Two New Cytotoxic and Virucidal Trisulfated Triterpene Glycosides from the Antarctic Sea Cucumber *Staurocucumis liouvillei*

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Two new trisulfated triterpene glycosides, liouvillosides A (**1**) and B (**2**), have been isolated from the Antarctic sea cucumber *Staurocucumis liouvillei*. Their structures have been elucidated by spectroscopic analysis (NMR and FABMS) and chemical transformations. Liouvillosides A (**1**) and B (**2**) are two new examples of a small number of trisulfated triterpene glycosides from sea cucumbers belonging to the family Cucumariidae. Both glycosides were found to be virucidal against herpes simplex virus type 1 (HSV-1) at concentrations below 10 μg/mL.

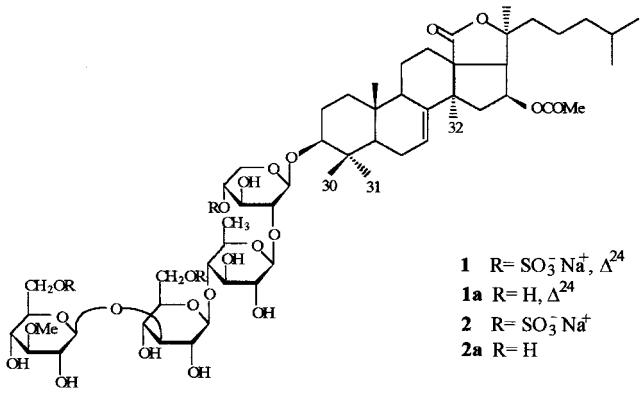
Triterpene glycosides are secondary metabolites typically found in sea cucumbers (class Holothuroidea). The majority of these saponins present a sugar chain of up to six monosaccharide units linked to the C-3 of the aglycon, which is usually a triterpene of the lanosterol type with a 18(20) lactone.[1] These substances are specific for different taxonomic groups of sea cucumbers and represent good models for studies on biochemical evolution.[2] They have a wide spectrum of biological effects: antifungal, cytotoxic, hemolytic, cytostatic, and immunomodulatory activities.[3] These biological activities and the membranotropic action of triterpene glycosides of sea cucumbers are indicative of their role in external defensive action. Among echinoderms, saponins and other steroidal compounds isolated from starfish and ophiuroids have shown antiviral activity against various pathogenic viruses of humans.[4−6] However, little is known about the antiviral activity of triterpene glycosides from sea cucumbers.

As part of our research on secondary metabolites of biological significance from echinoderms of cold waters of the South Atlantic,[7,8] we have investigated the polar extracts of the Antarctic sea cucumber *Staurocucumis liouvillei* (Vaney 1914) (Dendrochirotida, Cucumariidae). We report here the isolation and structure elucidation of two new trisulfated triterpene glycosides, liouvillosides A (**1**) and B (**2**), as well as the results of the cytotoxicity and virucidal activity of these compounds.

Results and Discussion

The ethanolic extract of *S. liouvillei* was sequentially submitted to Amberlite XAD-2 and Si gel C_{18} reversed-phase chromatography. Final separation and isolation was achieved by reversed-phase HPLC on Bondclone C_{18} to give two new trisulfated triterpene glycosides, liouvillosides A (**1**) and B (**2**). Both compounds present the same oligosaccharide moiety and differ in the side chain of the aglycon.

An examination of the ^{1}H and ^{13}C NMR spectra of liouvilloside A (**1**), the major component of *S. liouvillei*, suggested the presence of a triterpenoid aglycon with two olefinic bonds, one ester, and one lactone carbonyl group bonded to an oligosaccharide chain composed of four sugar



units. The assignments of the NMR signals associated with the aglycon moiety (Table 1) showed a close similarity to those reported for 16β-acetoxyholosta-7,24-diene-3β-ol, the aglycon of neothyonidioside C, isolated from the sea cucumber *Neothyonidium magnum*.[9]

The ^{1}H, ^{13}C NMR, and DEPT spectra of **1** showed resonances for a 7(8)-double bond [δ_C 145.2 (s, C-8) and 119.5 (d, C-7); δ_H 5.51 (1H, bs, H-7)] and those due to an acetoxy group [δ_C 169.4 (s) and 21.1 (q); δ_H 1.95 (3H, s)]. The location of the acetoxy group at C-16 was deduced from the chemical shift of the H-16 signal (δ 5.63), which showed coupling to signals at δ 2.74 (H-17), 2.40 (H-15α), and 1.24 (H-15β) in the ^{1}H−^{1}H COSY spectrum. The 16β configuration of the acetoxy group was confirmed by NOESY experiments and by coupling constant analysis for the C-16 proton with the C-17α and C-15 protons. Calculated coupling constant values of 8.9 ($J_{15\alpha,16\alpha}$), 7.4 ($J_{15\beta,16\alpha}$), and 8.9 Hz ($J_{16\alpha,17\alpha}$) for the most stable conformation of 16β-acetoxyholosta-7,24-diene-3β-ol obtained by molecular mechanics (Hyperchem) were coincident with experimental and reported[10] values and differed considerably from those calculated for the 16α-isomer (4.1, 6.9, and 1.2 Hz, respectively).

