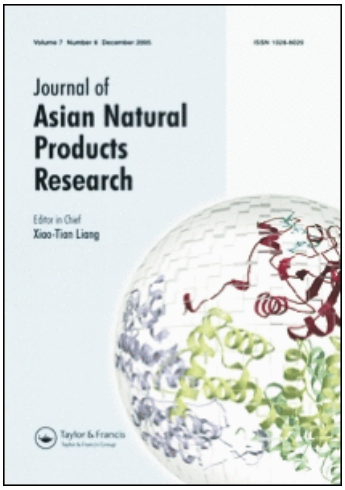

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Two new bioactive triterpene glycosides from the sea cucumber *Pseudocolochirus violaceus*

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By activity-guided fractionation, two new triterpene glycosides, violaceusides A (**1**) and B (**2**), were isolated from the sea cucumber *Pseudocolochirus violaceus* as active compounds causing morphological abnormality of *Pyricularia oryzae* mycelia. By extensive 2D NMR techniques and chemical evidence, the structures of the two new glycosides were established as 16β-acetoxy-3-*O*-[3-*O*-methyl-β-D-glucopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-β-D-quinovopyranosyl-(1 → 2)-4-*O*-sodiumsulphate-β-D-xylopyranosyl]-holosta-7,24-diene-3β-ol (**1**) and 16β-acetoxy-3-*O*-[3-*O*-methyl-β-D-glucopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 2)-4-*O*-sodiumsulphate-β-D-xylopyranosyl]-holosta-7,24-diene-3β-ol (**2**), respectively. The two glycosides also exhibited significant cytotoxicity against HL-60 and BEL-7402 cancer cell lines.

Keywords: *Pseudocolochirus violaceus*; Triterpene glycoside; Violaceuside A; Violaceuside B; *Pyricularia oryzae*

1. Introduction

Triterpene glycosides are the predominant metabolites of sea cucumbers (class Holothuroidea) and are responsible for their general toxicity. These glycosides have been reported to have a wide spectrum of biological effects, including anti-fungal, cytotoxic, haemolytic, and immunomodulatory activities [1]. There are about 500 species of sea cucumbers in China. Sea apple (*Pseudocolochirus violaceus* Theel) is one of them, distributed abundantly in the South China Sea, and on which no chemical and pharmacological studies have been reported to date. As part of our search for new bioactive compounds from echinoderms [2,3], we have investigated the n-BuOH fraction of the ethanolic extract of *Pseudocolochirus violaceus*, which showed a significant activity causing morphological abnormality of *Pyricularia oryzae* mycelia [minimum morphological deformation concentration (MMDC) = 64 μg/ml, 5-fluorouracil (5-FU) as positive control with MMDC = 5 μg/ml] [4]. We report herein the isolation, structural elucidation and

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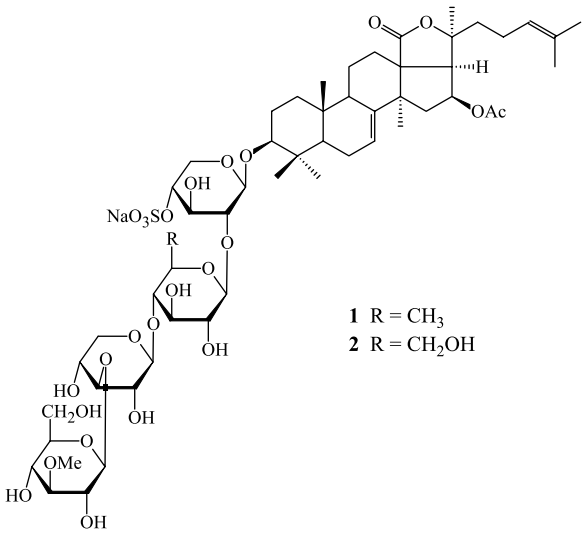

Figure 1. Structures of **1** and **2**.

biological activity of two new triterpene glycosides, violaceuside A (**1**) and B (**2**), isolated from this sea cucumber (see figure 1).

