

Antineoplastic Agents. 386. Isolation of Sesterstatins 1–3 from the Marine Sponge *Hyrtios erecta*^{1†}

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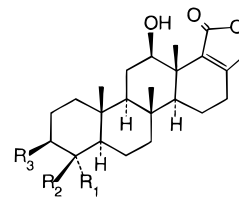
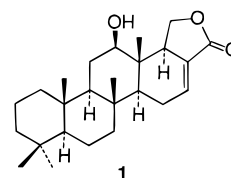
The Republic of Maldives' black marine sponge *Hyrtios erecta* has been found to contain three cancer cell-line inhibitory pentacyclic sesterterpenes designated sesterstatins 1–3 (2–4). One of the sesterterpenes, sesterstatin 2, specifically inhibited the Gram-positive opportunist *Staphylococcus aureus*. All three of the P-388 lymphocytic-leukemia-active (ED₅₀ 0.46 to 4.3 μg/mL) sesterstatins were obtained in trace quantities (3.0 × 10⁻⁷ to 5.4 × 10⁻⁷% yields) and represent structural variations on the more usual scalarin-type porifera sesterterpenes. The structures were elucidated by highfield (500 MHz) 2D NMR techniques augmented by HRMS results.

The marine porifera genus *Hyrtios* (order Dictyoceratida, family Thorectidae) has proven to be an especially valuable source of structurally diverse substances with potentially useful biological properties.¹ Recent examples include the remarkable anticancer spongistatin series,^{2,3} 15-oxopuupehenol (cancer cell line and malarial inhibitory),⁴ sesterterpene **1** (P-388 leukemia cell line inhibitory)¹ and dipuupehedione (cancer cell line inhibitory).⁵ The parent, puupehenone, and its related metabolites also show antibacterial, antiviral, antifungal, cytotoxic, and immunomodulatory activities.⁶ We have continued to pursue (from 1986) an extensive investigation of the Republic of Maldives' *H. erecta* that led to our discovery of spongistatins 1–3.^{1–3} By utilizing a bioassay- (murine P-388 lymphocytic leukemia) directed separation of certain other cancer-cell growth inhibitory fractions we have uncovered three new P-388 active sesterterpene constituents. Isolation and structure elucidation proceeded as follows with a 1994 recollection of the Maldives' *H. erecta*.

Results and Discussion

The wet sponge (500 kg) was extracted with MeOH followed by CH₂Cl₂–MeOH. The CH₂Cl₂ solution was subjected to a 9:1→3:2 MeOH–H₂O/hexane→CH₂Cl₂ solvent partition sequence. The final CH₂Cl₂ P-388 active fraction was carefully separated by an extensive series of Sephadex LH-20 gel permeation and partition (Si gel) chromatographic procedures, followed by final isolation on reversed-phase HPLC columns (Prepex C-8 and LiChrospher 100 RP-18) with 1:1 MeCN–H₂O as eluent. These procedures afforded colorless sesterterpenes **2–4** in 3.0 × 10⁻⁷, 3.0 × 10⁻⁷, and 5.0 × 10⁻⁷% yields, respectively, as amorphous powders designated, respectively, sesterstatins 1–3.

All three terpenes (**2–4**) exhibited a molecular ion peak (HRMS) at *m/z* 402 corresponding to molecular formula C₂₅H₃₈O₄. The ¹H- and ¹³C-NMR spectra of