The ^{1}H NMR spectrum showed two vinyl methyl signals at δ 1.54 and 1.64 and one olefinic proton signal at δ 5.03, assigned to the terminal isopropenyl group of the side chain. The ^{13}C NMR spectrum confirmed the presence of this group: two methyl groups at δ 25.6 (C-26) and 17.8 (C-27) attached to olefinic carbons at δ 131.3 (C-25) and 123.9 (C-24).

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Table 1. ^{1}H and ^{13}C NMR Data for the Aglycon Moieties of Liouvillosides A (**1**) and B (**2**)

	1		**2**	
carbon	δ_C mult[a]	δ_H mult[b] (J in Hz)	δ_C mult[a]	δ_H mult[b] (J in Hz)
1	35.5 t	1.34 m	35.5 t	1.34 m
2	26.5 t	1.72 m	26.5 t	1.72 m
3	88.6 d	3.08 m	88.6 d	3.08 m
4	38.9 s		38.9 s	
5	47.2 d	0.9 m	47.2 d	0.9 m
6	22.7 t	1.90 m	22.6 t	1.90 m
7	119.5 d	5.51 bs	119.5 d	5.49 bs
8	145.2 s		145.2 s	
9	46.5 d	3.10 m	46.5 d	3.10 m
10	35.0 s		35.0 s	
11	21.9 t	1.55 m; 1.78 m	21.9 t	1.55 m; 1.78 m
12	30.6 t	2.25 m	30.6 t	2.25 m
13	58.6 s		58.6 s	
14	47.0 s		47.0 s	
15	43.2 t	1.24 m; 2.40 dd(7.5, 12.1)	43.2 t	1.25 m; 2.37 dd (7.5, 12.1)
16	74.4 d	5.63 m (8.3, 8.6)	74.4 d	5.62 m (8.3, 8.6)
17	53.4 d	2.74 d (8.8)	53.5 d	2.78 d (8.7)
18	179.1 s		179.1 s	
19	23.7 q	0.92 s	23.7 q	0.91 s
20	84.8 s		85.0 s	
21	28.2 q	1.45 s	28.3 q	1.42 s
22	38.2 t		38.6 t	1.68 m; 2.21 m
23	23.1 t		22.2 t	1.26 m; 1.42 m
24	123.9 d	5.03 bt	39.2 t	1.10 m; 1.25 m
25	131.3 s		27.7 d	1.48 m
26	25.6 q	1.54 s	22.3 q	0.85 d (6.6)
27	17.8 q	1.64 s	22.8 q	0.83 d (6.6)
30	16.8 q	1.10 s	16.8 q	1.09 s
31	28.3 q	0.86 s	28.3 q	0.86 s
32	32.2 q	1.02 s	32.2 q	1.01 s
CH_3COO	169.4 s		169.4 s	
CH_3COO	21.1 q	1.95 s	21.3 q	2.00 s

[a] Recorded at 125 MHz in DMSO-d_6; multiplicity by DEPT. [b] Recorded at 500 MHz in DMSO-d_6.

In addition to the aglycon signals, the ^{1}H NMR spectrum (Table 2) showed four anomeric protons at δ 4.32 (d, J = 7.3 Hz, xylose), 4.40 (d, J = 7.8 Hz, glucose), 4.47 (d, J = 7.9 Hz, 3-*O*-methylglucose), and 4.49 (d, J = 8.0 Hz, quinovose), a doublet at δ 1.25 (J = 5.3 Hz) due to the methyl group of the quinovose unit, and a singlet at δ 3.49 ppm corresponding to the methoxy group of 3-*O*-methylglucose. The β stereochemistries at the anomeric carbons were deduced from the coupling constant values (J = 7.1−7.9 Hz). The presence of xylose, quinovose, glucose, and 3-*O*-methylglucose in a 1:1:1:1 ratio was confirmed by acid hydrolysis with aqueous 2 N trifluoroacetic acid followed by GC analysis of the corresponding alditol peracetates.

The molecular formula of liouvilloside A (**1**) was determined as $C_{56}H_{85}O_{32}S_3Na_3$ (*m/z* 1434) by pseudomolecular ions at *m/z* 1457 $[M + Na]^+$ in the FABMS (positive ion mode) and at *m/z* 1411 $[M - Na]^-$ in the FABMS (negative ion mode). Fragment ion peaks at *m/z* 1355 $[M - SO_3Na + Na + H]^+$, 1253 $[M - 2SO_3Na + 2H + Na]^+$, and 1151 $[M - 3SO_3Na + 3H + Na]^+$ indicated the presence of three sulfate groups in the glycoside. This was also confirmed by solvolytic desulfation of liouvilloside A (**1**) to give the desulfated derivative **1a**. The sequence of monosaccharide units in the carbohydrate chain of glycoside **1** was determined by fragments at *m/z* 1179, 915, 768, and 534, corresponding to the sequential losses of sulfated 3-*O*-methylglucosyl, sulfated glucosyl, quinovosyl, and sulfated xylosyl units, respectively (scheme in Supporting Information).