2. Results and discussion

Violaceuside A (**1**), a white amorphous powder with mp 224–225°C (dec), $[\alpha]_D^{20}$ -22 (*c* 0.2, MeOH), was positive to Liebermann-Burchard and Molish tests. The IR spectrum of **1** showed the presence of hydroxyl (3470 cm^{-1}), carbonyl (1742 cm^{-1}), olefinic bond (1650 cm^{-1}) and sulphate groups (1244, 1210 cm^{-1}). The positive ion mode HRESI-MS showed pseudomolecular ion peak at *m/z* 1223.4890 $[M + Na]^+$, which, together with the pseudomolecular ion peak at *m/z* 1176.94 $[M\text{-}Na]^-$ in negative-ion mode ESI-MS and NMR data, enable the molecular formula to be determined as $C_{55}H_{85}O_{25}SNa$.

An examination of the ^1H NMR and ^{13}C NMR spectra of **1** suggested the presence of a triterpene aglycone with seven methyls, two olefinic bonds, one acetoxyl and one lactone carbonyl group, together with an oligosaccharide chain composed of four sugar units [5]. The position of two double bonds at $\Delta^{7(8)}$ and $\Delta^{24(25)}$ were deduced from the NMR signals at δ_C 145.8 (s, C-8), 120.4 (d, C-7); δ_H 5.56 (1H, brs, H-7), and δ_C 124.2 (d, C-24), 131.9 (s, C-25); δ_H 5.00 (m, H-24) together with the analysis of the TOCSY and HMBC experiments. The NMR spectra of **1** also showed resonances due to an acetoxy group [δ_C 169.7 (s) and 21.1(q); δ_H 1.90 (3H, s)]. The location of the acetoxy group at C-16 was deduced from the chemical shift of the H-16 signal (δ_H 5.84), which showed coupling to signals at δ 2.54 (H-17), 2.49 (H-15α), 1.65 (H-15β) in the TOCSY spectrum. The 16β configuration of the acetoxy group was confirmed by NOESY experiments and from the coupling constants for H-16 with H-17α (8.2 Hz), H-15α (8.2 Hz) and H-15β (8.0 Hz). All the NMR signals associated with the aglycone moiety were unambiguously assigned by DQCOSY, TOCSY, HMQC and HMBC experiments (see table 1). These data were identical to 16β-acetoxyholosta-7,24-diene-3β-ol [6].

Table 1. ^{1}H, NMR, ^{13}C NMR data and key HMBC correlations of **1** (in pyridine-d_5, 600/150 MHz).

No.	δ_H *m (J in Hz)*	δ_C	*HMBC*	*No.*	δ_H *m (J in Hz)*	δ_C	*HMBC*
1	1.34 brs	36.1		XylI			
2	2.00 m, 1.80 m	27.1		1	4.61 d (7.2)	105.1	C-3, XylI C-3
3	3.15 dd (10.8, 2.4)	89.2		2	3.88 m	83.5	XylI C-3
4		39.6		3	4.17 m	75.3	XylI C-2, -4
5	0.92 brt (7.6)	48.1	C-4, -6, -10, -19, -30, -31	4	4.96 d (6.0)	75.7	
6	1.94 m	23.3	C-7	5	4.57d (6.8),3.59m	64.2	
7	5.56 s	120.4		Qui			
8		145.8		1	4.92 d (7.6)	105.3	XylI C-2
9	3.37 d (14.0)	47.2		2	3.85 m	76.2	Qui C-3
10		35.6		3	3.95 t (9.2)	75.2	Qui C-2
11	1.70 m	22.6		4	3.47 t (9.2)	86.0	XylII C-1, Qui C-3, -5
12	2.07 m, 1.89 m	31.5	C-9, -18	5	3.60 m	71.8	
13		59.3		6	1.57 d (6.8)	17.9	Qui C-4, -5
14		47.5		XylII			
15	2.49 dd (12.0, 8.0), 1.65 dd (12.0, 8.2)	43.7	C-13, -14, -16, -17, -32	1	4.72 d (7.6)	105.1	Qui C-4
16	5.84 ddd (8.2, 8.2, 8.0)	75.0	CH_3**CO**	2	3.89 m	73.7	
17	2.54 d (8.2)	54.7	C-12,-13,-18,-21	3	4.05 m	87.3	MeGlc C-1, XylII C-2, 4
18		179.4		4	4.02 m	68.9	
19	1.12 s	24.0	C-1, -9, -10	5	4.17 m, 3.54 m	66.5	
20		84.9		MeGlc			
21	1.42 s	28.3	C-17, -20, -22	1	5.19 d (7.6)	105.5	XylII C-3
22	2.45 m, 1.82 m	38.8	C-17	2	3.88 m	74.9	
23	1.94 m	23.7		3	3.56 m	87.9	MeGlc C-2, -4, OCH_3
24	5.00 m	124.2	C-26, -27	4	3.91 m	70.6	MeGlc C-5, -6
25		131.9		5	3.86 m	78.1	
26	1.56 s	25.5	C-24, -25, -27	6	4.33 d (9.6), 4.11 dd (11.2, 6.0)	62.0	
27	1.50 s	17.7	C-24, -25, -26	OMe	3.73 s	60.7	MeGlc C-3
30	1.01 s	17.3	C-4, -5, -31				
31	1.16 s	28.7	C-4, -5, -30				
32	1.06 s	32.2	C-8,-13, -14, -15				
CH_3**CO**		169.7					
CH_3CO	1.90 s	21.1	CH_3**CO**				

