New Quinone Sulfates from the Crinoids *Tropiometra afra macrodiscus* and *Oxycomanthus japonicus*

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Quinone pigments in the crinoids were investigated. New quinone sulfates 1 and 3 were isolated from *Tropiometra afra macrodiscus* and *Oxycomanthus japonicus*, respectively. The structures 1 and 3 were determined to be 1,6,8-trihydroxy-3-propylanthraquinone-2-carboxylic acid 6-*O*-sulfate (ptilometric acid 6-*O*-sulfate) and 2-butanoyl-3,6,8-trihydroxy-1,4-naphtoquinone 6-*O*-sulfate, respectively, by chemical and spectroscopic analysis. Ptilometric acid 6-*O*-sulfate (1) showed an antifeedant activity on fish.

Key words ptilometric acid 6-*O*-sulfate; crinoid; quinone sulfate; 2-butanoyl-3,6,8-trihydroxy-1,4-naphtoquinone 6-*O*-sulfate; *Tropiometra afra macrodiscus*; *Oxycomanthus japonicus*

Biologists have been attracted by the bright external color patterns of crinoids, leading to the isolation and structural elucidation of many kinds of pigments from crinoids. The color is mainly caused by quinones and carotenoids. Various reports on the quinone pigments of crinoids have been published.[1)] At present, the following four groups of polyketide-derived pigments, linear and angular naphthopyrones,[2—4)] 4-acylanthraquinones,[5—7)] 3-alkylanthraquinones,[6,8)] and diametric bianthrones (bianthraquinones and phenanthroperylenequinones)[9,10)] are known to occur in crinoids. Some of the quinone pigments in crinoid are present in sulfate form.[1,7)] This prompted us to search for water-soluble quinone pigments in crinoids. This paper reports the isolation and structural elucidation of new quinone sulfates from *Tropiometra afra macrodiscus* and *Oxycomanthus japonicus* by chemical and spectroscopic analysis. Furthermore, antifeedant activity of anthraquinone sulfate on fish is reported.

Structure of a New Anthraquinone Sulfate from *T. afra macrodiscus* The acetone extract of *T. afra macrodiscus* was partitioned with Et_2O–*n*-hexane (1 : 1) and water to remove fat and carotenoids from the aqueous acetone solution. The purple-red colored aqueous layer was extracted with EtOAc to remove free anthraquinones. The aqueous layer was evaporated, and the dark purple residue was subjected successively to column chromatography on ODS and preparative HPLC on ODS to yield a water-soluble yellow pigment **1**.

Compound **1** showed UV–vis absorption maxima at 225, 262, and 435 nm, indicating a hydroxyanthraquinone chromophore and IR absorption maxima at 1620 and 1481 cm^{-1}, which are characteristic absorption bands of anthraquinone.[8)] Furthermore, **1** showed strong IR absorptions at 1249 and 1057 cm^{-1}, suggesting the presence of a sulfate group.[7)] Negative ion FAB-MS gave a quasi-molecular ion at *m*/*z* 443, corresponding to the formula $[C_{18}H_{14}O_{10}SNa-H]^-$. The characteristic fragment ions at *m*/*z* 421 $[M-Na]^-$ and 341 $[M-SO_3Na]^-$ were compatible with the presence of a sodium sulfate group. Furthermore, the presence of sulfur and sodium atoms in **1** was confirmed by inductively coupled plasma (ICP) spectra. Acid hydrolysis of **1** gave ptilometric acid (**2**). Therefore, **1** was determined to be a sodium sulfate of ptilometric acid (**2**).

