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(2*S*,3*S*)-Sulfated Pterosin C, a Cytotoxic Sesquiterpene from the Bangladeshi Mangrove Fern *Acrostichum aureum*

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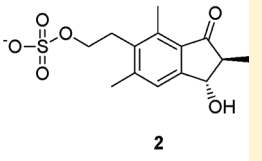
Supporting Information

ABSTRACT: Two new sesquiterpenes, (2*R*,3*S*)-sulfated pterosin C (**1**) and (2*S*,3*S*)-sulfated pterosin C (**2**), along with two known derivatives, (2*S*,3*S*)-pterosin C and (2*R*)-pterosin P, were isolated from a methanolic extract of the aerial parts of *Acrostichum aureum*. The structures of **1** and **2** were determined by the interpretation of their spectroscopic data. The isolated pterosins were evaluated for their cytotoxic activity against the AGS, HT-29, MDA-MB-231, and MCF-7 human cancer cell lines and the NIH3T3 normal mouse fibroblast cell line, using the MTT assay. Compound **2** showed IC_{50} values in the range 23.9−68.8 μM. The lowest IC_{50} value (23.9 μM) was recorded against AGS gastric adenocarcinoma cells. Compound **2** was found to exert an apoptotic effect on AGS cells within 24 h of treatment, which increased with time and was greater than the positive control, cycloheximide. The cytotoxicity of **2** seems to be due in part to the sulfate group on C-14 and the configuration at C-2.

Acrostichum aureum



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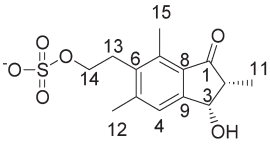
Acrostichum aureum L. (Pteridaceae) is a mangrove fern that occurs in tropical and subtropical areas worldwide, especially in Southeast Asia, Central America, and Africa.[1,2] It is found in the Sundarban and other coastal regions of Bangladesh and is named "tiger fern", due to providing a hiding place for tigers.[1] In Bangladesh, preparations from the rhizomes and leaves are used to cure wounds, ulcers, and boils, while the leaves are also used to stop bleeding.[2] In mainland China, the rhizomes are used to treat worm infections.[2] In Fiji, the plant is used to treat asthma, constipation, elephantiasis, and chest pain,[3] whereas the leaves are employed as emollients in Costa Rica.[4]

A Japanese specimen of *A. aureum* showed strong antioxidant and tyrosinase-inhibitory activity,[5] while the crude extract of a Chinese specimen showed cytotoxic activity against HeLa cells.[6] Recently, we reported on the cytotoxic effects of water and methanolic extracts from a Bangladeshi specimen of *A. aureum* for gastric, colon, and breast cancer cells.[7] So far, 19 compounds have been isolated from *A. aureum*, belonging to several common natural product classes, namely, sterols, flavonoids, fatty acids, and long-chain hydrocarbons.[8−10]

Herein, we report on the isolation from *A. aureum* of two new pterosins, (2*R*,3*S*)-sulfated pterosin C (**1**) and (2*S*,3*S*)-sulfated pterosin C (**2**), and two known derivatives, (2*S*,3*S*)-pterosin C and (2*R*)-pterosin P. These compounds were evaluated for cytotoxicity against normal mouse fibroblasts (NIH3T3) and four human cancer cell lines (AGS, MDA-MB-231, MCF-7, and HT-29). Compound **2** was further evaluated for its apoptosis and necrosis-inducing potential on gastric adenocarcinoma (AGS) cells.



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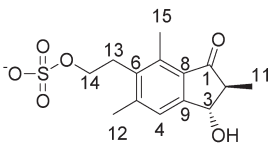

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Compound **1** was obtained as light yellow, crystalline needles. The negative mode HRESIMS data showed a quasimolecular ion peak at m/z 313.0721 $[M - H]^-$, suggesting a molecular ion at m/z 314.0824 and thus a molecular formula of $C_{14}H_{18}O_6S$. The IR spectrum showed major absorption bands at 3366 cm^{-1} (OH), 1688 cm^{-1} (C═O), and 1599 cm^{-1} (aromatic C═C). The ^1H NMR data (Table 1) of **1** indicated a pentasubstituted benzene ring with a single aromatic proton signal at δ_H 7.35 (1H, s). Two coupled methylene groups at δ_H 3.99 (2H, t, J = 7.8 Hz) and 3.17 (2H, t, J = 7.8 Hz) suggested the attachment of an ethylene chain to the aromatic ring. The remaining ^1H NMR signals were consistent with a C_{14} pterosin sesquiterpene structure.[11−13] The ^{13}C NMR data of **1** (Table 1) showed 14 distinct carbon signals, including one signal under the CD_3OD solvent peak at 49.4 ppm. The carbon signals also supported the presence