The presence of four β-sugar units in **1** was deduced from the ^{13}C NMR and ^{1}H NMR spectra, which showed four anomeric carbon resonances and four anomeric protons with coupling constants (*J* values) 7.2-7.6 Hz. The presence of D-xylose, D-quinovose and 3-*O*-methyl-D-glucose in a 2:1:1 ratio was confirmed by acid hydrolysis followed by GC-MS analysis of the corresponding aldononitrile peracetates. The ^{1}H NMR and ^{13}C NMR signals attributable to the different sugar units were assigned by the two-dimensional (2D) NMR experiments. By comparing the ^{13}C NMR chemical shifts of the sugar units of **1** with those of the corresponding methyl glycopyransides, a 2-glycosidated xylopyranosyl unit, a 4-glycosidated quinovopyranosyl unit, a 3-glycosidated xylopyranosyl unit and a terminal 3-*O*-methyl-glucopyranose unit were identified. The sequence of the sugar residues in **1** was determined by careful analysis of HMBC correlations. In the HMBC spectrum, cross peaks at δ_H 5.19/δ_C 87.3 (MeGlc H-1/XylII C-3), δ_H 4.72/δ_C 86.0 (XylII H-1/Qui C-4), δ_H 4.92/δ_C 83.5 (Qui H-1 XylI C-2), δ_H 4.61/δ_C 89.2 (XylI H-1/C-3 of aglycone) revealed that the sequence of sugar residues must be 3-O-MeGlc-(1 → 3)-Xyl-(1 → 4)-Qui-(1 → 2)-Xyl-(1 → 3)-aglycone. The fragment ion peak at *m/z* 1103 $[M + Na/NaSO_4/H]^+$ in ESIMS of **1** indicated the presence of a sulphated group in the glycoside. Esterification shifts was observed for the signal of XylI C-4 (from δ 70.3 to 75.7) [5], indicating that the sulphate group was located at C-4 of the inner xylose unit. Hence, the structure of violaceuside A (**1**) was determined as 16β-acetoxy-3-*O*-[3-*O*-methyl-β-D-glucopyranosyl-(1 → 3)-β-D-xylo pyranosyl-(1 → 4)-β-D-quinovopyranosyl-(1 → 2)-4-*O*-sodiumsulphate-β-D-xylopyranosyl]-holosta-7, 24-diene-3β-ol.

Violaceuside B (**2**), mp 258–260°C, $[\alpha]_D^{20}$ -29 (*c* 0.2, MeOH), was also obtained as a white amorphous powder which was shown to have the molecular formula $C_{55}H_{85}O_{26}SNa$ by HRESI-MS. Fragment ion peaks at *m/z* 1119 $[M + Na/NaSO_4/H]^+$ and 1065 $[M/NaSO_4/OCH_3/H]^+$ indicated the presence of a sulphated group in the glycoside. The ^{1}H NMR, ^{13}C NMR and DEPT spectra of **2** (see table 2) revealed signals due to aglycone protons and carbons identical with those observed in violaceuside A (**1**). This suggested that **2** contained the same holosta-7,24-diene-3β-ol nucleus with a 16β-acetoxy group as that of **1**. The NMR spectrum of **2** also indicated the presence of four β-sugar units and suggested that they were two D-xylose, one D-glucose and one 3-*O*-methyl-D-glucose. This was confirmed by acid hydrolysis followed by GC-MS analysis of the corresponding aldononitrile peracetates. The DQCOSY and TOCSY experiments allowed the sequential assignment of the resonances for each sugar unit, starting from the easily distinguished signals due to anomeric protons. The HMQC experiment correlated all proton resonances with those of their corresponding carbons. The exact sequence of the sugars and their attachment points were solved by the HMBC experiment. The following cross peaks in HMBC spectrum supported that the sequence of sugar residues must be 3-*O*-MeGlc-(1 → 3)-Xyl-(1 → 4)-Glc(1 → 2)-Xyl (1 → 3)-aglycone: δ_H 4.72 (XylI H-1)/δ_C 89.9 (C-3 of aglycone), δ_H 5.15 (Glc H-1)/δ_C 82.0 (XylI C-2), δ_H 4.94 (XylII H-1)/δ_C 81.1 (Glc C-4) and δ_H 5.17 (MeGlc H-1)/δ_C 87.4 (XylII C-3). Esterification shift was observed for the signal of XylI C-4 (from δ 70.3 to 76.5) [5], so the sulphate group was also located at C-4 of the inner xylose unit. Consequently, the structure of **2** was established as 16β-acetoxy-3-*O*-[3-*O*-methyl-β-D-glucopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 2)-4-*O*-sodiumsulphate-β-D-xylopyranosyl]-holosta-7,24-diene-3β-ol.