- 2, R₁ = R₂ = CH₃, R₃ = OH
 3, R₁ = CH₃, R₂ = CH₂OH, R₃ = H
 4, R₁ = CH₂OH, R₂ = CH₃, R₃ = H

substance **2** taken in DMSO-*d*₆ showed the presence of five singlets belonging to methyl groups at 0.66, 0.77, 0.81, 0.86, and 0.98 ppm; eight methylene groups; five methine groups; four quaternary carbons; and two fully substituted (165.2, 133.4 ppm) sp² carbon atoms incorporated into an α,β-unsaturated lactone ring (C=O at 176.3 ppm). The ¹H-NMR spectrum also revealed the presence of two hydroxyl groups, a singlet at 5.65 ppm and a doublet at 4.27 ppm, which accounted for a total of 38 hydrogen atoms, including 36 nonexchangeable ones. Analysis of the COSY and TOCSY spectra allowed us to assemble four partial structural units: C-1 to C-3 with a hydroxyl group attached to C-3; C-5 to C-7; C-9 to C-12 with another hydroxyl group attached to C-12; and C-14 to C-16. Interpretation of the HMBC spectrum indicated that carbons C-24 and C-16 were connected through the sp² carbon C-17, which participates, along with C-18 and C-25, in the α,β-unsaturated lactone ring. The three remaining segments were connected by quaternary carbons, which showed distinctive cross peaks with a methyl group attached. For example, protons of the methyl group at C-23 had cross peaks with the quaternary carbon at C-13, as well as with neighboring carbons at C-12, C-14, and C-18.

The stereochemistry of sesterterpene **2** was estab-

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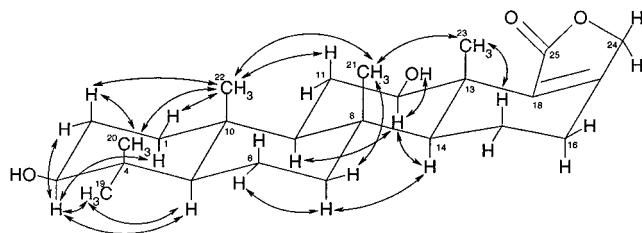


Figure 1. Sesterstatin 1(2).

lished by a ROESY experiment. The hydroxyl group at C-3 was assigned as equatorial because of intense cross peaks between the proton at H-3 (2.95 ppm) and the methyl protons at C-19 (0.86 ppm), H-5 (0.69 ppm), and H-1 (0.86 ppm). The proton at H-12 (3.50 ppm) was coupled with protons H-9 (0.77 ppm) and H-14 (1.01 ppm) and indicated that the 12-OH group was equatorial. The ROESY correlations for compound **2** are shown in Figure 1.

As noted above, sesterterpenes **3** and **4** were found to be isomers of structure **2**. The NMR spectra of both compounds (**3** and **4**) showed four methyl groups (0.83, 0.77, 0.79, 0.98 ppm for **3** and 0.65, 2 × 0.81, 0.98 ppm for **4**); 10 methylenes; four methines; four quaternary carbons; and an α,β -unsaturated carbonyl group. The $^1\text{H-NMR}$ spectra taken in $\text{DMSO-}d_6$ indicated the presence of two hydroxyl groups (5.65 ppm, s, 4.11 ppm, t, for **3** and 5.65 ppm, s, 4.36 ppm, s, for **4**). Interpretation of the COSY and HMBC spectra indicated that the hydroxyl groups were attached to C-12 and a methylene group in each compound. Detailed analysis of their HMBC, COSY, and TOCSY spectra revealed that the hydroxylated methylene group was connected to C-4 in both terpenes **3** and **4**, and led to the conclusion that terpenes **3** and **4** must have the same skeleton. Direct comparison of the NMR data showed several significant differences between substances **3** and **4** at C-5 (methine group), C-19, and C-20 (methyl group, hydroxylated methylene group). In the $^{13}\text{C-NMR}$ spectrum of terpene **3** the methyl group at C-19 appeared at lower field (27.3 ppm) than that in the spectrum of compound **4** (16.9 ppm at C-20). However, the methylene group at C-20 in terpene **3** resonated at a higher field (62.4 ppm) than that in the spectrum of terpene **4** (70.0 ppm). An analysis of the other known sesterterpenes of the scalarane family^{1,7} that lack a hydroxyl group at C-19 or C-20 led to the conclusion that in compound **3** the hydroxylated methylene group was at C-20 (axial) and in compound **4** at C-19 (equatorial). Such an assignment was supported by the ROESY spectra of terpenes **3** and **4**. For compound **3** the ROESY correlation was found between the C-20 methylene protons (3.19; 3.46 ppm) and the methyl protons at C-22 (0.77 ppm, axial) and between H-20 (3.19 ppm) and H-6 (1.40 ppm, axial). With compound **4** ROESY correlations were observed between the protons of C-20 (methyl, 0.65 ppm, axial) and C-22 (methyl, 0.81 ppm, axial) and H-2 (1.34 ppm, axial). The unusual positioning of the butenolide ring represents a significant structural departure from the usual scalarin-type porifera sesterterpenes.^{7b}

