Halistanol Disulfate B, a Novel Sulfated Sterol from the Sponge *Pachastrella* sp.: Inhibitor of Endothelin Converting Enzyme

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As part of a search for novel inhibitors of endothelin converting enzyme (ECE), the MeOH extract of a South African sponge, *Pachastrella* sp., was shown to be active. Bioassay-guided fractionation of the extract yielded a novel sterol sulfate, halistanol disulfate B (**1**). The structure and stereochemistry of **1** was established mainly by interpretation of spectral data. Disulfate (**1**) was found to be active at a micromolar concentration in the ECE assay.

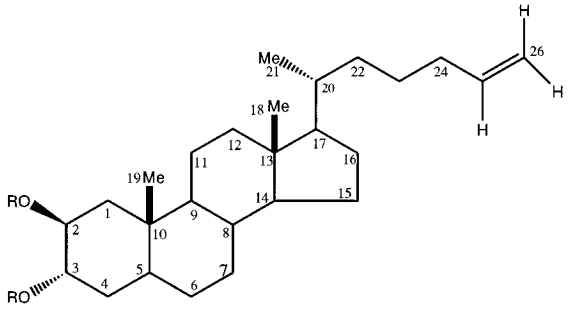
Endothelin converting enzyme (ECE) is a membrane-bound neutral metalloprotease that catalyzes the conversion of a 38-residue inactive intermediate big-endothelin (B-ET) to a 21-residue potent vasoconstrictive peptide endothelin-1 (ET-1). Overproduction of ET is associated with numerous disorders, including hypertension and renal failure. An ECE inhibitor, by interfering with the ET biosynthesis pathway, should reduce production of endothelin and may have therapeutic utility for hypertension or renal failure.

In the last two decades, the search for marine-derived natural products with useful pharmacological properties has been extended to all oceans of the world; the results of this search have been reported in numerous reviews.[1] Sulfated sterols have been described from a wide variety of marine organisms, particularly sponges and echinoderms,[2] and several of these steroidal sulfates have exhibited a broad range of activities. For example, halistanol sulfate, the most common sulfated sterol, isolated from the marine sponge *Halichondria moori* was antimicrobial, hemolytic, ichthyotoxic,[3] and active against HIV,[4] weinbersterol disulfate, isolated from the sponge *Petrosia weinbergi*,[5] exhibited in vitro activity against both feline leukemia virus and HIV-1, ibisterol sulfate, isolated from *Topsentia* sp., was active against HIV-1,[6,7] and halistanol sulfates A−E were found to be active in thrombin receptor assay.[8]

As part of a continuing search for biologically active natural products with potential utility in the treatment of hypertension, we initiated a high throughput screen to evaluate the ability of natural product extracts to inhibit ECE. One of the sponge extracts collected in South Africa was found to be active in the ECE inhibition screen and hence was selected for fractionation. We now report the isolation and structure determination of a novel sterol sulfate, halistanol disulfate B (**1**), from the sponge *Pachastrella* sp.

The freeze-dried sponge extract was sequentially extracted with EtOAc and MeOH. Bioassay-directed fractionation of the *n*-BuOH-soluble portion of MeOH extract, which exhibited ECE inhibitory activity, by column chromatography on Sephadex LH-20 followed by RP-18 PTLC and HPLC yielded halistanol disulfate B (**1**).