The position of the sulfate group in **1** was determined by using sulfation shifts in ^{1}H-NMR[7)] and ^{13}C-NMR.[11)] Because no ^{1}H- or ^{13}C-NMR data were available for ptilometric acid (**2**), we carried out complete ^{1}H- and ^{13}C-NMR assignments of **2** in DMSO-d_6 solution using two dimensional (2D)-NMR experiments. Double quantum filtered correlation spectroscopy (DQF-COSY) and nuclear Overhauser spectroscopy (NOESY) experiments revealed the assignment of aromatic protons at H-4, H-5, H-7, protons of the propyl chain (H-1′, H-2′, and H-3′), and a hydrogen bonded phenolic proton at 8-OH. The assignment of all proton-attached carbons was made from a ^{13}C–^{1}H COSY spectrum. ^{13}C signals of quaternary carbons at C-2, C-3, C-6, C-8, C-10, C-11, C-12, and C-14 were unambiguously assigned by correlation of proton signals in a ^{13}C–^{1}H long range COSY experiment. Furthermore, the remaining seven carbon signals (δ 132.4, 167.2, 180.8, 189.3) were assigned to C-13, C-1, 2-COOH, and C-9, respectively, by comparison with polyhydroxyanthraquinones.[12)] The assignment of ^{1}H- and ^{13}C-NMR data for **1** was carried out in the same manner described above. All proton-attached carbons were assigned by ^{13}C–^{1}H COSY. Quaternary carbons at C-6, C-8, C-10, C-11, and C-14 were also assigned by ^{13}C–^{1}H long range COSY. The remaining seven carbon signals at δ 125.2, 133.8, 134.0, 153.6, 168.8, 182.5, and 187.2 were assigned to C-2, C-12, C-13, C-3, C-1, 2-COOH, and C-9, respectively, by comparison with polyhydroxyanthraquinones[12)] and ptilometric acid (**2**). The total ^{1}H- and ^{13}C-NMR assignments for **1** and **2** are compiled in Table 1.

It is known that, in the ^{13}C-NMR spectra of phenol compounds, the introduction of *O*-sulfate, an electron withdrawing group, results in an increased electron density of the carbon carrying the sulfate group and a decreased electron density of the carbons *ortho* and *para*, thus resulting in an upfield shift for the former carbons and a downfield shift for the latter carbons.[11)] Sulfate **1** showed an upfield shift of 6.6 ppm at C-6, and downfield shifts of 4.9 ppm at C-7 and 0.8 ppm at C-5 compared with **2**. These results indicated that the hydroxy group at C-6 in **1** was present as the sulfate ester. Furthermore, about 0.4 ppm downfield shifts of H-5 and H-7 in **1** relative to **2** agreed with the 6-*O*-sulfate structure.[7)] Therefore, a new anthraquinone sulfate **1**, isolated from *T. afra macrodiscus*, was determined to be a sodium salt of ptilometric acid 6-*O*-sulfate (1,6,8-trihydroxy-3-propylanthraquinone-

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Table 1. ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) Data for Ptilometric Acid 6-*O*-Sulfate (**1**) and Ptilometric Acid (**2**) in DMSO-d_6

Carbon No.	Ptilometric acid 6-*O*-sulfate (**1**)			Ptilometric acid (**2**)		
	^{13}C mult.	^{1}H mult.	*J* (Hz)	^{13}C mult.	^{1}H mult.	*J* (Hz)
1	168.8 s			167.2 s		
2	125.2 s			130.4 s		
3	153.6 s			147.6 s		
4	117.4 d	7.23 s		118.6 d	7.52 s	
5	109.7 d	7.45 d	2.3	108.9 d	7.10 d	1.8
6	159.1 s			165.7 s		
7	112.8 d	7.04 d	2.3	107.9 d	6.57 d	1.8
8	163.2 s			164.4 s		
9	187.2 s[a)]			189.3 s[a)]		
10	182.5 s			180.8 s		
11	112.5 s			113.8 s		
12	133.8 s			135.0 s		
13	134.0 s			132.4 s		
14	115.9 s			113.8 s		
2-COOH	182.5 s[a)]			180.8 s[a)]		
1′	37.1 t	3.16 t	7.5	35.2 t	2.65 t	7.3
2′	23.9 t	1.58 tq	7.5	23.2 t	1.63 tq	7.3
3′	14.1 q	0.94 t	7.5	13.7 q	0.93 t	7.3
8-OH		13.3 br s			12.0 br s	

a) Assignments may be interchangeable within the same column.

