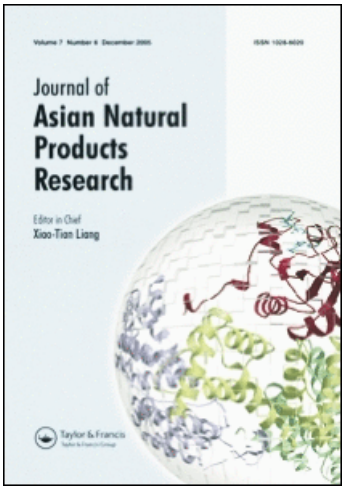

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Lecanorosides A and B, two new triterpene glycosides from the sea cucumber *Actinopyga lecanora*

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Two new sulphated triterpene glycosides, lecanorosides A (**1**) and B (**2**), along with the known compounds holothurins A (**3**), A_1 (**4**), and B (**5**), were isolated from the sea cucumber *Actinopyga lecanora*. Their structures were deduced from extensive spectral analysis (NMR and MS) and chemical evidence. Glycosides **1** and **4** showed marginal *in vitro* cytotoxicity against two human tumour cell lines.

Keywords: *Actinopyga lecanora*; Triterpene glycosides; Cytotoxicity; Lecanoroside A; Lecanoroside B

1. Introduction

Sea cucumber (class Holothuroidea) has been shown to contain a variety of lanosterol-type triterpene glycosides, characteristic with 18(20) lactone and a sugar chain of up to six monosaccharide units, linked to C-3 of the algycon. These substances showed wide biological effects, including antifungal, cytotoxic, hemolytic, cytostatic, and immunomodulatory activities [1]. As a part of our ongoing investigation on biologically active triterpene glycosides from sea cucumbers [2,3], we decided to focus our attention on the saponins of the South China sea cucumber *Actinopyga lecanora*, collected near Hainan province, China. In this paper, we report the isolation and structural elucidation of five sulphated glycosides: lecanorosides A (**1**) and B (**2**), along with the known compounds holothurins A (**3**), A_1 (**4**), and B (**5**).

2. Results and discussion

An ethanolic extract of *A. lecanora* was suspended in H_2O and extracted successively with petroleum ether and *n*-BuOH. The *n*-BuOH layer was dried and subjected to several chromatographic purification steps to afford **1–5**. Structures of these glycosides (Figures 1 and 2) were elucidated by extensive analyses of 1 D NMR (^{13}C, DEPT, ^{1}H) and 2 D NMR (^{1}H–^{1}H COSY, NOESY, HMQC, HMBC) and MS spectra.

^{13}C NMR spectral data of the aglycone in the glycosides **1** and **2** (Table 1) were found to be identical to those of holothurins A (**3**) and B (**5**) [4,5]. Combining the ^{1}H NMR spectrum and ^{1}H–^{1}H COSY, HMBC, and NOESY experiments (Table 1), the aglycone structure of compounds **1** and **2** was confirmed as holothurigenol, the same as that of **3** and **5**.

Lecanoroside A (**1**) was obtained as a colourless amorphous powder, positive to Liebermann–Burchard and Molish tests. The molecular formula was established as $C_{41}H_{62}O_{20}Na_2S_2$ from the $[M + Na]^+$ ion peak at *m/z* 1007.2973 in the positive ion mode HRESI-MS and $[M - Na]^-$ ion peak at

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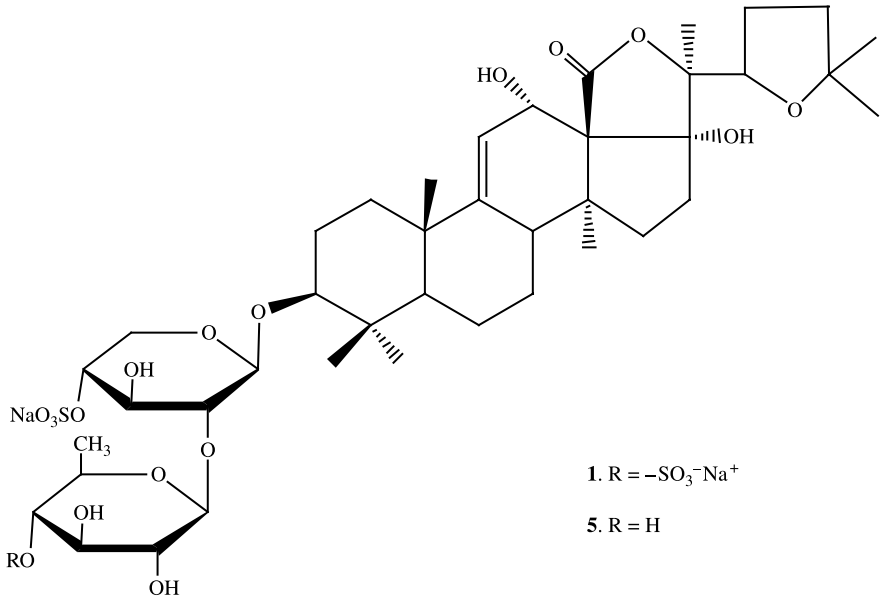

Figure 1. Structures of **1** and **5**.