The site of linkage of the sulfate groups in the sugar units was determined by comparison of ^{13}C NMR data of **1** and those of its desulfated derivative **1a** (Table 2). Esterification shifts were observed at the signals of C-4′ (xylose) (from 69.5 to 74.4), C-6‴ (glucose) (from 61.0 to 65.9), and C-6⁗ (3-*O*-methylglucose) (from 61.0 to 65.6).

Table 2. ^{1}H and ^{13}C NMR Data for the Sugar Moieties of Liouvillosides A (**1**) and B (**2**) and ^{13}C NMR Data for the Sugar Moieties of Desulfated Analogues **1a** and **2a**

	1 and **2**		**1a** and **2a**	
carbon	δ_C[a]	δ_H[b] (J in Hz)	δ_C	Δ_C[c]
1′	104.2	4.32 d (7.3)	104.4	0.2
2′	82.0	3.36 m	82.8	0.8
3′	74.7	3.54 m	76.7	2.0
4′	74.4	3.97	69.5	−4.9
5′	63.2	3.19 m	65.5	2.3
1″	103.8	4.49 d (8.0)	104.2	0.4
2″	74.9	3.10	75.0	0.1
3″	74.2	3.32 m	74.5	0.3
4″	86.2	3.03	86.3	0.1
5″	70.4	3.34 m	70.3	−0.1
6″	17.4	1.25 d (5.3)	17.8	0.4
1‴	103.1	4.40 d (7.8)	103.1	0
2‴	72.6	3.25 m	72.3	−0.3
3‴	85.9	3.49 m	87.8	1.9
4‴	68.7	3.20 m	68.6	−0.1
5‴	74.2	3.53 m	76.3	2.1
6‴	65.9	4.04 dd (18.0, 10.0); 3.78 m	61.0	−4.9
1⁗	103.8	4.47 d (7.9)	104.0	0.2
2⁗	73.6	3.15 m	73.6	0
3⁗	85.8	3.00	86.1	0.3
4⁗	69.3	3.21 m	69.5	0.2
5⁗	75.1	3.34 m	76.9	1.8
6⁗	65.6	4.04 dd (18.0, 10.0); 3.83 m	61.0	−4.6
OCH_3	60.1	3.49 s	60.0	−0.1

[a] Recorded at 125 MHz in DMSO-d_6. [b] Recorded at 500 MHz in DMSO-d_6. [c] $\Delta_C = \delta_C$(**1**,**2**) − δ_C(**1a**,**2a**).

The position of the interglycosidic attachments was determined using a combination of ^{1}H−^{1}H COSY, relayed COSY, and HETCOR experiments. From the ^{1}H−^{1}H COSY spectrum of **1** the anomeric proton signals at δ 4.32, 4.40, 4.47, and 4.49 were coupled to the H-2 proton signals at δ 3.36, 3.25, 3.15, and 3.10, respectively. These assignments were confirmed by the relayed COSY spectrum, which also showed the correlations of the anomeric proton signals with the corresponding H-2 and H-3 protons, as evidenced by cross-peaks at δ 4.32/3.36 (H-1′/H-2′) and 4.32/3.54 (H-1′/H-3′) for the sulfated xylose unit, δ 4.49/3.10 (H-1″/H-2″) and 4.49/3.32 (H-1″/H-3″) for the quinovose unit, δ 4.40/3.25 (H-1‴/H-2‴) and 4.40/3.49 (H-1‴/H-3‴) for the sulfated glucoe unit, and δ 4.47/3.15 (H-1⁗/H-2⁗) and 4.47/3.00 (H-1⁗/H-3⁗) for the sulfated 3-*O*-methylglucose unit. Further correlations of H-4, H-5, and H-6 in the ^{1}H−^{1}H COSY and relayed COSY spectra allowed assignment of all the protons of each sugar unit. In particular, the correlation of the quinovose methyl group (δ 1.25) with the proton signal at δ 3.34 (H-5″) in the ^{1}H−^{1}H COSY spectrum and the cross-peaks at δ 1.25/3.34 (H_3-6″/H-5″) and 1.25/3.03 (H_3-6″/H-4″) in the relayed COSY spectrum allowed assignments of H-4″ and H-5″ of the quinovose unit. The ^{13}C NMR signals of each sugar moiety were assigned by means of direct H−C correlations in the HETCOR spectrum. The cross-peaks between δ_H 3.49 (OCH_3) and δ_C 60.1 (OCH_3) and at δ 3.00/85.8 (H-3⁗/C-3⁗) were consistent with the presence of a methoxy group at C-3 of the terminal sulfated glucose unit.