Compounds **1** and **2** were evaluated for morphological deformation of *Pyricularia oryzae* mycelia [4], and cytotoxic activity against two cancer cell lines. The results showed that both of

Table 2. ^{1}H NMR, ^{13}C NMR data and key HMBC correlations of **2** (in pyridine-d_5, 600/150 MHz).

No.	δ_H *m (J in Hz)*	δ_C	*HMBC*	*No.*	δ_H *m (J in Hz)*	δ_C	*HMBC*
1	1.36 brs	36.6		XylI			
2	α = 2.00 m, β = 1.87 m	27.6		1	4.72 d (6.4)	105.6	C-3
3	3.22 d (9.2)	89.9	XylI C-1	2	4.14 m	82.0	Glc C-1, XylI C-3
4		40.0		3	4.30 m	75.9	XylI C-2
5	0.93 m	48.4	C-4, -10, -19, -30	4	5.04 m	76.5	
6	1.97 m	23.8		5	4.77 m, 3.75 m	64.7	XylI C-1, -3, -4
7	5.60 brs	121.0		Glc			
8		146.4		1	5.15 d (7.2)	105.1	XylI C-2
9	3.35 d (12.4)	47.8		2	4.07 m	75.9	Glc C-3, -4
10		36.1		3	3.95 m	69.8	Glc C-2
11	1.76 m, 1.49 m	23.2	C-8	4	4.09 m	81.1	XylII C-1, Glc C-2, -3
12	2.12 m	32.0		5	3.73 m	76.9	
13		60.1		6	4.36 m, 4.34 m	61.8	Glc C-5
14		48.0		XylII			
15	α = 2.58 m, β = 1.64 m	44.3	C-13, -14, -17, -32	1	4.94 d (7.6)	105.0	Glc C-4
16	5.89q-like (7.2)	75.9		2	3.90 m	74.2	XylII C-1
17	2.71 d (8.4)	55.2	C-13, -18, -21	3	4.08 m	87.4	MeGlc C-1, XylII C-2
18		180.3		4	3.94 m	71.2	
19	1.12 s	24.5	C-1, -5, -9, -10	5	4.12 m, 3.58 m	66.8	XylII C-1, -3, -4
20		86.1		MeGlc			
21	1.57 s	28.9	C-17, -20, -22	1	5.17 d (7.2)	105.3	XylII C-3
22	2.47 m, 1.90 m	39.3		2	3.91 m	75.3	MeGlc C-1, -5
23	2.05 m, 1.94 m	24.2		3	3.68 m	87.8	O**C**H$_3$
24	5.04 m	124.4		4	3.92 m	71.2	MeGlc C-5
25		132.8		5	3.89 m	78.3	MeGlc C-1, -4
26	1.64 s	26.2	C-24, -25, -27	6	4.39 m, 4.06 m	62.6	MeGlc C-5
27	1.58 s	18.4	C-24, -25, -26	OCH$_3$	3.83 s	61.3	MeGlc C-3
30	1.06 s	17.9	C-3, -4, -5, -31				
31	1.18 s	29.4	C-3, -4, -5, -30				
32	1.17 s	32.9	C-8, -13, -14, -15				
CH$_3$**C**OO		170.6					
CH$_3$COO	2.00 s	21.8	CH$_3$**C**OO				

them exhibited significant deforming effects against *Pyricularia oryzae* with MMDC < 8 μg/ml (5-FU as positive control with MMDC < 8 μg/ml). When tested by using the MTT colorimetic assay [7], **1** and **2** showed 84.7 and 87.9% cell division inhibitions of the leukaemia HL-60 cells at a concentration of 0.01 μM, respectively. **1** and **2** also showed noteworthy cytotoxicity toward human hepatoma BEL-7402 cells with $IC_{100} = 100$ and 10 μM, respectively.