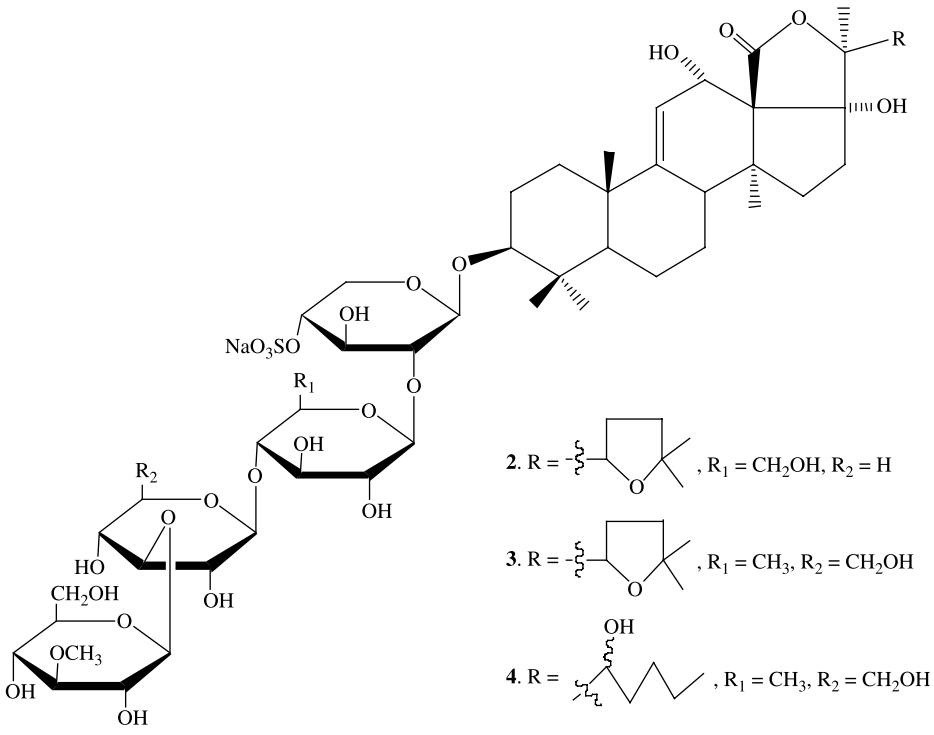

Figure 2. Structures of **2–4**.

Table 1. ^{13}C and ^{1}H NMR spectral data for the aglycone moieties of lecanorosides A (**1**) and B (**2**).

Position	δ_C mult[a]	δ_H mult[b] (J in Hz)	HMBC	NOESY correlation
1	36.9 t	1.38 m		
		1.86 m		
2	27.4 t	1.86 m		H-19
3	89.3 d	3.05 dd (4.0, 11.6)		H-5, H-31,H-1 Xyl_1
4	40.4 s			
5	52.3 d	0.85 m		H-3, H-31
6	21.7 t	1.42 m		
7	28.8 t	1.78 m		
8	41.4 d	3.22 m		H-19
9	154.5 s			
10	40.2 s			
11	115.9 d	5.63 d (4.0)	C-8, -10, -13	H-12
12	71.9 d	4.94 d (4.0)	C-9, -11, -14,-18	H-11, H-21
13	59.4 s			
14	46.4 s			
15	37.3 t	1.78 m		
16	35.9 t	2.92 dd (9.0, 14.6)		H-32
		2.35 m		
17	90.2 s			
18	175.5 s			
19	23.0 q	1.30 s	C-1, -5, -9, -10	H-2, H-8
20	87.6 s			
21	19.5 q	1.73 s	C-17, -20, -22	H-12
22	81.1 d	4.30 m	C-17, -20, -21	
23	28.6 t	1.98 m	C-20, -24, -25	
24	38.5 t	1.66 m	C-22, -23, -26, 27	
25	83.0 s			
26	29.1 q	1.26 s	C-24, -25, -27	
27	27.9 q	1.22 s	C-24, -25, -26	
30	17.2 q	0.98 s	C-3, -4, -5, -31	
31	28.6 q	1.13 s	C-3, -4, -5, -30	
32	20.7 q	1.53 s	C-8, -13,-14, -15	

