO_2]^-$ 679.32 $[M_{2Na} - MeQui - Glc - NaSO_3 - Qui + H - Na]^-$, 379.06 $[M_{2Na} - OAgl - 2Na - H]^{2-}$, 547.13 $[M_{2Na} - Xyl - NaSO_3 - OAgl - H - Na]^-$, and 401.08 $[M_{2Na} - Qui - Xyl - NaSO_3 - OAgl - H - Na]^-$ (also see Figure 2).

Liouvilloside A_3 (3): mp 225−228 °C, $[\alpha]_D^{20}$ −21 (*c* 0.1 pyridine); see Tables 2 and 4 for NMR data; ESI-FTMS, *m/z* 1293.4678 (calc 1293.4650) $[M_{2Na} - Na]^-$, 1271.4810 (calc 1271.4831) $[M_{H,Na} - Na]^-$, 1191.5251 (calc 1191.5263) $[M_{H,Na} - NaSO_3]^-$, and 635.2339 (calc 635.2379) $[M_{2Na} - 2Na]^{2}$; ESI-FTMS/MS (−) of the ion of $[M_{2Na} - 2Na]^{2-}$ at *m/z* 635.24, *m/z* 1173.51 $[M_{2Na} - NaSO_4 - Na - H]^-$, 1031.45 $[M_{2Na} - MeQui - NaSO_3 + 2H - Na]^-$, 1013.44 $[M_{2Na} - OMeQui - NaSO_3 - Na]^-$, 869.40 $[M_{2Na} - MeQui - Glc - NaSO_3 + H - Na]^-$, 809.38 $[M_{2Na} - MeQui - Glc - NaSO_3 + H - Na - AcOH]^-$, 723.34 $[M_{2Na} - MeQui - Glc - NaSO_3 - Qui + H - Na]^-$, 605.23 $[M_{2H} - AcOH - 2H]^{2-}$, 583.23 $[M_{2H} - AcOH - CO_2 - 2H]^{2-}$, 547.13 $[M_{2Na} - Xyl - NaSO_3 - OAgl - H - Na]^-$, 401.08 $[M_{2Na} - Qui - Xyl - NaSO_3 - OAgl - H - Na]^-$, and 379.06 $[M_{2Na} - OAgl - 2Na - H]^{2-}$ (also see Figure 1, Supporting Information).

Liouvilloside B_1 (4): mp 239−241 °C, $[\alpha]_D^{20}$ −51 (*c* 0.1 pyridine); see Tables 1 and 5 for NMR data; ESI-FTMS (−) *m/z* 1367.3729 (calc 1367.3725) $[M_{3Na} - Na]^-$, 1345.3900 (calc 1345.3905) $[M_{H,2Na} - Na]^-$, 1323.4063 (calc 1323.4086) $[M_{2H,Na} - Na]^-$, 1243.4464 (calc 1243.4518) $[M_{H,2Na} - Na - NaSO_3]^-$, 672.1898 (calc 672.1917) $[M_{3Na} - 2Na]^{2-}$, 661.1983 (calc 661.2007) $[M_{H,2Na} - 2Na]^{2-}$, 621.2201 (calc 621.2223) $[M_{3Na} - NaSO_3 - 2Na + H]^{2-}$, 440.4627 (calc 440.4647) $[M_{3Na} - 3Na]^{3-}$; ESI-FTMS/MS (−) of the ion $[M_{3Na} - 3Na]^{3-}$ at *m/z* 440.46, *m/z* 1127.49 $[M_{3Na} - 2NaSO_4 - Na - 2H]^-$, 969.42 $[M_{3Na} - MeGlc - NaSO_3 - NaSO_3]^{2-}$, 825.38 $[M_{3Na} - MeGlc - NaSO_3 - Glc - NaSO_3 - Na + H]^-$, 781.38 $[M_{3Na} - MeGlc - NaSO_3 - GlcNaSO_3 - Na - CO_2 + H]^-$, 679.32 $[M_{3Na} - MeGlc - NaSO_3 - Glc - NaSO_3 - Qui - Na + H]^-$, 612.22 $[M_{3Na} - NaSO_4 - 2Na - H]^{2-}$, 533.19 $[M_{3Na} - MeGlc - NaSO_3]^{2-}$, 417.07 $[M_{3Na} - Qui - Xyl - NaSO_3 - OAgl - NaSO_3 - Na]^-$, 321.04 $[M_{3Na} - Xyl - NaSO_3 - OAgl - H - 2Na]^{2-}$, 284.36 $[M_{3Na} - OAgl - 3Na - H]^{3-}$, 273.03 $[M_{3Na} - Glc - NaSO_3 - Qui - Xyl - NaSO_3 - OAgl - Na]^-$, and 255.02 $[M_{3Na} - GlcNaSO_3 - Qui - Xyl - NaSO_3 - OAgl - H - Na]^-$ (also see Figure 3).