Compound **1**, $[\alpha]^{22}_{D}$ +20.1° (*c* = 1.43, MeOH), which



1. R = SO_3H
2. R = H

was obtained as a colorless powder, displayed a molecular ion at *m*/*z* 548.2463 by HRDCIMS corresponding to $C_{26}H_{44}O_8S_2$. An intense fragmentation ion at *m*/*z* 467 in the negative ESIMS of **1** was interpreted as loss of sulfate from the molecular ion. The IR spectrum exhibited strong absorption at 1233 and 1213 cm^{-1}, consistent with the presence of a sulfate. The ^{13}C GASPE NMR spectrum of **1** (Table 1) indicated the presence of 26 carbons including three methyls, 12 methylenes, nine methines, and two quaternary carbons, among which two methines (δ 76.1 and 76.4) were oxygen bearing. The ^1H NMR spectrum (Table 1) supported the existence of two oxygenated methines (δ 4.72 and 4.69); the spectrum also contained two methyl singlets (δ 0.97 and 0.67) and a methyl doublet (δ 0.92, *J* = 6.5 Hz) as well as multiplets at δ 5.79 (ddt, *J* = 10.2, 17.0, 6.8 Hz), 4.96 (ddt, *J* = 2.2, 17.0, 1.6 Hz), and 4.90 (ddt, *J* = 2.2, 10.2, 1.3 Hz) that were indicative of a vinyl group. In the $^1H-^1H$ COSY spectrum, these three multiplets correlated to each other, establishing the presence of a terminal double bond in the side chain of **1**. The COSY data showed a correlation between the resonances at δ 5.79 (H-25) and the equivalent H-24 methylene pair (δ 2.00). The H-24 protons correlated to the H-23 (δ 1.44, 1.26) and H-22 (δ 1.40, 1.03) resonances, which further showed correlation to the H-20 methine (δ 1.40) and CH_3-21 (δ 0.92), establishing the C-20−C-26 network. The positions of the two sulfate groups in **1** at C-2 and C-3 were established from the correlations observed between the H-2 methine (δ 4.72) and the H-1 methylene pair (δ 2.06, 1.40) and the broad vicinal H-3 methine (δ 4.69), which further coupled to the H-4 methylene (δ 1.80, 1.58), which in turn correlated to the H-5 methine (δ 1.56).

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Table 1. ^{13}C and ^{1}H NMR Assignments in CD_3OD

	1	
C	^{13}C δ	^{1}H δ
1	39.2	2.06 (1H, m)
		1.40 (1H, m)
2	76.4	4.72 (1H, bm)
3	76.1	4.69 (1H, bm)
4	30.5	1.80 (1H, m)
		1.58 (1H, m)
5	40.2	1.56 (1H, m)
6	29.2	1.24 (2H, m)
7	33.2	1.66 (1H, m)
		0.90 (1H, m)
8	36.3	1.40 (1H, m)
9	56.6	0.69 (1H, m)
10	36.4	
11	22.0	1.51 (1H, m)
		1.30 (1H, m)
12	41.4	1.98 (1H, m)
		1.12 (1H, m)
13	43.8	
14	57.9	1.04 (1H, m)
15	25.2	1.57 (1H, m)
		1.05 (1H, m)
16	29.2	1.82 (1H, m)
		1.24 (1H, m)
17	57.6	1.11 (1H, m)
18	12.6	0.67 (3H, s)
19	14.3	0.97 (3H, s)
20	37.0	1.40 (1H, m)
21	19.2	0.92 (3H, d, $J = 6.5$)
22	36.6	1.40 (1H, m)
		1.03 (1H, m)
23	26.6	1.44 (1H, m)
		1.26 (1H, m)
24	35.3	2.00 (2H, m)
25	140.2	5.79 (1H, ddt, $J = 10.2, 17.0, 6.8$)
26	114.7	4.96 (1H, ddt, $J = 2.2, 17.0, 1.6$)
		4.90 (1H, ddt, $J = 2.2, 10.2, 1.3$)

HMQC and HMBC experiments established connections between quaternary and protonated resonances leading to the proposed structure for halistanol disulfate B (**1**). For example, the C-19 methyl protons (δ 0.97) correlated with C-1, C-5, C-9, and C-10, which placed this methyl substituent on C-10 (δ 36.4). The C-18 methyl protons (δ 0.67) correlated to the quaternary C-13, C-14, C-12 (δ 43.8, 57.9, 41.4) in ring C and C-17 (δ 57.6) in ring D. The H-12_{eq} proton (δ 1.98) shared a transperiplanar correlation with C-9 (δ 56.6) and with C-14 (δ 57.9). The H-2 signal (δ 4.72) correlated to the vicinal C-3 (δ 76.1) and the quaternary C-10. The H-3 resonance (δ 4.69) correlated to C-2 (δ 76.4) and C-5 (δ 40.2). Finally, the C-21 methyl doublet showed correlations to C-17, C-20, and C-22 (δ 57.6, 37.0, 36.6), which established that the seven-carbon side chain was attached to C-17, typical of a conventional steroid.

The relative streochemistry for **1** was established by a series of NOE experiments and coupling constant analysis. The NOE saturation of H-8 (δ 1.40) and the axial H-11 (δ 1.30) enhanced both the H-18 and H-19 methyls, suggesting that all four of these groups were situated on the same face of the steroid ring system. H-9 and H-14 shared a small NOE enhancement with each other but none with the angular methyls or with H-8, indicating both of these protons were on the opposite face of the steroid. The protons on C-2 and C-3 were assigned as equatorial due to the absence of any large vicinal couplings, and this placed the sulfate groups in axial positions, common with 2,3-disulfated sterols.[6] Irradiation of H-12_{eq} caused an NOE enhancement of the H-21 methyl, and saturation of the C-18 methyl caused an NOE enhancement at H-20, indicating that the C-21 methyl occupied an α position near H-12_{eq} and that H-20 occupied a β position.