[a] Recorded at 100 MHz in C_5D_5N/D_2O (4:1), multiplicity by DEPT.
[b] Recorded at 400 MHz in C_5D_5N/D_2O (4:1).

m/z 961 in the negative ion mode ESI-MS. The fragment ion peak at *m/z* 887 $[M + Na\text{-}NaHSO_4]^+$ and 767 $[M + Na\text{-}2NaHSO_4]^+$ in the positive ion mode ESI-MS/MS indicated the presence of two sulphate groups in the glycoside. This was confirmed by solvolytic desulphation of **1**, which rendered the same desulphated derivative (DS-holothurin B) as holothurin B (**5**) [4], indicating that both glycosides differed only in the degree of sulphation of oligosaccharide chain. Comparision of ^{13}C NMR spectral data of **1** with those of its desulphated derivative DS-holothurin B allowed the determination of the site of linkage of the sulphate groups. Esterification shifts were observed at signals of C-4′ (xylose) (from 70.9 to 76.7) and C-4 (quinovose) (from 77.1 to 82.6).

The sugar unit in **1** was deduced from the ^{13}C NMR and ^{1}H NMR spectra, which showed two anomeric carbons at near δ 105.0 and corresponding anomeric proton signals at δ 4.96 (d, $J = 7.3$ Hz, quiniovose) and 4.66 (d, $J = 7.2$ Hz, xylose) (Table 2). The β configurations of the anomeric protons were deduced from the coupling constant values ($J = 7.3$ and 7.2 Hz). The interglycosidic linkages in disaccharide chain of **1** and its connectivity to aglycone were confirmed by HMBC experiment (Table 2), which

Table 2. ^{1}H NMR and ^{13}C NMR spectral data for the sugar moieties of lecanoroside A (**1**).

Position	δ_C mult[a]	δ_H mult[b] (J in Hz)	HMBC	NOESY correlation
Xyl(1 → C-3)				
1′	105.1	4.96 d (7.3)	C-3	H-3, H-31, H-3,5 Xyl
2′	82.9	3.98 m	C-1 Qui, C-3Xyl	H-1 Qui
3′	75.6	4.23 m	C-4 Xyl, C-2 Xyl	H-1,5 Xyl
4′	76.7	3.66 m	C-2 Xyl	
5′	64.5	3.76 m	C-1 Xyl	H-1,3 Xyl
		4.75 m		
Qui (1 → 2Xyl)				
1″	105.0	4.66 d (7.2)	C-2 Xyl	H-2 Xyl, H-3,5 Qui
2″	76.7	3.94 m	C-3 Qui	
3″	81.2	4.30 m	C-4 Qui	H-1,5 Qui
4″	82.6	3.72 m	C-3,5 Qui	
5″	71.9	3.84 m		H-1,3 Qui
6″	18.8	1.70 s	C-4,5 Qui	H-30

[a] Recorded at 100 MHz in C_5D_5N/D_2O (4:1), multiplicity by DEPT.
[b] Recorded at 400 MHz in C_5D_5N/D_2O (4:1).

showed correlations between H-1 of xylose and C-3 of the aglycone, and H-1 of quinovose and C-2 of xylose. These data were also confirmed by the NOESY cross-peaks between H-1 of the first xylose residue and H-3 of aglycone; between H-2 of the xylose residue and H-1 of quinovose (Table 2). Compound **1** was treated with 2 M trifluoroacetic acid to give D-xylose and D-quivonose in the ratios 1:1. The sugars were identified by GC-MS in the form of the corresponding aldononitrile peracetates.

All these data confirmed the structure of lecanoroside A as 3β-*O*-[4″-*O*-sodium sulphate-β-*D*-quinovopyranosyl- (1 → 2)-4′-*O*-sodium sulphate-β-D-xylopyranosyl]-22,25-epoxyholost-9-en-12α,17α-diol (**1**).