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Table 1. ^{1}H and ^{13}C NMR Data for Compounds 1 and 2

	1		2	
position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1		208.2		209.2
2	2.71 (1H, m)	49.4	2.34 (1H, m)	54.7
3	5.07 (1H, d, 6.3)	70.3	4.58 (1H, d, 4.2)	75.9
4	7.35 (1H, s)	126.8	7.25 (1H, s)	125.7
5		146.6		146.2
6		137.7		137.4
7		138.4		138.2
8		132.6		131.0
9		154.4		154.8
11	1.10 (3H, d, 7.5)	10.6	1.30 (3H, d, 7.2)	13.3
12	2.49 (3H, s)	21.4	2.39 (3H, s)	21.4
13	3.17 (2H, t, 7.8)	29.8	3.07 (2H, t, 7.8)	29.7
14	3.99 (2H, t, 7.8)	67.1	3.97 (2H, t, 7.8)	67.2
15	2.66 (3H, s)	14.0	2.56 (3H, s)	14.1

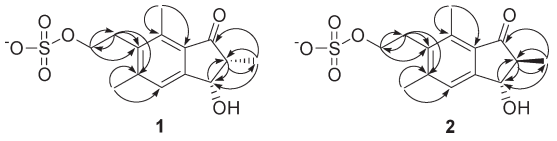

Figure 1. Key HMBC correlations observed in 1 and 2.

of a pentasubstituted aromatic ring, including a low-field signal at 208.2 ppm (C=O), as assigned for C-1 of a pterosin-type sesquiterpene skeleton (Table 1, Figure 1).[13] HMBC proton–carbon correlations confirmed a pterosin C unit (Figure 1). However, an 80 amu difference in mass was evident between **1** (314 amu) and pterosin C (234 amu).[13−15] The mass difference between these two compounds strongly suggested the hydroxy group in pterosin C to be replaced with a sulfate substituent ($-SO_4$), which is a common functional group in marine natural products[16] and is consistent with *A. aureum* being a marine mangrove fern.

Further, the IR bands for **1** at 1203, 1075, 897, and 573 cm^{-1} indicated a sulfate group, and the ^{13}C NMR shift for C-14 (+6 ppm downfield relative to C-14 in pterosin C) confirmed the presence of a sulfate group in the molecule.[12,13,17,18] In **1**, the protons at H-11 and H-3 appeared as doublets at δ_H 1.10 (3H, d, J = 7.5 Hz) and 5.09 (1H, d, J = 6.3 Hz), respectively, which differed from the data published for (2*S*,3*S*)- and (2*R*,3*R*)-pterosin C, but were very similar to (2*R*,3*S*)-pterosin C.[11,14,19] The optical rotation of **1** was positive ($[\alpha]_D$ +10.8) and similar to (2*S*,3*S*)-pterosin C,[14,20] but opposite in sign to values published for (2*R*,3*R*)- and (2*S*,3*R*)-pterosin C.[11,14] Confirmation of the structure proposed was obtained from the CD spectrum.[14] The sign of the CD spectra for these types of compounds depends mainly on the configuration at the C-3 position, regardless of the configuration at C-2 and the solvent.[21] Thus, 3*S* and 3*R* pterosin sesquiterpenes exhibit positive and negative Cotton effects, respectively. A positive Cotton effect ($\Delta\varepsilon$ +8599) for the CD data of **1** indicated that the C-3 position is *S*. Therefore, the structure of **1** was deduced as (2*R*,3*S*)-sulfated pterosin C.

From the HRESIMS, compound **2** gave the same molecular formula as **1** ($C_{14}H_{18}O_6S$). The UV, IR, NMR, and MS data of **2** were very similar to those of **1**. However, a difference was found in the configuration at the C-3 position for these two compounds. Confirmation of the configuration at C-2 and C-3 came from reference to published data (^{1}H NMR and CD) for pterosin C.[11,14,19,21] This indicated that **2** has a *trans*-like configuration as in (2*S*,3*S*)-pterosin C. The CD spectrum of **2** exhibited a positive Cotton effect ($\Delta\varepsilon$ +4890), indicating that the C-3 position is *S*. On the basis of the data obtained, **2** was assigned as (2*S*,3*S*)-sulfated pterosin C.