Evaluation of sesterstatins 1–3 against the P-388 leukemia showed, respectively, ED_{50} values of 0.46, 4.2, and 4.3 $\mu\text{g/mL}$. As suggested in the previous contribution¹ the cancer-cell growth-inhibitory activity is quite likely due to the butenolide-type lactone groups in these

sesterterpenes. Sesterstatin 2 inhibited growth of the Gram-positive bacterium *S. aureus*, with a minimum inhibitory concentration of 50–100 $\mu\text{g/disk}$. Several biosynthetically related sesterterpenes have similar antibacterial activity. Puupehenone inhibits *S. aureus*,^{6a} and palauolol from the Palau marine sponge *Fascaplysinopsis* is antimicrobial for *S. aureus* and the Gram-positive bacterium *Bacillus subtilis*.⁸ Thus, the sesterterpenes have potential as a new class of narrow-spectrum, anti-Gram-positive antibiotics.

Experimental Section

General Experimental Procedures. All TLC plates were viewed with UV light and developed with a ceric sulfate- H_2SO_4 acid spray (heating to approximately 150 $^\circ\text{C}$ for 10 min). The Sephadex LH-20 (25–100 μ) employed for gel permeation chromatography was obtained from Pharmacia Fine Chemicals AB Uppsala, Sweden. The Prepex C-8 HPLC column was provided by Phenomenex, Torrance, CA. A LiChrospher 100 RP-18 column was obtained from E. Merck, Darmstadt, Germany.

The UV spectra were recorded with a Perkin-Elmer Lambda 3B spectrophotometer. Nuclear magnetic resonance spectra were recorded in $\text{DMSO-}d_6$ with a Varian 500 MHz instrument. The MS data were obtained using a Finnigan-MAT model 312 (70 eV electron ionization). Other experimental conditions were as previously described.⁹

Extraction and Initial Separation of *H. erecta*. Details of the 1994 recollection and taxonomy of the Republic of Maldives' black marine sponge *H. erecta* have been summarized in a preceding report.¹ A 500-kg (wet wt) portion of the 1994 sponge recollection was extracted with MeOH followed by CH_2Cl_2 . The second CH_2Cl_2 extract (1004 g; P-388 ED_{50} 1.2 $\mu\text{g/mL}$) was dissolved in MeOH- H_2O (9:1) and the solution filtered to remove insoluble material (91 g; ED_{50} 53 $\mu\text{g/mL}$). After the filtration the solution was partitioned four times between hexane and 9:1 MeOH- H_2O . The hexane layer was removed and concentrated to yield 437 g (ED_{50} 35 $\mu\text{g/mL}$) of black-brown material. The MeOH- H_2O phase was diluted to give a ratio of 3:2 (by addition of H_2O) and extracted four times with CH_2Cl_2 . The CH_2Cl_2 layer was concentrated to afford a black oily P-388-active (502 g, ED_{50} 0.53 $\mu\text{g/mL}$) fraction. The remaining MeOH- H_2O solution was discarded as inactive.

Isolation of Sesterstatins 1–3 (2–4). A 283-g aliquot of the P-388-active CH_2Cl_2 fraction was partially dissolved in MeOH, and the solution was filtered and separated on a Sephadex LH-20 column with MeOH as eluent. Seven fractions were obtained. One of the fractions (38 g, ED_{50} 0.34 $\mu\text{g/mL}$) was further separated on a Sephadex LH-20 column in hexane-toluene-MeOH (3:1:1). This separation yielded 10 fractions, and a 1.9 g-fraction with an ED_{50} of 0.0045 $\mu\text{g/mL}$ was subjected to chromatographic separation on a Si gel Si 60 (40–63 μm) column with a solvent mixture of gradually increasing polarity: hexane- CH_2Cl_2 -MeOH (6:9:1)→methanol. All 13 fractions obtained from this step showed P-388 activity and a 53.7-mg fraction with ED_{50} 0.25 $\mu\text{g/mL}$, a dark-brown material, was purified on a preparative reversed-phase HPLC column (Prepex C-8) with 1:1 MeCN- H_2O (a flow rate of 3 mL/min). A