Location of the interglycosidic linkages in the oligosaccharide chain was deduced from the chemical shifts of C-2′ (δ 82.0), C-4″ (δ 86.2), and C-3‴ (δ 85.9) assigned on the basis of cross-peaks at δ 3.36/82.0 (H-2′/C-2′), 3.03/86.2 (H-4″/C-4″), and 3.49/85.9 (H-3‴/C-3‴) in the HETCOR spectrum. The carbons involved in the interglycosidic

linkages gave values shifted downfield from those expected for the corresponding methyl glycopyranosides.[11] The linkage positions for the sugar units were confirmed by methylation of the desulfated derivative **1a** followed by acid hydrolysis and GC−MS analysis of the partially methylated alditol acetates derived from 2-linked xylopyranose, 4-linked quinovopyranose, 3-linked glucopyranose, and terminal 3-*O*-methylglucopyranose.

On the basis of these data we conclude that the oligosaccharide part of **1** is identical to the sugar chain of cucumechinosides D and F, isolated from the sea cucumber *Cucumaria echinata*.[12]

The four carbohydrate units belong to the D-series, as determined by GC analysis of the mixture of 1-[(*S*)-*N*-acetyl-(2-hydroxypropylamino)]-1-deoxyalditol acetate derivatives following the procedure of Cases et al.[13]

On the basis of the data discussed above, the structure of liouvilloside A (**1**) was determined as 16β-acetoxy-3-*O*-{6‴″-*O*-sodium sulfate-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}-holosta-7,24-diene-3β-ol.

The molecular formula of liouvilloside B (**2**) was deduced to be $C_{56}H_{87}O_{32}S_3Na_3$ on the basis of pseudomolecular ions at *m/z* 1413 $[M + Na]^-$ in the FABMS (negative ion mode) and *m/z* 1459 $[M + Na]^+$ in the FABMS (positive ion mode), which also showed fragment ions at *m/z* 1357 $[M - SO_3Na + H + Na]^+$, 1255 $[M - 2SO_3Na + 2H + Na]^+$, and 1153 $[M - 3SO_3Na + 3H + Na]^+$, indicating the presence of three sulfate groups. The presence of 3-*O*-methylglucose, glucose, quinovose, and xylose in a 1:1:1:1 ratio was established by acid hydrolysis with aqueous 2 N trifluoroacetic acid followed by GC analysis of the corresponding alditol peracetates. Analysis of ^1H and ^{13}C NMR data (Table 2) and FABMS spectra showed that liouvilloside B (**2**) had the same tetrasaccharide chain as liouvilloside A (**1**). The position of the sulfate groups in the sugar chain was confirmed by comparison of ^{13}C NMR data of **2** and **1** and those of desulfated derivatives **1a** and **2a** (Table 2).

The ^1H and ^{13}C NMR data (Table 1) of the aglycon moieties of **1** and **2** indicated that both glycosides differed only in the side chain. The presence of two doublets at δ 0.83 (J = 6.6 Hz) and 0.84 (J = 6.6 Hz) in the ^1H NMR spectrum of **2** and the signals at δ 22.3 and 22.8 in the ^{13}C NMR spectrum confirmed the presence of 16β-acetoxyholost-7-en-3β-ol as the aglycon of glycoside **2**, identical to that of frondoside A from *Cucumaria frondosa*.[10]

Hence, the structure of liouvilloside B (**2**) was determined as 16β-acetoxy-3-*O*-{6‴″-*O*-sodium sulfate-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}-holost-7-ene-3β-ol.

This work represents the first study of the glycosidic content of an Antarctic sea cucumber belonging to the genera *Staurocucumis* (family Cucumariidae, order Dendrochirotida). Liouvillosides A (**1**) and B (**2**) are new triterpene trisulfated glycosides. The presence of three sulfate groups in the carbohydrate chain of these compounds is very rare. The only such substances previously reported are cucumechinosides D, E, and F from the sea cucumber *Cucumaria echinata*,[12] cucumariosides A_7-1, A_7-2, and A_7-3 from *Cucumaria japonica*,[14] and koreoside A, isolated from the sea cucumber *Cucumaria koraiensis*.[15]

The cytotoxicity of saponins **1** and **2** was evaluated by the MMT method in Vero cells at concentrations ranging from 6.25 to 50 μg/mL with increasing time periods. Little or no cytotoxicity was detected within 8 h of cell exposure to the compounds, but both saponins were cytotoxic following prolonged incubation periods.

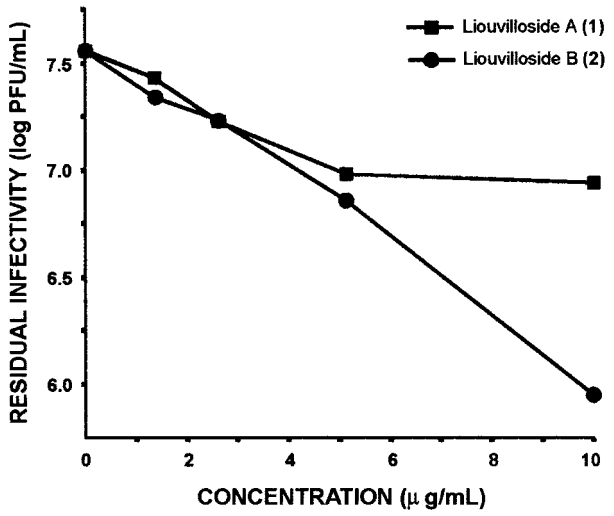

Figure 1. Dose−response curves for virucidal activity of liouvillosides A (**1**) and B (**2**) against HSV-1. The data represent the average values from duplicate independent experiments.