The identities of the known compounds (2*S*,3*S*)-pterosin C and (2*R*)-pterosin P were established by comparison of their physical and spectroscopic data with published values.[11−13,21,22]

In this study, compound **2** was the only cytotoxic compound among the four isolated pterosins tested and gave IC_{50} values of 46.8, 23.9, 68.8, >100, and 64.4 μM against NIH3T3, AGS, HT29, MDA-MB-231, and MCF-7 cells, respectively. The structural difference between the noncytotoxic compound, (2*S*,3*S*)-pterosin C, and **2** is a sulfate group on the hydroxyethylene chain (C-14). This indicates that the sulfate group contributes to the cytotoxic activity of **2**. Previous research indicates that the presence of a sulfate group in some molecules such as holothurioid,[23] chondroitin,[24] dictyodendrins A–E,[25] and sokotrasterol[26] is important for their biological activity. The noncyotoxic compound **1** has the same structure as **2** apart from a configurational difference at C-2, which indicates that this may be important for mediating the cytotoxic activity of the latter compound.

Investigation into the apoptosis- and necrosis-inducing potential of **2** showed a significant ($p < 0.05$) increase in the number of cells that undergo early apoptosis (AV^+/PI^-), from 6.8% to 25.2% following 24 and 48 h treatment, respectively (Figure S1, Supporting Information). The number of cells showing early and late apoptosis was significantly higher ($p < 0.05$) at 48 h of treatment with **2** (15 μg/mL) than following 48 h of treatment with cycloheximide (150 μg/mL).

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a JASCO P-1010 polarimeter. The UV spectra were obtained on a Shimadzu BioSpec-mini UV spectrophotometer. IR spectra were recorded on a Bruker Optics alpha-QuickSnap (A220/D-01) FT-IR spectrophotometer. CD spectra were recorded at 300–380 nm on a JASCO J-715 CD spectrometer. NMR spectra were recorded on either a Bruker Avance 300 or 600 MHz spectrometer in CD_3OD. The chemical shifts (δ) are given in ppm, with reference to tetramethylsilane (TMS) as internal standard, and coupling constants (J) are in Hz. LRMS and HRESIMS were obtained on Bruker Daltonics Esquire 3000 and Bruker Daltonics Apex III 4.7e mass spectrometers, respectively. Analytical HPLC was performed on a Varian Prostar instrument with a 335 DAD using a RP (Luna C_{18}, 5 μm, 250 × 4.6 mm) column. Preparative HPLC was performed on a Waters instrument equipped with a Waters 600E pump, Rheodyne 7725i injector, and Waters 2487 dual-wavelength detector using a RP (Luna C_{18}, 5 μm, 150 × 21.2 mm) column. SPE cartridges (Alltech, 10 g, RP-C_{18}) were used to fractionate the extract.

Plant Material. The aerial parts of *A. aureum* were collected from tidal forests in coastal Sundarban (a swamp region in the Ganges delta) of Bangladesh in February 2007. The plant material was identified by Dr. Momtaz Mahal Mirza, Principal Scientific Officer, Bangladesh National Herbarium, Dhaka, and shade-dried. A specimen was deposited in the Bangladesh National Herbarium, Dhaka (voucher no.: DACB 31538).

Extraction and Isolation. The dried and pulverized plant material of *A. aureum* (150 g) was extracted with methanol (1 L) by soaking overnight at room temperature with continuous stirring. The extract was filtered, and the residue was further extracted three times with methanol

(1 L each) for 1 h under sonication. All of the resulting extracts were combined and concentrated under reduced pressure to give 7.57 g of extract (5.04% w/w). The resulting MeOH extract was partitioned between *n*-hexane and MeOH (1:1) to give two fractions. The MeOH fraction (6.7 g) was further subfractionated into four fractions using RP SPE columns with a H_2O/MeOH stepwise gradient (100:0, 80:20, 60:40, and 0:100). The SPE fractions were analyzed by analytical RP-HPLC, and three fractions were further separated (SPE2, -3, and -4) by semipreparative RP-HPLC using a H_2O/MeOH gradient system containing 0.05% TFA. Semipreparative HPLC of the SPE2 fraction (0.42 g) yielded **1** (1.5 mg), and SPE3 (0.34 g) yielded **2** (9.0 mg) and (2*S*,3*S*)-pterosin C (10.0 mg; $[\alpha]^{25}_{D}$ +66, *c* 0.53, MeOH), while SPE4 (0.40 g) yielded (2*R*)-pterosin P (2.0 mg; $[\alpha]^{25}_{D}$ −7.7, *c* 0.27, MeOH).