3. Experimental

3.1 General experimental procedures

Melting points were determined on a XT5 micromelting point apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. The IR spectra were taken on a Bruker vector 22 spectrometer with KBr pellets. The 1D and 2D NMR spectra were measured in pyridine-d_5, with TMS as internal standard, on a Varian Inova-600 spectrometer. ESIMS and HRESI-MS were taken on a Micromass Quattromass spectrometer. GC were run on a Finnigan Voyager GC-MS chromatograph. Semi-preparative HPLC was carried out on an Agilent 1100 liquid chromatograph equipped with a refractive index detector using a Zorbax 300 SB-C_{18} column (25 cm × 9.4 mm i.d.). Chromatographic materials were silica gel (10–40 μm), reversed-phase silica gel (Merck RP-18, 40–63 μm) and Sephadex LH-20 (Pharmacia). TLC detection was achieved by spraying the Si gel plates with 10% H_2SO_4 followed by heating.

3.2 Animal material

Specimens of *Pseudocolochirus violaceus* were collected by hand using scuba or by trawl from offshore waters of the Sanya Bay in the South China Sea in March 2003. Taxonomic identification was carried out by Dr H.U. Dahms of the Oldenburg University, Germany. A voucher specimen (No. HN-0303) is deposited in the Research Centre for Marine Drugs, School of Pharmacy, Second Military Medical University, Shanghai, China.

3.3 Extraction and isolation

The sea cucumbers (60 kg, wet weight) were cut into small pieces, homogenised and extracted three times with 85% EtOH, and centrifuged. The EtOH extract was concentrated and the residue was suspended in water (3 L), and then partitioned successively with petroleum ether (3 L × 3), $CHCl_3$ (3 L × 3) and n-BuOH (3 L × 3). The combined n-BuOH phase was evaporated under reduced pressure to give a glassy material (30 g) which was shown to be bioactive against *Pyricularia oryzae*. The n-BuOH extract was subjected to column chromatography over silica gel (1000 g, 10–40 μm), developing with $CHCl_3$/MeOH/H_2O (8:2:1 to 7:3:1 to 6.5:3.5:1, lower phase, 10,000 ml each) and divided into ten major fractions based on TLC analysis. Fractions 6 and 8 mainly contained triterpene glycosides. Fraction 6 (500 mg) was chromatographed on a silica-gel column (3 × 30 cm) eluting with $CHCl_3$/MeOH/H_2O (7.5:3:1, lower phase, 3,000 ml) to yield crude glycoside **1**. The crude glycoside **1** was submitted to HPLC to give the pure glycoside **1** (101 mg, $t_R = 25.8$ min) using MeCN/MeOH/H_2O (30:6:64) as the mobile phase with a flow rate of 2 ml/min. Fraction 8 (310 mg) was chromatographed over 100 g silica gel with $CHCl_3$/MeOH/H_2O (7:3.5:1) to give crude glycoside **2** which was purified by Sephadex LH-20 and RP-18 chromatography to remove the

pigments and carbohydrates. Final purification of **2** was achieved by HPLC with MeOH/H_2O (48.5:51.5) as mobile phase and a flow rate of 1.9 ml/min, 18 mg of pure glycoside **2** was obtained (t_R = 34.1 min).