Liouvilloside B_2 (5): mp 240−243 °C, $[\alpha]_D^{20}$ −18 (*c* 0.1 pyridine); see Tables 2 and 5 for NMR data; ESI-FTMS (−) *m/z* 1411.3992 (calc 1411.3987) $[M_{3Na} - Na]^-$, 1389.4145 (calc 1389.4168) $[M_{H,2Na} - Na]^-$, 1367.4356 (calc 1367.4349) $[M_{2H,Na} - Na]^-$, 694.2048 (calc 694.2048) $[M_{3Na} - 2Na]^{2-}$, 683.2134 (calc 683.2138) $[M_{H,2Na} - 2Na]^{2-}$, and 455.1396 (calc 455.1401) $[M_{3Na} - 3Na]^{3-}$; ESI-FTMS/MS (−) of the ion $[M_{3Na} - 3Na]^{3-}$ at *m/z* 455.14, *m/z* 555.20 $[M_{3Na} - MeGlc - NaSO_3]^{2-}$, 969.42 $[M_{3Na} - MeGlc - NaSO_3 - NaSO_3]^{2-}$, 869.40 $[M_{3Na} - MeGlc - NaSO_3 - Glc - NaSO_3 - Na + H]^-$, 809.38 $[M_{3Na} - MeGlc - NaSO_3 - Glc - NaSO_3 - Na - AcOH + H]^-$, 765.39 $[M_{3Na} - MeGlc - NaSO_3 - Glc - NaSO_3 - Na - AcOH - CO_2 + H]^-$, 723.34 $[M_{3Na} - MeGlc - NaSO_3 - Glc - NaSO_3 - Qui - Na + H]^-$, 634.23 $[M_{3Na} - NaSO_4 - H - 2Na]^{2-}$, 435.13 $[M_{3Na} - CO_2 - 3Na]^{3-}$, 420.47 $[M_{3Na} - AcOH - CO_2 - 3Na]^{3-}$, 321.04 $[M_{3Na} - Xyl - NaSO_3 - OAgl - H - 2Na]^{2-}$, 284.36 $[M_{3Na} - OAgl - H - 3Na]^{3-}$, 273.03 $[M_{3Na} - Glc - NaSO_3 - Qui - Xyl - NaSO_3 - OAgl - Na]^-$, 255.02 $[M_{3Na} - Glc - NaSO_3 - Qui - Xyl - NaSO_3 - OAgl - H - Na]^-$, and 248.01 $[M_{3Na} - Qui - Xyl - NaSO_3 - OAgl - H - 2Na]^{2-}$ (also see Figure 2, Supporting Information).

Liouvilloside A (6): 2.1 mg, isolated from fraction B of trisulfated glycosides as described above; physical constants and ^{13}C NMR spectrum were identical with literature data.[8]

Hemoiedemoside B (7): 2.9 mg, isolated from fraction B of trisulfated glycosides as described above; physical constants and ^{13}C NMR spectrum were identical with literature data.[12]

Hemoiedemoside A (8): 2.8 mg, isolated from fraction A of disulfated glycosides as described above; physical constants and ^{13}C NMR spectrum were identical with literature data.[12]

Acid Hydrolysis and Determination of the Absolute Configuration of Monosaccharides in Liouvilloside A_1 (1). The acid hydrolysis of liouvilloside A_1 (**1**) (1 mg) was carried out in a solution of 0.2 M trifluoroacetic acid (TFA) (0.3 mL) in a stoppered vial on a H_2O bath at 100 °C for 30 min. The H_2O layer was washed with $CHCl_3$ (3 × 0.5 mL) and concentrated *in vacuo*. One drop of concentrated TFA and 0.2 mL of (−)-2-octanol (Aldrich) were added to the sugar mixture, and the ampule was sealed and then heated on a glycerol bath at 130 °C for 6 h. The mixture was evaporated *in vacuo* and treated with a mixture of pyridine/acetic anhydride (1:1, 0.6 mL) for 24 h at room temperature. The acetylated (−)-2-octylglycosides were analyzed by

GLC using the corresponding authentic samples: D-xylose, D-quinovose, D-glucose, and 3-*O*-methyl-D-glucose treated by the same procedure. The following peaks were detected: D-xylose (retention times 24.57, 24.74, and 25.01 min), D-quinovose (retention times 24.04, 24.26, 24.68, and 24.88 min), D-glucose (retention times 28.26, 28.92, 29.13, and 29.37 min), and 3-*O*-methyl-D-glucose (retention times 28.29, 28.63, 28.92, and 29.12 min). Retention times of authentic samples were as follows: D-xylose (retention times 24.57, 24.74, and 25.01 min), D-quinovose (retention times 24.04, 24.26, 24.68, and 24.88 min), D-glucose (retention times 28.26, 28.92, 29.13, and 29.37 min), and 3-*O*-methyl-D-glucose (retention times 28.29, 28.63, 28.92, and 29.12 min).

Acid Hydrolysis and Determination of the Absolute Configuration of Monosaccharides in Liouvilloside A_2 (2). The acid hydrolysis of liouvilloside A_2 (**2**) and determination of the sugar absolute configurations were carried out as described above. The following peaks were detected: D-xylose (retention times 24.57, 24.74, and 25.01 min), D-quinovose (retention times 24.04, 24.26, 24.68, and 24.88 min), D-glucose (retention times 28.26, 28.92, 29.13, and 29.37 min), and 3-*O*-methyl-D-quinovose (retention times 22.50, 23.20, 23.42, and 23.76 min). Retention times of authentic samples were as follows: D-xylose (retention times 24.57, 24.74, and 25.01 min), D-quinovose (retention times 24.04, 24.26, 24.68, and 24.88 min), D-glucose (retention times 28.26, 28.92, 29.13, and 29.37 min), and 3-*O*-methyl-D-quinovose (retention times 22.50, 23.20, 23.42, and 23.76 min).

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Supporting Information Available: MS/MS spectra of **1**−**5**, 2D NMR spectra of **1**−**5**, as well as MS/MS fragmentations for **3** and **5** (Figures 1 and 2) are provided free of charge via the Internet at http://pubs.acs.org.

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