According to the results obtained in the cytotoxicity test, the virucidal activity of glycosides **1** and **2** was then evaluated by incubation of a suspension of HSV-1 with the compounds at concentrations below 10 μg/mL during 1 h at 37 °C. As shown in Figure 1, both saponins exerted an irreversible virucidal effect on HSV-1, but with different effectiveness: liouvilloside A (**1**) produced a weak inactivation of HSV-1 since at the maximum concentration tested the residual infectivity was 24% with respect to the control virus sample, whereas after treatment with liouvilloside B (**2**) in the same experimental conditions the remaining infectivity was 10-fold lower (2.5%).

These results are in agreement with previous reports about the activity of triterpenoid compounds against several viruses, including important pathogens such as HSV and human immunodeficiency virus.[16,17] In particular, naturally occurring saponins with different structures were found to be inhibitory of HSV either through a direct virucidal effect or by interference with an early step of the viral replicative cycle.[18,19]

Experimental Section

General Experimental Procedures. ^1H and ^{13}C NMR spectra were recorded in DMSO on a Bruker AM 500 spectrometer. The FABMS (positive and negative ion modes) were obtained on a VG-ZAB mass spectrometer, on a glycerol matrix. Optical rotations were measured on a Perkin-Elmer 343 polarimeter. Preparative HPLC was carried out on a SP liquid chromatograph equipped with a Spectra Series P100 solvent delivery system, a Rheodyne manual injector, and a refractive index detector using a C_{18} Bondclone 10μ column (30 cm × 21.2 mm i.d.). TLC was performed on precoated Si gel F254 (*n*-BuOH−HOAc−H_2O (4:1:5) (upper layer)) and C_{18} reversed-phase plates (65% MeOH−H_2O). GC was performed on a Hewlett-Packard 5890A chromatograph equipped with a flame-ionization detector, an SP-2330 column (25 m × 0.2 mm i.d.) (for analysis of peracetylated alditols), and an ULTRA-2 column (50 m × 0.2 mm i.d.) (for analysis of 1-[(*S*)-*N*-acetyl-(2-hydroxypropylamino)]-1-deoxyalditol acetate derivatives). GC−MS was performed on a TRIO-2 VG mass spectrometer coupled to a Hewlett-Packard 5890A chromatograph.

Animal Material. Specimens of *S. liouvillei* were collected at different locations around South Georgias Islands during a scientific cruise of the *BIP Dr. Holmberg*. The organisms were identified by Prof. Ahmed S. Thandar of the Department of

Zoology of the University of Durban Wetsville, South Africa, where a voucher specimen is preserved.

Extraction and Isolation. The sea cucumbers (3.2 kg) were defrosted, homogenized in EtOH, and centrifuged. The EtOH extract was evaporated, and the aqueous residue was passed through an Amberlite XAD-2 column and eluted with distilled water (until a negative reaction of chloride was observed) followed by MeOH. The MeOH eluate was evaporated under reduced pressure to give a glassy material (8 g), which was subjected to vacuum dry column chromatography on Davisil C-18 reversed phase (35−75μ) using H_2O, H_2O−MeOH mixtures with increasing amounts of MeOH, and finally MeOH as eluents. Fractions eluted with 70% and 80% MeOH contained the sulfated triterpene glycosides. These fractions were combined and subjected to vacuum dry column chromatography on SiO_2 gel, eluting with CH_2Cl_2−MeOH−H_2O, acetone, and MeOH. Fractions eluted with CH_2Cl_2−MeOH−H_2O (5:5:1) (lower phase) and acetone were combined (0.5 g) and submitted to repeated reversed-phase HPLC (ODS, MeOH−H_2O 50%) to give the pure glycosides **1** (62 mg) and **2** (15 mg).

Liouvilloside A (1): white amorphous powder, mp 191−193 °C, $[\alpha]^{20}_D$ −4.9° (*c* 0.5, pyridine); ^{1}H and ^{13}C NMR, see Tables 1 and 2; FABMS (positive ion mode), see Supporting Information; FABMS (negative ion mode) *m/z* 1411 [M − Na]$^-$, 1309 [M − SO_3Na + H − Na]$^-$, 1207 [M − 2SO_3Na + 2H − Na]$^-$, 1115 [M − 3-*O*-Me-Glc-OSO$_3$Na-*O* − H − Na]$^-$, 869 [M − 3-*O*-Me-Glc-OSO$_3$Na-*O*-Glc-SO$_3$Na + H − Na]$^-$, 852 [M − 3-*O*-Me-Glc-OSO$_3$Na-*O*-Glc-OSO$_3$Na-*O* − H − Na]$^-$, 722 [M − 3-*O*-Me-Glc-OSO$_3$Na-*O*-Glc-OSO$_3$Na-*O*-Qui − H − Na]$^-$, 706 [M − 3-*O*-Me-Glc-OSO$_3$Na-*O*-Glc-OSO$_3$Na-*O*-Qui-*O* − H − Na]$^-$, 538 [3-*O*-Me-Glc-OSO$_3$Na-*O*-Glc-OSO$_3$Na-*O* + 2H − Na]$^-$.