Hydrolysis of **1** with methanolic HCl in a sealed tube at 80 °C for 3 h yielded the diol **2** (m/z 388, M^+). The ^{1}H and ^{13}C NMR spectra of **2** revealed that desulfurization occurred at C-2 and C-3, leaving the corresponding diol. The H-2 and H-3 multiplets resonated at δ 3.90 and 3.87, respectively.

Halistanol disulfate B (**1**) had an IC_{50} of 2.1 μM for inhibition of ECE while the diol **2** was totally inactive. Many sulfated marine natural products including halistanol trisulfate, psammaplin A disulfate, and bisaprasin disulfate have shown potent ECE inhibitory activity. These compounds have little likelihood of being able to enter cells, so, even with potent activity, pachasterol disulfate will not be considered as an ECE lead.

Experimental Section

General Experimental Procedures. IR spectra were recorded on a Nicolet Model 20 DXB FTIR spectrometer. All homonuclear and heteronuclear one- and two-dimensional NMR data were recorded on a Bruker AMX-400 spectrometer in $CDCl_3$. ESIMS was obtained in the negative mode on a Perkin-Elmer Sciex API-III triple quadruple mass spectrometer. The HRDCIMS was acquired on a VG-70SE using CH_4 and NH_3 gases. Analytical and preparative TLC were carried out on precoated reversed-phase (Whatman KC18F) plates. A Rainin HPXL solvent delivery system equipped with a refractive index detector, Model 156, was used for HPLC separations employing a Magnum-9 ODS-3 column. Optical rotation was recorded on a Perkin-Elmer 241 MC polarimeter. Reagent-grade chemicals (Fisher and Baker) were used.

Biological Assays. The assay consisted of an incubation of human B-ET in the presence of solubilized recombinant ECE expressed and purified from COS cells. Each reaction mixture contained rECE, B-ET (0.2 μM), buffer (0.1 M Na_2PO4 pH 6.8, 0.5 M NaCl), and test compound and was incubated for 3 h at 37 °C. Endothelin was the end product of the reaction and was quantitated using an EIA assay (Cayman Chemical). The microtiter plates were precoated with a monoclonical antibody specific for endothelin. Following the binding of endothelin to the plate, a second ET-antibody was added which bound specifically to a different ET epitope. The second ET-antibody was conjugated to an acetylcholinesterase (AchE). The concentration of ET was then determined by measuring the AchE activity with Ellman's reagent. The intensity of color at 412 nm is proportional to the endothelin concentration in the assay.

Biological Material. The sponge (SAF94-091) was collected from between 25 and 30 m deep on a rocky substrate on Aliwol Shol, off Umokomos, South Africa. The sponge was partially buried; the only visible portions visible above the substrate were slender fistules 3−6 cm high, arising from a large platelike base. The sponge is extremely siliceous in texture and incompressible, and the color in life is white externally and the interior is khaki. The spiculation is large four-rayed calthrops, relatively small oxeas, streptasters, microacanthostronglyes, and characteristic tiny rhabds with

central thickening. The sample is an undescribed species of *Pachastrella* (Family Pachastrellidae, Order Astrophorida). A voucher specimen has been deposited at the Natural History Museum, London, U.K. (BMNH 1995:10:4:7).

Extraction and Isolation. The freeze-dried sponge (528 g) was extracted with EtOAc (1 L × 2) and MeOH (1 L × 2) to give 1.07 and 28 g extracts, respectively. The pale brown MeOH extract, which showed ECE inhibitory activity, was dissolved in H_2O (250 mL) and extracted with *n*-BuOH (250 mL × 3). The *n*-BuOH layer was evaporated under reduced pressure, and an aqueous layer was lyophilized to yield 1.14 and 26.2 g extracts, respectively. An ECE active *n*-BuOH-soluble portion (IC_{50} = 35.7 μg/mL) was applied to a column of Sephadex LH-20 and eluted with MeOH. Several fractions (15 mL each) were collected and monitored by TLC (RP-18, H_2O:CH_3CN 10:90). Like fractions were combined to yield six (A−F) individual fractions. RP-18 PTLC (H_2O:CH_3CN 10:90) followed by RP-18 HPLC (H_2O:CH_3CN:TFA 9:91:0.1; flow rate 2.5 mL; RI detection, t_R 14.5 min) of fraction F (72 mg), which showed ECE inhibitory activity (IC_{50} = 4 μg/mL), afforded halistanol disulfate B (**1**) as a white powder (16.5 mg).

