Notes

Sulfated Alkenes from the Echinus *Temnopleurus hardwickii*

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Three new sulfated alkenes (**1**−**3**), trimethylammonium (5*R*)-5,9-dimethyl-(3*E*)-3,8-decadienyl-1-sulfate, dimethylammonium (5*R*)-5,9-dimethyl-(3*E*)-3,8-decadienyl-1-sulfate, and 2′-methyl-4′-oxobutan-2-ylammonium (5*R*)-5,9-dimethyl-(3*E*)-3,8-decadienyl-1-sulfate, were isolated from an echinoderm, *Temnopleurus hardwickii*. Their structures were elucidated by spectral analysis and chemical degradation methods.

The echinoderms, which include crinoids (sea lilies), ophiuroids (brittle stars), asteroids (sea stars), holothuroids (sea cucumbers), and echinoids (sea urchins), are a rich source of sulfated metabolites. Most of the metabolites are saponins,[1] and only a small number of them contain nitrogen. These compounds have shown a broad spectrum of biological activities, such as antiviral properties, cytotoxicity, and inhibition of protein tyrosine kinases.[2]

In the course of our search for antitumor constituents from the echinoderms, cytotoxicity was detected in the crude extract of the echinoderm *Temnopleurus hardwickii* (Gray, 1855). Bioassay-guided fractionation of this extract afforded three novel sulfated alkenes, compounds **1**−**3**. Here we report the isolation, structure elucidation, and cytotoxic activities of these three compounds.

Specimens of *T. hardwickii* were collected in Qingdao in 2004. The tissue was homogenized and extracted in ethanol. The concentrated aqueous residue of this extract was partitioned between water and ethyl acetate. Then the water layer was re-extracted with *n*-BuOH, and this extract was sequentially subjected to Si gel column chromatography, Sephadex LH-20 gel filtration, and repeated RP-18 HPLC (CH_3CN/H_2O, 1:3) to give compounds **1** (80 mg), **2** (10 mg), and **3** (15 mg).

Compound **1** was obtained as a colorless oil. Negative HRESIMS of **1** showed an anion peak at *m*/*z* 261.1162 (calcd for $C_{12}H_{21}O_4S^-$ 261.1161), and the negative ESIMSMS exhibited an anion peak at *m*/*z* 97.0, suggesting the presence a sulfate group in the structure.[3] The presence of the sulfate moiety was further corroborated by a strong IR (KBr) absorption band at 1228 cm^{-1}.[2] The ^{13}C NMR spectrum of compound **1** showed 15 signals. The ^1H NMR spectrum and DEPT experiments revealed the presence of six methyls, four methylenes, four methines, one trisubstituted double bond, and one disubstituted double bond. The COSY experiment readily established the carbon skeleton from C1 to C8. The two singlets in the ^1H NMR spectrum at δ 1.58 (3H, s) and 1.67 (3H, s) were attributed to an isopropenyl group connected to C-8 on the basis of the HMBC correlations of CH_3-12 with C-9 and CH_3-10 with C-8. Furthermore, the HMBC correlations of CH_3-11 with C-5, C-6, and C-4 indicated that CH_3-11 was attached to C-5. The ^{13}C shift of C-1 (δ 68.8) suggested that it was connected to the sulfate group through an ester bond. Thus the sulfated alkene core structure of **1** was established.

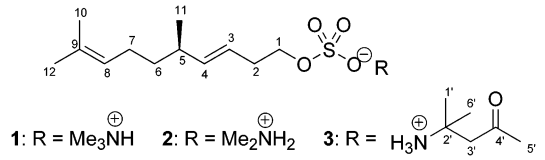
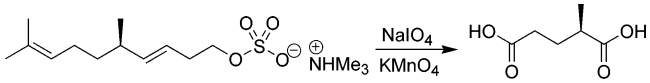


Figure 1. Structures of compounds **1**−**3**.