Table 1. ^{13}C - and ^1H -NMR Assignments (Recorded in $\text{DMSO}-d_6$) for Sesterstatins 1 (**2**), 2 (**3**), 3 (**4**)

	2		3		4	
	^{13}C	^1H (mult, J in Hz)	^{13}C	^1H (mult, J in Hz)	^{13}C	^1H (mult, J in Hz)
1	37.8	0.86 (1H, m) 1.60 (1H, m)	39.6	0.76 (1H, m) 1.63 (1H, m)	38.9	0.72 (1H, m) 1.60 (1H, m)
2	27.1	1.49 (1H, m) 1.58 (1H, m)	17.9	1.31 (1H, m) 1.50 (1H, m)	17.6	1.34 (1H, m) 1.39 (1H, m)
3	76.8	2.95 (1H, m)	35.4	0.76 (1H, m) 1.73 (1H, m)	35.0	1.09 (1H, m) 1.73 (1H, m)
3-OH		4.28 (1H, d, 5.0)				
4	38.5		38.2		37.2	
5	55.0	0.69 (1H, m)	56.7	0.85 (1H, m)	48.8	1.11 (1H, m)
6	17.6	1.41 (1H, m) 1.50 (1H, m)	17.9	1.40 (1H, m) 1.54 (1H, m)	17.3	1.31 (1H, m) 1.43 (1H, m)
7	41.1	0.86 (1H, m) 1.77 (1H, m)	41.4	0.81 (1H, m) 1.75 (1H, m)	40.7	0.88 (1H, m) 1.71 (1H, m)
8	36.6		36.7		36.5	
9	57.1	0.77 (1H, m)	57.4	0.80 (1H, m)	57.4	0.84 (1H, m)
10	36.5		36.7		36.6	
11	25.6	1.35 (1H, m) 1.59 (1H, m)	26.6	1.34 (1H, m) 1.58 (1H, m)	25.5	1.34 (1H, m) 1.61 (1H, m)
12	75.4	3.50 (1H, dd, 11.0, 4.5)	75.3	3.50 (1H, dd, 11.0, 4.5)	75.4	3.51 (1H, dd, 11.0, 4.0)
12-OH		5.65 (1H, s)		5.65 (1H, s)		5.65 (1H, s)
13	41.6		41.5		42.0	
14	54.3	1.01 (1H, m)	54.3	1.01 (1H, m)	54.4	1.02 (1H, m)
15	16.0	1.56 (1H, m) 1.79 (1H, m)	15.9	1.55 (1H, m) 1.79 (1H, m)	15.9	1.55 (1H, m) 1.81 (1H, m)
16	24.7	2.26 (1H, ddd, 19.5, 11.5, 7.0) 2.46 (1H, dd, 19.5, 5.5)	24.7	2.27 (1H, ddd, 19.5, 11.5, 7.0) 2.45 (1H, dd, 19.5, 5.5)	24.7	2.26 (1H, ddd, 19.0, 11.0, 7.0) 2.45 (1H, dd, 19.0, 5.5)
17	165.2		165.1		165.1	
18	133.4		133.3		133.4	
19	28.0	0.86 (3H, s)	27.3	0.83 (3H, s)	70.0	2.84 (1H, dd, 10.5, 3.0) 3.17 (1H, dd, 10.5, 3.0) 4.36 (1H, s)
19-OH						0.65 (3H, s)
20	15.6	0.66 (3H, s)	62.4	3.19 (1H, dd, 10.5, 5.0) 3.46 (1H, dd, 10.5, 5.0) 4.11 (1H, t, 5.0)	16.9	
20-OH						
21	16.8	0.81 (3H, s)	16.7	0.79 (3H, s)	17.2	0.81 (3H, s)
22	15.8	0.77 (3H, s)	16.2	0.77 (3H, s)	16.6	0.81 (3H, s)
23	16.6	0.98 (3H, s)	16.5	0.98 (3H, s)	16.5	0.98 (3H, s)
24	72.5	4.81 (1H, d, 18.0) 4.86 (1H, d, 18.0)	72.4	4.81 (1H, d, 18.0) 4.86 (1H, d, 18.0)	72.5	4.81 (1H, d, 18.0) 4.86 (1H, d, 18.0)
25	176.3		176.2		176.2	