(2*R*,3*S*)-Sulfated pterosin C (1): light yellow, crystalline needles (MeOH); $[\alpha]^{25}_{D}$ +10.8 (*c* 0.21, MeOH); UV (MeOH) λ_{max} (log ε) 341 (2.26), 289 (3.0), 257 (3.71), 215 (4.17) nm; CD (MeOH, *c* 0.021) λ_{max} ($\Delta\varepsilon$) 328.4 (+8599) nm; IR (film) ν_{max} 3366, 1688, 1599, 1203, 1075, 897, 573 cm^{-1}; ^1H (300 MHz, CD_3OD) and ^{13}C NMR (300 or 600 MHz, CD_3OD) data, see Table 1; negative HRESIMS *m*/*z* 313.0721 $[M - H]^-$ (calcd for $C_{14}H_{17}O_6S$ $[M - H]^-$, 313.0745).

(2*S*,3*S*)-Sulfated pterosin C (2): light yellow, crystalline needles (MeOH); $[\alpha]^{25}_{D}$ +22.8 (*c* 0.53, MeOH); UV (MeOH) λ_{max} (log ε) 340 (3.4), 300 (3.6), 250 (4.2), 215 (4.4) nm; CD (MeOH, *c* 0.003) λ_{max} ($\Delta\varepsilon$) 329 (+4890) nm; IR (film) ν_{max} 3366, 1688, 1599, 1203, 1075, 897, 573 cm^{-1}; ^1H and ^{13}C NMR (300 MHz, CD_3OD) data, see Table 1; negative HRESIMS *m*/*z* 313.0724 $[M - H]^-$ (calcd for $C_{14}H_{17}O_6S$ $[M - H]^-$, 313.0746).

MTT Cytotoxicity Assay. The cytotoxicity of the compounds isolated was tested against normal mouse fibroblast (NIH3T3) cells and four human cancer cell lines, inclusive of gastric adenocarcinoma (AGS), colon adenocarcinoma (HT-29), and two breast ductal carcinoma (MCF-7 and MDA-MB-231) cell lines, using the MTT assay performed according to the method described by Uddin et al.[7] Briefly, cells were seeded in 96-well plates at a density of 1.0×10^4 to 3.5×10^4 cells/well. Following 24 h incubation at 37 °C with 5% CO_2, cells were treated with different concentrations of compounds **1**−**4** for 48 h. Following washing and incubation with MTT solution for 2 h, cells were lysed. The absorbance was measured after 45 min using a microplate reader (Wallac 1420 Multilevel counter, Perkin-Elmer) at a wavelength of 560 nm. The IC_{50} values were calculated with probit analysis software (LdP Line Software, Doki, Cairo). Cycloheximide was used as a positive control, generating IC_{50} values of 1.1, 3.6, 12.8, 1.2, and >100 μM against NIH3T3, AGS, HT29, MDA-MB-231, and MCF-7 cells, respectively.

Annexin V-FITC Apoptosis Measurement. The FITC annexin V apoptosis assay was used to measure apoptosis of the isolated cytotoxic compounds from *A. aureum* against a human gastric adenocarcinoma (AGS) cell line, according to Jason et al.[27] Briefly, cells were seeded in a six-well plate at a density of 40×10^4 cells/well and incubated at 37 °C with 5% CO_2 for 24 h. The following day, cells were treated with 15 μg/mL of **2** for 24 and 48 h. Cells were rinsed with PBS, trypsinized rapidly, and centrifuged to pellet the cells. The supernatant was removed, and the cells were resuspended in 1× binding buffer (0.1 M Hepes/NaOH, 1.4 M NaCl, 25 mM $CaCl_2$), and 5 μL aliquots of the staining solutions (FITC annexin V and PI) was added. After 15 min incubation in the dark at room temperature, the cell suspension was diluted with binding buffer and analyzed within 1 h using a BD FACSCalibur flow cytometer (BD Bioscience, San Jose, CA), and data were recorded using BD CellQuest Pro software. Cells with no treatment served as a negative control, and cycloheximide (150 μg/mL) was used as a positive control.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Results of the apoptosis- and necrosis-inducing potential assay of **2** and the ^1H and ^{13}C NMR spectra for **1** and **2**. This information is available free of charge via the Internet at http://pubs.acs.org.

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