The presence of 3-*O*-methylglucose, glucose and xylose in a 1:1:2 ratio in **2** was established by acid hydrolysis with 2 M trifluoroacetic acid and GC-MS analysis of the corresponding aldononitrile peracetates, that was confirmed by the ^{13}C NMR and DEPT spectra of **2** (Table 3) by showing four anomeric carbons at δ 105.0–106.2 and their corresponding anomeric protons at δ 4.68 (1H, d, $J = 7.8$ Hz), 5.03 (1H, d, $J = 7.2$ Hz), 4.95 (1H, d, $J = 7.8$ Hz), and 5.25 (1H, d, $J = 7.2$ Hz) (Table 3), indicating the β configurations for the glycosidic bonds. The NMR spectral data of the sugar part of **2** were coincident with those of intercedenside C previously isolated from *Mensamaria intercedens* [2], having a sulphate at C-4 of the first xylose residue. The HMBC correlations between H-1′ (δ 4.68) and C-3 (δ 89.3); H-1″ (δ 5.03) and C-2′ (δ 83.2); H-1″ (δ 4.95) and C-4″ (δ 81.7); H-1″ (δ 5.25) and C-3″ (δ 88.5) indicated the sequence of the sugar residue of **2** should be 3-*O*-methyl-glu-(1 → 3)-xly-(1 → 4)-glu-(1 → 2)-xyl-(1 → 3)-aglycone, which were confirmed by the NOESY spectrum on showing NOE corrections between H-1′ (δ 5.03) and H-3 (δ 3.05); H-1″ (δ 5.03) and H-2′ (δ 3.98); H-1″ (δ 4.95) and H-4″ (δ 3.94); H-1‴ (δ 5.25) and H-3″ (δ 4.20). Hence, the structure of lecanoroside B was elucidated as 3-*O*-[3″-*O*-Methy1-β-D-glucopyranosyl-(1 → 3)-β-D-xylopyranosy1-(1 → 4)-β-D-glucopyranosy1-(1 → 2)-4′-*O*-sodium sulphate-β-D-xylopyranosy1]-22,25-epoxyholost-9-en-12α,17α-diol (**2**).

Glycosides **1** and **4** were tested for *in vitro* cytotoxicity against human leukaemia LH-60 cells and human hepatoma BEL-7402 cells. The IC_{50} values of each saponin tested were detected after 72 h of cell exposure to compounds. Our results indicated that **1** and

Table 3. ^{1}H NMR and ^{13}C NMR spectral data for the sugar moieties of lecanoroside B (**2**).

Position	δ_C mult[a]	δ_H mult[b] (J in Hz)	HMBC	NOESY correlation
$Xyl_1(1 \rightarrow$ C-3)				
1′	106.2	4.68 (7.8)	C-3	H-3
2′	83.2	3.98 m	Glu C-1	Glu H-1
3′	75.7	4.13 m	Xyl_1 C-2	
4′	76.6	5.12 m		
5′	64.3	4.72 m		
		3.72 m		
Glu $(1 \rightarrow 2Xyl_1)$				
1″	105.2	5.03 d (7.2)	Xyl_1 C-2	Xyl_1 H-2
2″	76.3	4.28 m		
3″	69.7	4.04 m		
4″	81.7	3.94 m	Xyl_2 C-1	
5″	77.0	3.84 m		
6″	62.1	4.28 m		
		4.36 m		
Xyl_2 $(1 \rightarrow$ 4Glu)				
1‴	105.0	4.95 d (7.8)	Glu C-3	Xyl_2 H-3
2‴	73.8	4.02 m		
3‴	88.5	4.20 m	MeGlu C-1	Xyl_2 H-1
4‴	69.4	4.04 m		
5‴	64.7	4.40 m		
		3.60 m		
Meglu $(1 \rightarrow 3Xyl_2)$				
1⁗	105.5	5.25 d (7.2)	Xyl_2 C-3	MeGlu H-3
2⁗	75.1	3.98 m	MeGlu C-3	
3⁗	87.9	3.68 m	MeGlu C-2, -4, OMe	MeGlu H-1
4⁗	70.7	4.06 m	OMe	
5⁗	78.3	3.94 m	MeGlu C-4	
6⁗	62.3	4.4 2m		
		4.18 m		
O*CH*$_3$	60.7	3.84 s	MeGlu C-3	

[a] Recorded at 100 MHz in C_5D_5N/D_2O (4:1)
[b] Recorded at 400 MHz in C_5D_5N/D_2O (4:1)

4 were marginally cytotoxic against LH-60 (IC_{50}s 4.5 ± 0.1 and 1.68 ± 0.1 μg/ml, respectively) and BEL-7402 (IC_{50}s 17.5 ± 0.1 and 6.2 ± 0.1 μg/ml, respectively) cell lines.