Table 2. ^{1}H- (300 MHz) and ^{13}C-NMR (75 MHz) Data for 2-Butanoyl-3,6,8-trihydroxy-1,4-naphthoquinone 6-*O*-Sulfate (**3**) in D_2O, and ^{1}H-NMR (300 MHz) Data for 2-Butanoyl-3,6,8-trihydroxy-1,4-naphtoquinone (**4**) in Acetone-d_6

Carbon No.	**3**			**4**	
	^{13}C mult.	^{1}H mult.	*J* (Hz)	^{1}H mult.	*J* (Hz)
1	186.5 s[a)]				
2	115.9 s				
3	172.2 s				
4	184.4 s				
5	113.5 d	7.23 d	2.3	6.97 d	1.4
6	154.5 s				
7	111.5 d	6.93 d	2.3	6.56 d	1.4
8	160.9 s				
9	112.4 s				
10	132.3 s				
1′	184.4 s[a)]				
2′	45.5 t	2.64 t	7.5	2.86 t	7.5
3′	18.0 t	1.45 tq	7.5	1.58 tq	7.5
4′	12.9 q	0.73 t	7.5	0.86 t	7.5

a) Assignments may be interchangeable within the same column.

2-carboxylic acid 6-*O*-sulfate).

Structure of a New Naphthoquinone Sulfate from *O. japonicus* In the same manner as described above, a water-soluble reddish pigment **3** was isolated from the acetone extract of *O. japonicus*. Compound **3** showed UV–vis absorption maxima at 249, 295, and 374 nm, and an IR absorption maximum at 1630 cm^{-1}, characteristic of hydrogen-bonded quinones.[1)] Furthermore, **3** showed strong IR absorptions at 1275 and 1057 cm^{-1}, suggesting the presence of a sulfate group.[7)] A negative ion FAB-MS gave a quasi-molecular ion at *m*/*z* 378, corresponding to the formula $[C_{14}H_{11}O_9SNa]^-$. The characteristic fragment ions at *m*/*z* 355 $[M-Na]^-$ and 275 $[M-SO_3Na]^-$ were compatible with the presence of a sodium sulfate group. ^{1}H-NMR spectra, including a ^{1}H–^{1}H COSY experiment, revealed the presence of a propyl chain (δ 0.73, 1.45, 2.64) and two aromatic protons with an AX spin system (δ 6.93, 7.23). The coupling constant (J=2.3 Hz) between the aromatic protons indicated *meta*-orientation. The ^{13}C-NMR spectra, including a distortionless enhancement by polarization transfer (DEPT) experiment, showed 14 carbon signals due to one methyl, two methylenes, two methines and nine quaternary carbons. All proton-attached carbons were assigned by a ^{13}C–^{1}H COSY experiment. Of the nine quaternary carbons, three were ascribed to carbonyl carbons (δ 184.4×2C, 186.5) and three to phenol substituted carbons (δ 172.2, 160.9, 154.5) by their chemical shifts. These spectral data strongly suggested that **3** was a trihydroxy-1,4-naphthoquinone substituted with a butanoyl group.[13)] Acid hydrolysis of **3** gave a free naphthoquinone (**4**), which showed a molecular ion peak at 276.0649 compatible with the formula $C_{14}H_{12}O_6$. An electron impart (EI)-MS fragment ion at *m*/*z* 206 $[M-COC_3H_6]$ indicated the presence of a butanoyl group in **4**. The structure of **4** for 2-butanoyl-3,6,8-trihydroxy-1,4-naphthoquinone was postulated by UV–vis, IR, ^{1}H-NMR, and EI-MS spectral data comparison with those of flaviolin (3,6,8-trihydroxy-1,4-naphthoquinone).[14)] The position of the sulfate group in **3** was determined from sulfation shifts in the ^{1}H-NMR.[7)] The protons at H-5 and H-7 in **3** were shifted downfield by 0.26 and 0.37 ppm, respectively, relative to those of **4**. These data indicated that the hydroxy group at C-6 was present as the sulfate ester. Therefore, the structure of **3** was determined to be a sodium salt of 2-butanoyl-3,6,8-trihydroxy-1,4-naphthoquinone 6-*O*-sulfate.