resulting 41.3-mg fraction with ED_{50} 2.5 $\mu\text{g}/\text{mL}$ contained a mixture of four compounds. Each compound was finally isolated on a HPLC LiChrospher 100 RP-18 column with 1:1 MeCN– H_2O (a flow rate of 1 mL/min and the UV detector set at 230 nm). Sesterterpenes **2**–**4** were eluted in the following order: **3** (1.56 mg) at 12.4 min; **2** (1.5 mg) at 14.2 min; **4** (2.7 mg) at 15.9 min; and 16-*O*-deacetyl-16-*epi*-scalarobutenolide⁷ at 22.07 min. All four compounds were colorless and had very limited solubility in MeOH, CH_2Cl_2 , MeCN, and H_2O .

Sesterstatin 1 (2): 1.5 mg; $3.0 \times 10^{-7}\%$; ED_{50} 0.46 $\mu\text{g}/\text{mL}$; mp 297–298 $^\circ\text{C}$; $[\alpha]^{22}_{\text{D}} + 16.3^\circ$ (c 0.12, CHCl_3); UV (MeOH) λ_{max} 217 nm, ϵ 1550; IR (film) 3385, 2930, 2854, 1712, 1653 cm^{-1} ; EIMS 402 corresponding to $\text{C}_{25}\text{H}_{38}\text{O}_4$; and refer to Table 1 and Figure 1 (NOE results) for the NMR data.

Sesterstatin 2 (3): 1.5 mg; $3.0 \times 10^{-7}\%$; ED_{50} 4.2 $\mu\text{g}/\text{mL}$; mp 295–296 $^\circ\text{C}$; $[\alpha]^{22}_{\text{D}} + 13.8^\circ$ (c 0.09, CHCl_3); UV (MeOH) λ_{max} 216 nm, ϵ 8030; IR (film) 3380, 2924, 2854, 1716, 1653 cm^{-1} ; EIMS 402 corresponding to $\text{C}_{25}\text{H}_{38}\text{O}_4$; and see Table 1 for the NMR results.

Sesterstatin 3 (4): 2.7 mg; $5.4 \times 10^{-7}\%$; ED_{50} 4.3 $\mu\text{g}/\text{mL}$; mp 293–294 $^\circ\text{C}$; $[\alpha]^{22}_{\text{D}} + 27.2^\circ$ (c 0.22, CHCl_3); UV (MeOH) λ_{max} 217 nm, ϵ 8680; IR (film) 3380, 2924, 2854, 1716, 1653 cm^{-1} ; EIMS 402 corresponding to $\text{C}_{25}\text{H}_{38}\text{O}_4$; for the NMR conclusions refer to Table 1.

Disk Susceptibility Testing. Disk susceptibility tests were performed on a small panel of Gram-positive and Gram-negative bacteria and the fungus *Cryptococcus neoformans*.¹⁰ Sesterstatins 1–3 were reconstituted in sterile DMSO, and two-fold dilutions were applied to sterile disks. At 100 $\mu\text{g}/\text{disk}$, sesterstatins 1 and 3 did not inhibit *S. aureus*, *Micrococcus luteus*, *Neisseria gonorrhoeae*, or *C. neoformans*, and sesterstatin 2 did not inhibit *M. luteus*, *N. gonorrhoeae*, or *C. neoformans*.

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References and Notes

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