Scheme 1. Chemical Oxidation of Compound **1**



Additionally, the ^1H NMR signal at δ 2.96 (9H, d, J = 3.7 Hz) and its COSY correlation with an exchangeable proton at δ 9.78 (1H, brs) together with the ^{13}C NMR signal at δ 45.3 (CH_3) revealed a trimethylammonium ion in **1**. This is consistent with the positive HRESIMS pseudomolecular ion peak at *m*/*z* 381.2792 [calcd for $C_{12}H_{21}O_4S^-\cdot NH(CH_3)_3^+ + NH(CH_3)_3^+$, 381.2787]. It is interesting to note that despite the 1:1 NMR ratio between the trimethylammonium and the sulfate portions of the molecule, the positive HRESIMS pseudomolecular ion contained two trimethylammonium ions. This is because one trimethylammonium ion was required to form the neutral molecular complex of **1** with the sulfate anion, while a second trimethylammonium cation was captured from another molecule of **1** for ESIMS ionization. Therefore, compound **1** was established as a trimethylamium sulfate alkene with the molecular formula $C_{15}H_{31}NO_4S$ ($C_{12}H_{21}O_4S^-\cdot NH(CH_3)_3^+$). The *E*-configuration of the double bond in the alkene chain was assigned on the basis of the H-3/H-4 coupling constant (15.4 Hz) and the ^{13}C NMR chemical shifts of C-2 and C-5 (Table 2).[4,5] The absolute stereochemistry of **1** was determined through oxidative degradation. (2*R*)-2-Methylpentanedioic acid (**1a**) was obtained from the reaction mixture of **1** with $NaIO_4$ and $KMnO_4$.[6] The absolute configuration of **1a** was determined by comparison of its $[\alpha]^{25}_D$ (−16.8) with those of authentic samples, (2*R*)-2-methylpentanedioic acid ($[\alpha]^{28}_D$ −22.8)[7] and the (2*S*)-isomer ($[\alpha]^{28}_D$ +22.5).[8] Thus the structure of **1** was deduced as trimethylammonium (5*R*)-5,9-dimethyl-(3*E*)-3,8-decadienyl-1-sulfate.

Compound **2** displayed a pseudomolecular ion at *m*/*z* 353.2487 in the positive HRESIMS [calcd for $C_{12}H_{21}O_4S^-\cdot NH_2(CH_3)_2^+$ +

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$NH_2(CH_3)_2^+$, 353.2474], which was further corroborated by the NMR data. The structure of **2** was established by comparing its NMR data with those of compound **1**. The ^1H and ^{13}C NMR spectra of **2** were very similar to those of **1** except for the presence of one extra exchangeable proton (δ 8.15, 2H, brs) and two instead of three methyls attached to nitrogen (^1H δ 2.77, 6H, t, $J = 5.5$ Hz; ^{13}C δ 35.5), indicating the replacement of $NH(CH_3)_3^+$ in **1** by $NH_2(CH_3)_2^+$ in **2**. Therefore the structure of **2** is dimethylammonium 5,9-dimethyl-(3*E*)-3,8-decadienyl-1-sulfate.

Compound **3** exhibited an anion peak at *m*/*z* 261.1155 in the negative HRESIMS (calcd for $C_{12}H_{21}O_4S^-$ 261.1161) and a pseudomolecular ion at *m*/*z* 493.3322 in the positive HRESIMS [calcd for $C_{12}H_{21}O_4S^-\cdot C_6H_{14}NO^+ + C_6H_{14}NO^+$, 493.3311], which was further corroborated by the NMR data. On the basis of the ^1H NMR and ^{13}C NMR spectra, **3** contained the same sulfate alkene core structure as in **1** and **2**, with a different ammoniun counterion. The ^1H NMR spectrum of **3** revealed three exchangeable protons at δ 7.55 (3H, brs) and no $N-CH_3$ signals, as well as three additional methyls and one additional methylene, which were also observed by ^{13}C NMR (methyls at δ 31.0, 25.7, 25.7 and methylene at δ 49.9). Additionally, one carbonyl was observed at δ 208.7. The HMBC data revealed that these carbons belonged to a 2-methyl-4-oxobutan-2-yl ammonium ion ($C_6H_{14}NO$). Therefore the structure of compound **3** is 2′-methyl-4′-oxobutan-2′-ylammonium 5,9-dimethyl-(3*E*)-3,8-decadienyl-1-sulfate.

Since compounds **2** and **3** possess the same core structure and nearly identical optical rotations as **1**, they were assigned the same absolute stereochemistry as **1**. Compounds **1**−**3** were evaluated for their cytotoxicities against the P388 and A-549 cell lines by the MTT method.[9] Only **3** was found to be weakly active against the P388 cell line (IC_{50} 290 μM).

While sulfated alkenes are common in marine invertebrates, those that contain complex counterions are rare. To the best of our knowledge, this sulfated carbon skeleton is reported for the first time from nature and might represent a biodegradation product of a sesquiterpene. Alternatively, these metabolites could result from the addition of an acetate group to the oxidized end of a monoterpene.