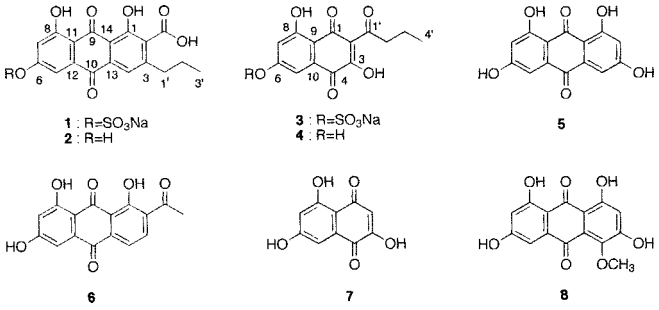
Furthermore, the sodium sulfate of 1,3,6,8-tetrahydroxyanthraquinone (**5**) was also obtained. A negative ion FAB-MS gave a quasi-molecular ion at *m*/*z* 373, compatible with the structure of a sodium sulfate of **5**. However, the position of the sulfate group could not be determined because of the small sample size.

In addition to these quinone sulfates, the following five known quinones, 1,3,6,8-tetrahydroxyanthraquinone (**5**), 2-acetylemodin (**6**), flaviolin (**7**), rhodolamprometrin (**8**), and

Table 3. The Inhibitory Effect of Ptilometric Acid 6-*O*-Sulfate (**1**) and Ptilometric Acid (**2**) on Feeding by Fish

Test substance	Concentration of test substance in diet	Species of fish		
		Poecilia reticulata (n=6)	*Oplegnathus fasciatus* (n=24)	*Parapristipomatriline atum* (n=10)
Ptilometric acid (**2**)	1.0%	−	−	−
Ptilometric acid 6-*O*-sulfate (**1**)	0.2%	±	+	±
Ptilometric acid 6-*O*-sulfate (**1**)	1.0%	+	+	+

+, Substantial preference for control diet; −, no statistically significant preference for either diet; ±, marginal preference for control diet.



ptilometric acid (**2**), were isolated from *O. japonicus*.

Antifeedant Activity of Ptilometric Acid (2) and Its Sulfate (1) in Fish It has been reported that quinone pigments, especially the sulfate forms, provide a chemical mechanism against predatory fish for some species of crinoids.[9] In this study, ptilometric acid 6-*O*-sulfate (**1**) showed concentration-dependent antifeedant activity in fish, but ptilometric acid (**2**) was ineffective (Table 3). Antifeedant activities of 2-butanoyl-3,6,8-trihydroxy-1,4-naphtoquinone (**4**) and its sulfate (**3**) could not be measured because of the small sample size.

Experimental

General The UV–vis spectra were recorded in aqueous MeOH or EtOH solution on a Shimadzu UV-240 spectrophotometer. The IR spectra were obtained by a Perkin Elmer FT-IR 1600 as KBr pellets. The EI- and FAB-MS spectra were recorded using a JMS-DX 300 mass spectrometer. FAB-MS were obtained using glycerin as a matrix. The ^{13}C- and ^{1}H-NMR spectra were measured with a Bruker ARX-500 spectrometer or Varian XL-300 spectrometer. The ICP spectra were recorded with a ICPS-8000 (Shimadzu) in water at a concentration of 300 μg/ml. HPLC was performed on a Shimadzu LC-6AD instrument with a Shimadzu SPD-6AV spectrophotometer set at 440 nm. The column used was a Shim-Pack PREP-ODS (20 mm×250 mm ID, particle size 5 μm, Shimadzu) using 20% MeOH as the mobile phase.

Animal Material Specimens of *T. afra macrodiscus* and *O. japonicus* were collected from the sea at Goza in Mie Prefecture in July 1992 and in June 1993, respectively. Voucher specimens have been deposited at Kyoto Pharmaceutical University.

Extraction and Isolation of Quinone Pigments from *T. afra macrodiscus* Seventeen fresh specimens of *T. afra macrodiscus* (800 g) were immediately immersed in acetone to yield a deep red solution, which was then partitioned between *n*-hexane–Et_2O (1 : 1) and water to remove fat and carotenoids from the acetone solution. The purple-red water layer was extracted with ethyl acetate to remove free anthraquinones. The aqueous layer was concentrated by a rotary evaporator. The obtained dark purple residue was subjected to column chromatography on ODS (Fuji Silysia Chemical, Ltd., DM-1020T, 100—200 mesh). The purple-red fraction eluted with 20% MeOH was further purified by preparative HPLC on ODS with 20% MeOH, and was crystallized from EtOH–EtOAc–water to afford compound **1** (25 mg).