Halistanol disulfate B (**1**): colorless powder; $[\alpha]_D$ +20.1° (*c* =1.43, MeOH); IR (KBr) ν_{max} 3470, 3075, 2934, 2866, 2853, 1685, 1445, 1233 and 1213 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; LRESIMS *m/z* 548 (M^+); HRDCIMS *m/z* 548.2463 (M^+, calcd for $C_{26}H_{44}O_8S_2$, 548.2477).

Hydrolysis of 1. A mixture of disulfate **1** (5 mg) and methanolic HCl (2 mL, prepared from acetyl chloride and MeOH) was heated in a sealed tube at 80 °C for 3 h. The solvent was evaporated, and the residue was purified by RP-18 HPLC (H_2O:MeOH 2:98) to yield desulfated diol **2** (3.6 mg) as a white powder: LRESIMS *m/z* 388; HRDCIMS *m/z* 388. 3329 (M^+, calcd for $C_{26}H_{44}O_2$, 388.3341); IR (KBr) ν_{max} 3300, 2950, 1686, 1375, 1030, 880 cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.82 (1H, ddt, *J* = 10.2, 17.0, 6.8 Hz, H-25), 4.99 (1H, ddt, *J* = 2.2, 17.0, 1.6 Hz, H-26), 4.93 (1H, ddt, *J* = 2.2, 10.2, 1.3 Hz, H-26), 3.90 (1H, m, H-2), 3.87 (1H, m, H-3), 2.06−0.90 (27 H, m), 0.99 (3H, s, CH_3-19), 0.91 (3H, d, *J* = 6.6 Hz, CH_3-21), 0.70 (1H, m, H-9), 0.65 (3H, s, CH_3-18); ^{13}C NMR ($CDCl_3$) δ 139.3 (CH), 114.1 (CH_2), 71.8 (CH), 70.6 (CH), 56.4 (CH), 56.1 (CH), 55.1 (CH), 42.6 (C), 40.6 (CH_2), 40.0 (CH_2), 38.9 (CH), 35.7 (C), 35.6 (CH), 35.4 (CH_2), 34.9 (CH), 34.2 (CH_2), 31.9 (CH_2), 31.7 (CH_2), 28.2 (2×CH_2), 25.4 (CH_2), 24.1 (CH_2), 20.9 (CH_2), 1 8.6 (CH_3), 14.6 (CH_3), 12.1 (CH_3).

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References and Notes

(1) Krebs, H. C. *Fortschr. Chem. Org. Naturst.* **1986**, *49*, 152−361. Faulkner, D. J. *Nat. Prod. Rept.* **1995**, *12*, 223−269 and previous reviews in this series and references cited therein.
(2) Kerr, R. G.; Baker, B. J. *Nat. Prod. Rep.* **1991**, *8*, 465−497.
(3) Fusetani, N.; Matsunga, S.; Konosu, S. *Tetrahedron Lett.* **1981**, *22*, 1985−1988.
(4) McKee, T. C.; Cardellina, J. H., II; Raffaele, R.; D'Auria, M. V.; Iorizzi, M.; Minale, L.; Moran, R. A.; Gulakowski, R. J.; MaMohan, J. B.; Buckheit, R. W.; Snader, K. M.; Boyd, M. R. *J. Med. Chem.* **1994**, *37*, 793−797.
(5) Sun, H. H.; Cross, S. S.; Gunasekera M.; Koehn, F. E. *Tetrahedron* **1991**, *47*, 1185−1190.
(6) McKee, T. C.; Cardellina, J. H., II; Tischler, M.; Snader, K. M.; Boyd, M. R. *Tetrahedron Lett.* **1993**, ***34***, 389−392.
(7) D'Auria, M. V.; Paloma, G.; Minale, L.; Riccio, R.; Zampella, A. *J. Nat. Prod.* **1995**, *58*, 189−196.
(8) Kanazawa, S.; Fusetani, N.; Matsunaga, S. *Tetrahedron* **1992**, *48*, 5467−5472.

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