Liouvilloside B (2): white amorphous powder, mp 192−194 °C, $[\alpha]^{20}_D$ −10.5° (*c* 0.4, pyridine); ^{1}H and ^{13}C NMR, see Tables 1 and 2; FABMS (positive ion mode) *m/z* 1459 [M + Na]$^+$, 1357 [M − SO_3Na + H + Na]$^+$, 1255 [M − 2SO_3Na + 2H + Na]$^+$, 1180 [M − 3-*O*-Me-Glc-OSO$_3$Na + Na]$^+$, 1163 [M − 3-*O*-Me-Glc-OSO$_3$Na-*O* − H + Na]$^+$, 1153 [M − 3SO_3Na + 3H + Na]$^+$, 945 [3-*O*-Me-Glc-OSO$_3$Na-*O*-Glc-OSO$_3$-Na-*O*-Qui-*O*-Xyl-OSO$_3$Na + H + Na]$^+$, 915 [M − 3-*O*-Me-Glc-OSO$_3$Na-*O*-Glc-OSO$_3$Na − H + Na]$^+$, 770 [M − 3-*O*-Me-Glc-OSO$_3$Na-*O*-Glc-OSO$_3$Na-*O*-Qui + Na]$^+$; FABMS (negative ion mode) *m/z* 1413 [M − Na]$^-$, 1311 [M − SO_3Na + H − Na]$^-$, 1206 [M − 2SO_3Na − H − Na]$^-$, 1117 [M − 3-*O*-Me-Glc-OSO$_3$-Na-*O* − H − Na]$^-$, 871 [M − 3-*O*-Me-Glc-OSO$_3$Na-*O*-Glc-OSO$_3$-Na + H − Na]$^-$, 724 [M − 3-*O*-Me-Glc-OSO$_3$Na-*O*-Glc-OSO$_3$Na-*O*-Qui − H − Na]$^-$, 708 [M − 3-*O*-Me-Glc-OSO$_3$Na-*O*-Glc-OSO$_3$Na-*O*-Qui-*O* − H − Na]$^-$, 540 [3-*O*-Me-Glc-OSO$_3$Na-*O*-Glc-OSO$_3$Na-*O* + 2H − Na]$^-$.

Acid Hydrolysis of Liouvilloside A (1) and Liouvilloside B (2). Each glycoside (5 mg) was heated in a screwcap vial with 2 N trifluoroacetic acid (1 mL) at 120 °C for 2 h. The aglycon was extracted with CH_2Cl_2, and the aqueous residue was evaporated under reduced pressure. Each sugar mixture was treated with 0.5 M NH_3 (0.5 mL) and $NaBH_4$ (5 mg) at room temperature for 18 h. After acidification with 1 M AcOH, each reaction mixture was treated with MeOH (0.5 mL) and evaporated under reduced pressure. Both alditol mixtures were peracetylated with Ac_2O (0.5 mL) and pyridine (0.5 mL) at 100 °C for 45 min. The reaction mixtures were cooled and poured into $CHCl_3$−H_2O (1:1), and the aqueous phases were extracted with $CHCl_3$. The combined chloroform extracts were washed with H_2O (0.5 mL), saturated $NaHCO_3$ solution (0.5 mL), and H_2O (0.5 mL) and evaporated to dryness under nitrogen. Each mixture of peracetylated alditols was analyzed by GC using standard peracetylated alditols as reference samples.

Desulfation of a Mixture of Liouvilloside A (1) and Liouvilloside B (2). A solution of glycosides **1** and **2** (80 mg) in anhydrous 0.15% HCl−MeOH (45 mL) was kept stirring at room temperature for 1 h. After neutralization with Ag_2CO_3, the reaction mixture was centrifuged and the supernatant was evaporated to dryness at reduced pressure. The mixture was dissolved in H_2O (5 mL), percolated through an Amberlite XAD-2 column, and eluted with distilled water (until a negative chloride reaction was observed) followed by MeOH. The MeOH eluate was evaporated to dryness to give the mixture of desulfated glycosides, which were separated by reversed-phase HPLC on a preparative C_{18} Bonclone column eluted with 82.5% MeOH−H_2O to give the desulfated glycosides **1a** (13 mg) and **2a** (4 mg).