On the other hand, the EtOAc extract was subjected to preparative TLC on silica gel G (Kieselgel 60, Merck) with benzene–EtOAc–AcOH (60 : 40 : 1). A yellow band (*Rf* 0.30) was extracted with 2% AcOH in EtOAc and was further chromatographed on Sephadex LH-20 (Pharmacia Fine Chemicals) with MeOH. Crystallization from aqueous AcOH afforded ptilometric acid (**2**) (2 mg).

Ptilometric Acid 6-*O*-Sulfate (**1**): Reddish crystal. UV–vis: (30% MeOH) nm (ε) 225 (11270), 262 (11240), 435 (4660). IR cm^{-1}: 3442, 2962, 1620, 1481, 1374, 1249, 1057. Negative ion FAB-MS: *m/z* 443 [M−H]$^-$, 421 [M−Na]$^-$, 341 [M−SO_3Na]$^-$, 297 [M−SO_3Na−CO_2]$^-$. ^{1}H- and ^{13}C-NMR (DMSO-d_6): Table 1. DQF-COSY correlations: between H-5 to H-7, H-1′ to H-3′. NOESY correlations: H-4 to H-1′, H-2′, and H-3′, H-1′ to H-2′, H-2′ to H-3′, OH-8 to H-7. ^{13}C–^{1}H long range COSY correlations: C-6 to H-7, C-8 to H-7, C-10 to H-4 and H-5, C-11 to H-7 and H-5, C-14 to H-4, C-2′ to H-3′. ICP-optical emission spectrum: 589.592 nm (Na), 180.731 nm (S).

Ptilometric Acid (**2**): Reddish crystal. UV–vis: (EtOH) nm (ε) 228 (34500), 277 (29240), 313 (9700) and 444 (14860). IR cm^{-1}: 3418, 2965, 1715, 1674, 1626. EI-MS *m/z*: 342 [M]$^+$, 324 [M−H_2O]$^+$, 298 [M−CO_2]$^+$, 270 [M−CO_2−CH_2CH_2]$^+$. High resolution (HR)-EI-MS: *m/z* 342.0731 ($C_{18}H_{14}O_7$ Calcd for 342.0737). ^{1}H- and ^{13}C-NMR (DMSO-d_6): Table 1. DQF-COSY correlations: between H-5 to H-7, H-1′ to H-3′. NOESY correlations: H-4 to H-1′, H-2′, and H-3′, H-1′ to H-2′, H-2′ to H-3′. ^{13}C–^{1}H long range COSY correlations: C-2 to H-14, C-3 to H-4 and H-1′, C-4 to H-1′, C-5 to H-7, C-6 to H-7, C-7 to H-5 and OH-8, C-8 to H-7 and OH-8, C-10 to H-14 and H-5, C-12 to H-5, C-14 to H-4, C-1′ to H-1′ and H-3′, C-3′ to H-1′ and H-3′.

Acid Hydrolysis of 1 Compound **1** (3 mg) was dissolved in 20 ml 1 N HCl and stored at 37 °C for 4 h. Then, the pigment was extracted with EtOAc and was purified by preparative TLC on silica gel G with benzene–EtOAc–AcOH (60 : 40 : 1) to give ptilometric acid (1.5 mg). The identification of ptilometric acid was based on UV–vis, IR, EI-MS, HR-EI-MS, ^{1}H-, and ^{13}C-NMR data.