3.3.1 Violaceuside A (1). White amorphous powder, mp 224–225°C (dec), $[\alpha]_D^{20}$ − 22 (*c* 0.2, MeOH); IR (KBr) ν_{max} 3470 (OH), 1742 (C═O), 1650 (C═C), 1244, 1210 (sulphate group); ^{1}H NMR and ^{13}C NMR data, see table 1; ESI-MS (positive ion mode) *m/z* 1223 $[M + Na]^+$, 1239 $[M + K]^+$, 1121 $[M + Na\text{-}SO_3Na + H]^+$, 945 $[1121 - MeGlc]^+$, 791 $[945\text{-}Xyl\text{-}Na + H]^+$, 609 $[MeGlc + Xyl + Qui + Xyl + Na]^+$, 513 $[791\text{-}Qui\text{-}Xyl]^+$, 477 $[MeGlc + Xyl + Qui + Na]^+$ ESI-MS/MS (positive ion mode, *m/z* 1223) *m/z* 1165 $[M + Na\text{-}OAc + H]^+$, 1103 $[M + Na\text{-}SO_4Na\text{-}H]^+$, 1044 $[1103\text{-}OAc]^+$, 1031 $[M + Na\text{-}MeGlc\text{-}O]^+$, 729 $[609 + SO_4Na + H]^+$, 477, 331 $[MeGlc + Xyl + Na]^+$; ESI-MS (negative ion mode) *m/z* 1177 $[M - Na]^-$; ESI-MS/MS (negative ion mode, *m/z* 1177) *m/z* 1049 $[M\text{-}SO_4Na\text{-}OCH_3\text{-}H]^-$, 1009 $[M\text{-}Me\text{-}MeGlc]^-$, 949 $[1009\text{-}OAc\text{-}H]^-$, 817 $[949 - Xyl]^-$; HRESI-MS (positive ion mode) *m/z* 1223.4890 $[M + Na]^+$ (calcd for $C_{55}H_{85}O_{25}SNa_2$, 1223.4896).

3.3.2 Violaceuside B (2). White amorphous powder, mp 258–260°C, $[\alpha]_D^{20}$ − 29 (*c* 0.2, MeOH); IR (KBr) ν_{max} 3500 (OH), 1748 (C═O), 1662 (C═C), 1220, 1218 (sulphate group); ^{1}H NMR and ^{13}C NMR data, see table 2; ESI-MS (positive ion mode) *m/z* 1239 $[M + Na]^+$, 1119 $[M + Na\text{-}SO_4Na\text{-}H]^+$, 619 $[\text{sugar moiety-}SO_4Na + H_2O]^+$, 493 $[MeGlc + Xyl + Glc + Na]^+$; ESI-MS/MS (positive ion mode, *m/z* 1239) *m/z* 1047 $[M + Na\text{-}MeGlc\text{-}O]^+$, 769 $[M + Na\text{-}MeGlc\text{-}Xyl\text{-}Glc]^+$, 625 $[MeGlc + Xyl + Glc + Xyl + Na]^+$, 493 $[MeGlc + Xyl + Glc + Na]^+$; ESI-MS (negative ion mode) *m/z* 1193 $[M - Na]^-$; ESI-MS/MS (negative ion mode, *m/z* 1193) *m/z* 1065 $[M\text{-}SO_4Na\text{-}OCH_3\text{-}H]^-$, 1025 $[M - Me\text{-}MeGlc]^-$, 965 $[1025 - HOAc]^-$, 885 $[M\text{-}2Na\text{-}MeGlc\text{-}Xyl]^-$, 723 $[885 - Glc]^-$; HRESI-MS (positive ion mode) *m/z* 1239.4840 $[M + Na]^+$ (calcd for $C_{55}H_{85}O_{26}SNa_2$, 1239.4845).

3.3.3 Acid hydrolysis of 1 and 2. Each glycoside (3 mg) was dissolved in 1 ml of 2 M trifluoroacetic acid and heated in an ampoule at 120°C for 2 h. The reaction mixture was cooled and poured into CH_2Cl_2/H_2O (1:1). The aqueous phase was evaporated under reduced pressure, and 0.8 ml of pyridine and 2 mg of $NH_2OH \cdot HCl$ were added to the dry residue. The mixture was heated at 90°C for 30 min, and then 0.8 ml of Ac_2O was added and the heating at 90°C was continued for 1 h. The solution was evaporated to dryness under reduced pressure. The resulting aldononitrile peracetates were analysed by GC-MS.

The GC-MS experiment was carried out on a Finnigan Voyager GC-MS chromatograph using a DB-5 capillary column (30 cm × 0.25 mm). Nitrogen was used as carrier gas. The initial column oven temperature was 150°C for 2 min; then the temperature was increased by 15°C/min to a final value of 300°C. The carbohydrates were determined by comparing the retention times and MS behaviour with standard aldononitrile peracetates prepared from authentic sugars by the same procedure performed for the sample. Xylose, quinovose and 3-*O*-Me-glucose were identified in a ratio of 2:1:1 for glycoside **1**, and xylose, glucose and 3-*O*-Me-glucose in a ratio of 2:1:1 for glycoside **2**.

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