Desulfated liouvilloside A (1a): white amorphous powder, $[\alpha]^{20}_D$ −28.9° (*c* 0.09, pyridine); ^{1}H NMR (DMSO, 500 MHz) aglycon δ 5.63 (1H, m, *J* = 8 Hz, H-16α), 5.50 (1H, bs, H-7), 5.03 (1H, bt, H-24), 2.74 (1H, d, *J* = 8.9 Hz, H-17α), 1.95 (3H, s, C*H*$_3$COO), 1.65 (3H, s, H$_3$-27), 1.54 (3H, s, H$_3$-26), 1.45 (3H, s, H$_3$-21), 1.01 (3H, s, H$_3$-32), 0.92 (3H, s, H$_3$-19), 0.86 (3H, s, H$_3$-31); sugars δ 4.48 (1H, d, *J* = 7.8 Hz, H-1 quinovose), 4.46 (1H, d, *J* = 7.9 Hz, H-1 3-*O*-methylglucose), 4.39 (1H, d, *J* = 7.8 Hz, H-1 glucose), 4.27 (1H, d, *J* = 7.1 Hz, H-1 xylose), 3.49 (1H, s, OC*H*$_3$), 1.24 (3H, d, *J* = 5.7 Hz, H$_3$-6 quinovose); ^{13}C NMR (DMSO, 125 MHz) aglycon δ 179.1 (C-18), 169.4 (CH$_3$*C*O$_2$), 145.2 (C-8), 131.3 (C-25), 123.8 (C-24), 119.4 (C-7), 88.5 (C-3), 84.8 (C-20), 74.4 (C-16), 58.3 (C-13), 53.3 (C-17), 47.2 (C-5), 47.0 (C-14), 46.5 (C-9), 43.2 (C-15), 38.9 (C-4), 38.2 (C-22), 35.5 (C-1), 34.9 (C-10), 32.2 (C-32), 30.6 (C-12), 28.3 (C-31), 28.2 (C-21), 26.5 (C-2), 25.5 (C-26), 23.7 (C-19), 23.1 (C-23), 22.6 (C-6), 21.9 (C-11), 21.1 (*C*H$_3$CO$_2$),17.7 (C-27), 16.8 (C-30); sugars, see Table 2; FABMS (positive ion mode) *m/z* 1151 [M + Na]$^+$, 1110 [M + Na − CH$_3$CO + 2H]$^+$, 934 [1110−3-*O*-Me-Glc + H]$^+$, 771 [934 − Glc − H]$^+$, 624 [771 − Qui − H]$^+$.

Desulfated liouvilloside B (2a): white amorphous powder, $[\alpha]^{20}_D$ +36.7° [*c* 0.15, pyridine]; ^{1}H NMR (DMSO, 500 MHz) aglycon δ 5.62 (1H, m, *J* = 8 Hz, H-16α), 5.49 (1H, bs, H-7), 2.73 (1H, d, *J* = 8.9 Hz, H-17α), 2.00 (3H, s, C*H*$_3$COO), 1.43 (3H, s, H$_3$-21), 1.01 (3H, s, H$_3$-32), 0.92 (3H, s, H$_3$-19), 0.86 (3H, s, H$_3$-31), 0.85 (3H, d, *J* = 6.6 Hz, H$_3$-27), 0.83 (3H, d, *J* = 6.6 Hz, H$_3$-26); sugars δ 4.48 (1H, d, *J* = 7.8 Hz, H-1 quinovose), 4.46 (1H, d, *J* = 7.9 Hz, H-1 3-*O*-methylglucose), 4.39 (1H, d, *J* = 7.8 Hz, H-1 glucose), 4.27 (1H, d, *J* = 7.1 Hz, H-1 xylose), 3.49 (1H, s, OC*H*$_3$), 1.24 (3H, d, *J* = 5.7 Hz, H$_3$-6 quinovose); ^{13}C NMR (DMSO, 125 MHz) aglycon δ 179.1 (C-18), 169.4 (CH$_3$*C*O$_2$), 145.2 (C-8), 119.5 (C-7), 88.5 (C-3), 84.9 (C-20), 74.4 (C-16), 58.6 (C-13), 53.4 (C-17), 47.2 (C-5), 47.0 (C-14), 46.5 (C-9), 43.2 (C-15), 39.2 (C-24), 38.9 (C-4), 38.5 (C-22), 35.5 (C-1), 35.0 (C-10), 32.2 (C-32), 30.6 (C-12), 28.3 (C-31), 28.2 (C-21), 27.7 (C-25), 26.5 (C-2), 23.7 (C-19), 22.8 (C-27), 22.7 (C-6), 22.3 (C-26), 22.1 (C-23), 21.9 (C-11), 21.3 (*C*H$_3$CO$_2$), 16.8 (C-30); sugars, see Table 2; FABMS (positive ion mode) *m/z* 1153 [M + Na]$^+$, 1111 [M + Na − CH$_3$CO + H]$^+$, 936 [1111−3-*O*-Me-Glc + 2H]$^+$, 773 [936 − Glc − H]$^+$, 626 [773 − Qui − H]$^+$.