Extraction and Isolation of Quinone Pigments from *O. japonicus* Nineteen fresh specimens of *O. japonicus* (1100 g) were immediately immersed in acetone to yield a deep red solution which was then partitioned between *n*-hexane–Et_2O (1 : 1) and water to remove fat and carotenoid from the acetone solution. The water layer was extracted with EtOAc to remove free quinines. The aqueous layer was concentrated by a rotary evaporator. The dark red residue was subjected to column chromatography on ODS. The yellow fraction eluted with 20% MeOH was further purified by preparative HPLC on ODS with 25% MeOH to afford a sulfate of **5** (0.7 mg), and the red fraction eluted with 20% MeOH was further purified by preparative HPLC on ODS with 25% MeOH to afford a new compound **3** (2.5 mg). The EtOAc solution was also concentrated by a rotary evaporator. The dark red residue (1.8 g) was subjected to column chromatography on silica gel (Merck, 70—230 mesh) with mixed solvents of benzene–EtOAc–AcOH as eluents. The yellow fraction eluted with benzene–EtOAc–AcOH (95 : 5 : 1) was further purified by HPLC on ODS with 80% MeOH to afford 2-acetylemodin (**6**, 0.6 mg). The yellow fraction eluted with benzene–EtOAc–AcOH (90 : 10 : 1) was further purified with the same HPLC system to afford 1,3,6,8-tetrahydroanthraquinone (**5**, 0.7 mg) and flaviolin (**7**, 1.0 mg). The orange fraction eluted with benzene–EtOAc–AcOH (80 : 20 : 1) was further purified with the same HPLC system to afford rhodolamprometrin (**8**, 1.1 mg). The orange fraction eluted with benzene–EtOAc–AcOH (60 : 40 : 1) was further purified with the same HPLC system to afford ptilometric acid (**2**, 4 mg). Identification of these five known quinones, 2-acetylemodin (**6**),[15] 1,3,6,8-tetrahydroxyanthraquinone (**5**),[16] flaviolin (**7**),[14] rhodolamprometrin (**8**),[16] and ptilometric acid (**2**) were made by UV–vis, IR, EI-MS, and ^{1}H-NMR data and by comparison with authentic samples[14—16] on HPLC.

2-Butanoyl-3,6,8-trihydroxy-1,4-naphthoquinone 6-*O*-Sulfate (**3**): A reddish amorphous powder; UV–vis: (30% MeOH) nm (ε) 249 (12740), 295 (11980), 374 (2720). IR cm^{-1}: 3500—3300, 2950, 1630, 1275, 1057. Negative ion FAB-MS: *m/z* 378 [$C_{14}H_{11}O_9SNa$]$^-$, 355 [M−Na]$^-$, 275 [M−SO_3Na]$^-$. ^{1}H- and ^{13}C-NMR (D_2O): Table 2.

Acid Hydrolysis of 3 Compound **3** (1.2 mg) was dissolved in 8 ml 1 N HCl and stored at 37 °C for 4 h. The pigment was extracted with EtOAc and was purified by preparative HPLC on ODS with 65% MeOH to give **4** (0.6 mg).

2-Butanoyl-3,6,8-trihydroxy-1,4-naphthoquinone (**4**): An orange amorphous powder; UV–vis: (1% AcOH/MeOH) nm (ε) 234 (12640), 268 (14660), 300 (16180). IR cm^{-1}: 3443, 2965, 1621, 1479. HR-EI-MS: *m/z* 276.0649 ($C_{14}H_{12}O_6$, Calcd 276.0640). EI-MS *m/z* (rel. int. %) 276 $[M]^+$ (68), 234 $[M-C_3H_6]^+$ (45), 206 $[M-COC_3H_6]^+$ (80), 60 (100). ^{1}H-NMR (acetone-d_6): Table 2.

Sulfate of 1,3,6,8-Tetrahydroxyanthraquinone (**5**): A yellow amorphous powder; UV–vis: (30% MeOH) nm (ε) 248, 297, 428. IR cm^{-1}: 3444, 1633, 1614, 1283, 1100. Negative ion FAB-MS: *m/z* 373 $[M-H]^-$. ^{1}H-NMR (D_2O) δ: 6.77 (2H, br s, H-2, H-7), 7.06 (2H, br s, H-3, H-6). Acid hydrolysis of this compound with 1 N HCl at 37 °C for 4 h gave 1,3,6,8-tetrahydroanthraquinone (**5**). Identification of **5** was based on UV–vis and co-HPLC with an authentic sample.

Antifeedant Activity for Fish Commercial diets, Flake Food (Tetra Werke Co., Germany) and Nipai No. 1 (Nippon Formula Feed Manufacturing Co., Ltd., Japan) were used for fresh-water fish (*Poecilia reticulata*) and marine fish (*Oplegnathus fasciatus*, *Parapristipomatriline atum*), respectively, as control diets. The anthraquinone-containing diet was prepared by adding ptilometric acid and its sulfate, which was dissolved in acetone and 30% MeOH, to the control diet. The time required to eat each sample was recorded.

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