3. Experimental

3.1 General experimental procedures

Melting points were determined on a XT5-XMT apparatus and are uncorrected. Optical rotations were measured on a Perkin–Elmer-341 polarimeter. NMR spectra were recorded on Inova-400 spectrometer. ESI-MS and HRESI-MS were recorded on a Micromass Quatro mass spectrometer. GC-MS were performed on a Finnigan Voyager GC-MS spectrometer with a HP-5 column (30 m × 0.25 mm i.d.). Semipreperative HPLC was carried out on an Agilent 1100 liquid chromatograph equipped with a refractive index detector using a Zobax 300 SB-C_{18} column (250 × 9.4 mm i.d.). Column chromatographies were performed on silica gel H (10–40 μm, Qingdao Marine Chemical Inc.) and reversed-phase silica gel (Lichroprep RP–18, 40–63 μm). Fractions were monitored by TLC (precoated silica gel G60 F-254 plates from Yantai Zhifu Chemical Group Co.), and spots were visualised by heating silica gel plates sprayed with 15% H_2SO_4 in EtOH.

3.2 Animal material

Specimens of *Actinopyga lecanoria* were collected at different locations around the South China Sea near Hainan Province in spring 2004. The organisms were identified by Professor Y.L. Liao of Qingdao Institute of Oceanic Research, China. A voucher specimen (No. AL20041225) is preserved at the Research Center for Marine Drugs, School of Pharmacy, Second Military Medical University, China.

3.3 Extraction and isolation

The sea cucumbers (800 g, dry weight) were ground into fine powder and extracted twice with refluxing ethanol. The combined extracts were evaporated under reduced pressure, and the aqueous residue was dissolved in water. Desalting was carried out by passing this fraction through a DA 101 resin column (Nankai University, Tianjin, China), first eluting the inorganic salts and polar impurities with H_2O, and then the glycoside fraction (7.5 g) with 80% aqueous ethanol. The glycoside fraction was further chromatographed on silica gel column, eluting with $CHCl_3$/MeOH/H_2O (70:20:1 to 70:30:3) to furnish fractions A (1.36 g), B (58.6 mg), C (135.3 mg), and D (30.8 mg). Fraction A was chromatographed on a reversed-phase ODS column (3 × 40 cm) with MeOH-H_2O (45:55) to give **1** (57.1 mg) and fraction A_1 (962 mg). Final purification of these fractions was achieved by HPLC: part of fraction A_1 (240 mg) afforded glycoside **2** (16.2 mg), **3** (67.4 mg), and **4** (24.2 mg) using MeOH-H_2O (47:53); fraction C yielded **5** (41.3 mg) using MeOH-H_2O (55:45).

3.3.1 Lecanoroside A (1)

White amorphous powder, mp 248.5 ± 0.5°C, $[\alpha]_D^{20}$ −31.8 (*c* 0.5, pyridine); ^{1}H NMR and ^{13}C NMR spectral data, see Tables 1 and 2; HRESI-MS: *m/z* 1007.2973 (positive ion mode) $[M + Na]^+$ (calcd for $C_{41}H_{62}O_{20}S_2Na_3$, 1007.2969).

3.3.2 Lecanoroside B (2)

White amorphous powder, mp 236.0 ± 0.5°C, $[\alpha]_D^{20}$ −15.2 (*c* 0.5, pyridine); ^{1}H NMR and ^{13}C NMR, see Tables 1 and 3; HRESI-MS: *m/z* 1230.4670 (positive ion mode) $[M + Na]^+$ (calcd for $C_{53}H_{84}O_{27}SNa_2$, 1230.4716).

3.4 Acid hydrolysis of 1 and 2

Each compound (5 mg) was diluted in a screw-cap vial with 2 M trifluoroacetic acid (5 ml) and heated in ampoule at 120°C for 1 h. The aglycone was extracted with CH_2Cl_2, and the aqueous residue was evaporated to dryness. Each sugar mixture was treated with pyridine (1 ml) and $NH_2OH{\cdot}HCl$ (2 mg) at 100°C for 1 h. The reaction mixture was cooled and peracetylated with acetic anhydride (1 ml) at 100°C for 1 h. The resulting aldononitrile peracetate was evaporated under reduced pressure and submitted to analysis by GC-MS, using aldononitrile peracetates of standard D-3-*O*-Me-glucose, D-xylose, D-glucose, and D-quinovose as reference samples. Xylose and quinovose were identified for **1** in a ratio of 1:1. Xylose, glucose, and 3-*O*-Me-glucose were identified for the glycoside **2** in a ratio of 2:1:1.

3.5 Cytotoxicity assay

The cytotoxicity against human leukemia HL-60 cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay on 96-well microplates as previously reported [6]. The cytotoxicity data against human hepatoma BEL-7402 cells was evaluated by the sulphorhodamine B (SRB) protein assay described in a previous paper [7] using 10-hydroxycamptothecine as the positive control. Dose–response curves were plotted for the samples (**1** and **2**), and the IC_{50}s were calculated as the concentrations of the tested saponins resulting in 50% reduction of absorption compared to the control cells.

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