Table 1. ^1H NMR Data for Compounds **1**−**3**[a]

position	1	2	3
1	4.02 (t, 7.3)	4.02 (t, 7.1)	4.01 (t, 7.1)
2	2.37 (m)	2.39 (m)	2.38 (m)
3	5.36 (dt, 15.4, 6.1)	5.34 (dt, 15.4, 6.4)	5.34 (dt, 15.4, 6.5)
4	5.38 (dd, 15.4, 7.0)	5.40 (dd, 15.4, 7.3)	5.39 (dd, 15.4, 7.0)
5	2.06 (m)	2.07 (m)	2.06 (m)
6	1.27 (m)	1.27 (m)	1.26 (m)
7	1.92 (m)	1.92 (m)	1.92 (m)
8	5.07 (t, 6.5)	5.07 (t, 6.5)	5.07 (t, 6.4)
10	1.58 (s)	1.58 (s)	1.58 (s)
11	0.94 (d, 6.6)	0.95 (d, 6.9)	0.94 (d, 6.6)
12	1.67 (s)	1.68 (s)	1.67 (s)
NH	9.75 (brs)	8.15 (brs)	7.55 (brs)
$N-CH_3$	2.96 (d, 3.7)	2.77 (t, 5.5)	
3′			2.94 (s)
5′			2.21 (s)
1′, 6′			1.46 (s)

[a] Spectra were recorded in $CDCl_3$ at 600 MHz for ^1H using TMS as internal standard.

Table 2. ^{13}C NMR Data for Compounds **1**−**3**[a]

position	1	2	3
1	67.8 CH_2	68.3 CH_2	68.2 CH_2
2	32.6 CH_2	32.6 CH_2	32.5 CH_2
3	123.1 CH	123.0 CH	123.1 CH
4	139.1 CH	139.4 CH	139.3 CH
5	36.2 CH	36.3 CH	36.3 CH
6	36.9 CH_2	37.0 CH_2	37.0 CH_2
7	25.6 CH_2	25.7 CH_2	25.7 CH_2
8	124.5 CH	124.6 CH	124.6 CH
9	131.1 qC	131.2 qC	131.2 qC
10	17.6 CH_3	17.7 CH_3	17.7 CH_3
11	20.5 CH_3	20.5 CH_3	20.5 CH_3
12	25.5 CH_3	25.7 CH_3	25.7 CH_3
$N-CH_3$	45.3 CH_3	35.5 CH_3	
2′			53.2 qC
3′			49.9 CH_2
4′			208.7 qC
5′			31.0 CH_3
1′, 6′			25.7 CH_3

[a] Spectra were recorded in $CDCl_3$ at 150 MHz for ^{13}C using TMS as internal standard.

Experimental Section

General Experimental Procedures. Melting points were measured using a Yanaco MP-500D micro-melting point apparatus and were uncorrected. Optical rotations were obtained on a JASCO P-1020 digital polarimeter. UV spectra were recorded on a Beckmen DU 640 spectrophotometer. IR spectra were taken on a Nicolet Nexus 470 spectrophotometer in KBr disks. All NMR data were recorded on a JEOL Eclips-600 spectrometer using TMS as internal standard, and the chemical shifts were recorded as δ values. ESIMS was measured on a Q-TOF Ultima Global GAA076 LC mass spectrometer. Semi-prepartive HPLC was performed using a RP-18 column (YMC-Pack ODS-A, 10 × 250 mm, 5 μm, 4 mL/min).

Animal Material. *T. hardwickii* (5 kg) was collected in Jiaozhou Bay, Qingdao (−15 m), and was frozen at −18 °C until extracted. The animals were identified by Professor Xiaoqi Zeng, and a voucher specimen of the organism is preserved at Ocean University of China.

Extraction and Isolation. The frozen animals were homogenized in EtOH (15 L) and extracted for 12 h. After centrifugation of the extract the solvent was removed and the concentrated aqueous residue was partitioned against EtOAc and subsequently against *n*-BuOH. The *n*-BuOH extract was subjected to Si gel CC using a step gradient of $CHCl_3$/MeOH, and the 85:15 $CHCl_3$/MeOH fraction was collected. Further chromatography on Sephadex LH-20 (MeOH) followed by repeated C18 RP-HPLC (CH_3CN/H_2O, 1:3) gave compounds **1** (80 mg), **2** (10 mg), and **3** (15 mg).