Methylation of Glycoside 1a Followed by Hydrolysis. A solution of **1a** (5 mg) in anhydrous DMSO (1.3 mL) was treated with NaOH (63 mg) and stirred at room temperature for 20 min. The reaction mixture was treated with CH_3I (0.4 mL) and stirred for a further 30 min. After addition of water (4 mL) the mixture was extracted with $CHCl_3$ (5 mL) and evaporated to dryness under nitrogen. The permethylated glycoside was heated in a screwcap vial with 2 N trifluoroacetic acid (0.8 mL) at 120 °C for 1.5 h. After extraction with $CHCl_3$, the aqueous residue was evaporated and further coevaporated with H_2O (2 × 0.5 mL) and MeOH (0.5 mL). The sugar mixture was treated with 0.5 M NH_3 (0.5 mL) and $NaBH_4$ (5 mg) at room temperature for 18 h. After acidification with 1 M AcOH, the reaction mixture was treated with MeOH (0.5 mL) and evaporated under reduced pressure. The alditol mixture was acetylated with Ac_2O (0.4 mL) and pyridine (0.4 mL) at 100 °C for 45 min. The reaction mixture was cooled and poured into $CHCl_3$−H_2O (1:1) and the aqueous phase extracted with $CHCl_3$. The combined chloroform extracts were washed with H_2O (0.5 mL), saturated $NaHCO_3$ solution (0.5 mL), and H_2O (0.5 mL) and evaporated to dryness under nitrogen. The mixture of methylated alditol acetates was analyzed by GC−MS. The following carbohydrates could be detected: 2-linked xylose (1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylxylitol; t_R = 12.9 min; *m/z* (%) = 189 (1.3), 129 (8), 117 (13), 101 (4.6), 87 (9)); 4-linked quinovose (1,4,5-tri-*O*-acetyl-2,4-di-*O*-methylquinovitol; t_R = 11.6 min; *m/z* (%) = 203 (1), 143 (5.1), 117 (13), 101 (8.8), 87 (2.5)); 3-linked glucose (1,3,5-tri-*O*-acetyl-2,4-di-*O*-methylglu-

citol; t_R = 13.9 min; *m*/*z* (%) = 233 (1.2), 161 (3.8), 129 (9.9), 117 (15.9), 101 (7.7), 87 (3.5)); terminal 3-*O*-methylglucose (1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol; t_R = 10.8 min; *m*/*z* (%) = 205 (0.9), 161 (4.7), 145 (4.3), 129 (7.1), 117 (8.1), 101 (16.3), 87 (6)).

Determination of the Absolute Configuration of the Carbohydrate Subunits. A solution of **1** (9 mg) in 2 N trifluoroacetic acid (1.2 mL) was heated at 120 °C for 2 h. After extracting with EtOAc, the H_2O layer was concentrated to furnish the monosaccharides mixture. Then, the following solutions were added: (a) 1:8 (*S*)-1-amino-2-propanol in MeOH (20 μL), (b) 1:4 glacial AcOH−MeOH (17 μL), and (c) 3% Na-[BH_3CN] in MeOH (13 μL), and the mixture was allowed to react at 65 °C for 1.5 h. After cooling, 3 M aqueous CF_3CO_2H was added dropwise until the pH dropped to pH 1−2. The mixture was evaporated and further coevaporated with H_2O (2 × 0.5 mL) and MeOH (0.5 mL). The residue was acetylated with Ac_2O (0.5 mL) and pyridine (0.5 mL) at 100 °C for 0.75 h. After cooling, the derivatives were extracted with $CHCl_3$−H_2O (1:1) (2 × 1 mL). The chloroform extracts were washed with saturated $NaHCO_3$ solution (0.5 mL) and H_2O (2 × 0.5 mL) and evaporated. The mixture of 1-[(*S*)-*N*-acetyl-(2-hydroxypropylamino)]-1-deoxyalditol acetate derivatives of the monosaccharides were identified by co-GC analysis with standard sugar derivatives prepared under the same conditions. The derivatives of D-glucose, 3-*O*-methyl-D-glucose, D-xylose, and D-quinovose were detected with t_R (min) of 40.66, 37.87, 29.86, and 29.07, respectively.

Cells and Viruses. Vero (African green monkey kidney) cells were grown in minimum essential medium (MEM) supplemented with 5% bovine serum. For maintenance medium (MM), serum concentration was reduced to 1.5%. Herpes simplex virus type 1 (HSV-1) was received from Dr. E. De Clercq's laboratory (Rega Institute, Leuven, Belgium).

Cytotoxicity Test. Vero cell viability was measured by the MMT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method. Confluent cultures in 96-well plates were exposed to different concentrations of the triterpene glycosides, three wells for each concentration, and incubated for different periods at 37 °C. Then 10 μL of MM containing MTT (final concentration 5 μg/mL) was added to each well. After 2 h of incubation at 37 °C, the supernatant was removed and 200 μL of EtOH was added to each well to solubilize the formazan crystals. After vigorous shaking, absorbance was measured in a microplate reader at 595 nm.

Virucidal Assay. A virus suspension of HSV-1 strain KOS containing 4×10^7 plaque-forming units (PFU) was incubated with an equal volume of MM with or without different concentrations of the compounds for 1 h at 37 °C. The samples were then diluted in cold MM to determine residual infectivity in a plaque formation assay using Vero cells. Results are expressed as residual infectivity, i.e., the ratio of virus titer in compound-treated samples with respect to virus titer in control samples.

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Supporting Information Available: Scheme for positive FABMS fragmentation of liouvilloside A (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

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