Biological Assays. The fractions were assayed using the MTT method[9] with the mouse temperature-sensitive $p34^{cdc2}$ mutant cell line tsFT210. Cytotoxic activity of the three compounds was evaluated by the MTT method using P388 and A-549 cell lines. The cell lines were grown in RPMI-1640 supplemented with 10% FBS under a humidified atmosphere of 5% CO_2 and 95% air at 37 °C (tsFT210 cell line at 32 °C). A 200 μL portion of this cell suspension at a density of 5×10^4 cell mL^{-1} was plated in 96-well microtiter plates and incubated for 24 h at the above conditions. Then 2 μL of the test compound solutions (in DMSO) at different concentrations was added to each well and further incubated for 72 h in the same conditions. A 20 μL volume of the MTT solution (5 mg/mL in IPMI-1640 medium) was added to each well and incubated for 4 h. The medium (150 μL) containing MTT was then replaced with DMSO and gently mixed to dissolve any formazan crystals formed. UV absorbance was then measured on a Spectra Max Plus plate reader at 540 nm.

Oxidation of Compound 1. To a solution of 424 mg (1.98 mmol) $NaIO_4$ in 5 mL of water was added 7.0 mg (0.044 mmol) of $KMnO_4$.[6] After stirring for 30 min at 25 °C, 30.4 mg (0.22 mmol) of anhydrous K_2CO_3, 1.25 mL of *t*-BuOH, and 35 mg (0.11 mmol) of compound **1** in 1.25 mL of *t*-BuOH were added while maintaining the solution temperature at 20−30 °C. The resulting reddish-purple suspension was stirred for 2 h at 25 °C. After the addition of 0.1 mL (1.5 mmol) of ethylene glycol and further stirring for 2 h, the reaction was acidified to pH 4 with 1 M aqueous HCl and then extracted with Et_2O (20 mL × 3). The organic extract was washed with brine and dried over anhydrous Na_2SO_4. The solvent was removed in vacuo, and the residue was purified on RP-18 HPLC using CH_3OH/H_2O (4:6) as the mobile phase to afford (2*R*)-2-methylpentanedioic acid (**1a**, 4.9 mg): $[\alpha]^{25}_D$ −16.8 (*c* 0.067, ethanol); ^1H NMR ($CDCl_3$, 600 MHz) δ 11.4 (2H, brs, COOH), 2.55 (1H, m, H-2), 2.47 (2H, t, $J = 7.1$ Hz, H-4), 2.02 (1H, m, H-3a), 1.84 (1H, m, H-3b), 1.22 (3H, d, $J = 7.0$ Hz, CH_3); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 182.6, 179.7, 38.6, 31.7, 28.1, 16.8; negative ESIMS *m*/*z* 145 $[M - H]^-$.

Compound 1: colorless oil; $[\alpha]^{25}_{D}$ −17.0 (*c* 1.89, $CHCl_3$); IR (KBr) ν_{max} 3477, 2960, 2703, 1478, 1228, 1067, 987 cm^{-1}; ^1H and ^{13}C NMR (see Tables 1 and 2); positive HRESIMS *m*/*z* 381.2792 ([M + $HN(CH_3)_3$]$^+$ calcd for $C_{18}H_{41}N_2O_4S$, 381.2787); negative HRESIMS *m*/*z* 261.1162 ([M − $HN(CH_3)_3$]$^-$ calcd for $C_{12}H_{21}O_4S$, 261.1161).

Compound 2: colorless oil; $[\alpha]^{25}_{D}$ −17.2 (*c* 0.50, $CHCl_3$); IR (KBr) ν_{max} 3471, 2966, 2781, 1467, 1225, 1063, 1002 cm^{-1}; ^1H and ^{13}C NMR (see Tables 1 and 2); positive HRESIMS *m*/*z* 353.2487 ([M + $H_2N(CH_3)_2$]$^+$ calcd for $C_{16}H_{37}N_2O_4S$, 353.2474).

Compound 3: colorless oil; $[\alpha]^{25}_{D}$ −13.4 (*c* 0.75, $CHCl_3$); IR (KBr) ν_{max} 2964, 2925, 1715, 1467, 1377, 1245, 1209, 994 cm^{-1}; ^1H and ^{13}C NMR (see Tables 1 and 2); positive HRESIMS *m*/*z* 493.3322 ([M + $C_6H_{14}NO$]$^+$ calcd for $C_{24}H_{49}N_2O_6S$, 493.3311); negative HRESIMS *m*/*z* 261.1155 ([M − $C_6H_{14}NO$]$^-$ calcd for $C_{12}H_{21}O_4S$, 261.1161).

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