Antineoplastic Agents. 386. Isolation of Sesterstatins 1−3 from the Marine Sponge *Hyrtios erecta*[1†]

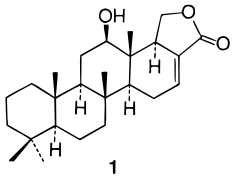
George R. Pettit,* Zbigniew A. Cichacz, Rui Tan, Michael S. Hoard, Noeleen Melody, and Robin K. Pettit

Cancer Research Institute and Department of Chemistry, Arizona State University, Tempe, Arizona 85287-2404

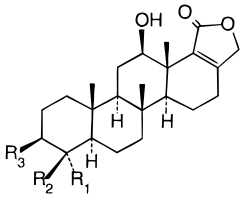
Received April 16, 1997[⊗]

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 (ED_{50} 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.*



1



2, $R_1 = R_2 = CH_3$, $R_3 = OH$
3, $R_1 = CH_3$, $R_2 = CH_2OH$, $R_3 = H$
4, $R_1 = CH_2OH$, $R_2 = CH_3$, $R_3 = H$

Results and Discussion

The wet sponge (500 kg) was extracted with MeOH followed by CH_2Cl_2−MeOH. The CH_2Cl_2 solution was subjected to a 9:1→3:2 MeOH−H_2O/hexane→CH_2Cl_2 solvent partition sequence. The final CH_2Cl_2 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−H_2O 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 $C_{25}H_{38}O_4$. The ^{1}H- and ^{13}C-NMR spectra of 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) sp^2 carbon atoms incorporated into an α,β-unsaturated lactone ring (C=O at 176.3 pm). The ^{1}H-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 sp^2 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-

* To whom correspondence should be addressed. Phone: (602) 965-3351. FAX: (602) 965-2747.

† Dedicated to Professor James P. Kutney on the occasion of his 65th birthday.

⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1997.

S0163-3864(97)00203-6 CCC: $15.00
Published on Web 01/23/1998

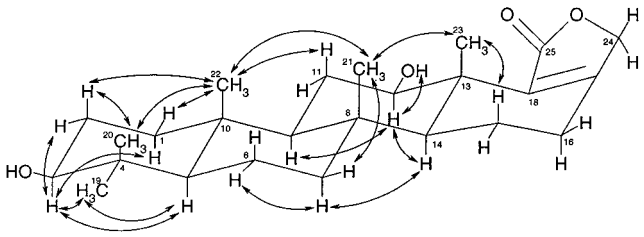


Figure 1. Sesterstatin 1(**2**).

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}H-NMR spectra taken in 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}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 μg/mL. As suggested in the previous contribution[1] 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 μg/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 °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 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 μg/mL) was dissolved in MeOH−H_2O (9:1) and the solution filtered to remove insoluble material (91 g; ED_{50} 53 μg/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 μg/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 μg/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 μg/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 μg/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 μg/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 ^{1}H-NMR Assignments (Recorded in DMSO-d_6) for Sesterstatins 1 (**2**), 2 (**3**), 3 (**4**)

	2		**3**		4	
	^{13}C	^{1}H (mult, *J* in Hz)	^{13}C	^{1}H (mult, *J* in Hz)	^{13}C	^{1}H (mult, *J* in Hz)
1	37.8	0.86 (1H, m)	39.6	0.76 (1H, m)	38.9	0.72 (1H, m)
		1.60 (1H, m)		1.63 (1H, m)		1.60 (1H, m)
2	27.1	1.49 (1H, m)	17.9	1.31 (1H, m)	17.6	1.34 (1H, m)
		1.58 (1H, m)		1.50 (1H, m)		1.39 (1H, m)
3	76.8	2.95 (1H, m)	35.4	0.76 (1H, m)	35.0	1.09 (1H, m)
				1.73 (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)	17.9	1.40 (1H, m)	17.3	1.31 (1H, m)
		1.50 (1H, m)		1.54 (1H, m)		1.43 (1H, m)
7	41.1	0.86 (1H, m)	41.4	0.81 (1H, m)	40.7	0.88 (1H, m)
		1.77 (1H, m)		1.75 (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)	26.6	1.34 (1H, m)	25.5	1.34 (1H, m)
		1.59 (1H, m)		1.58 (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)	15.9	1.55 (1H, m)	15.9	1.55 (1H, m)
		1.79 (1H, m)		1.79 (1H, m)		1.81 (1H, m)
16	24.7	2.26 (1H, ddd, 19.5, 11.5, 7.0)	24.7	2.27 (1H, ddd, 19.5, 11.5, 7.0)	24.7	2.26 (1H, ddd, 19.0, 11.0, 7.0)
		2.46 (1H, dd, 19.5, 5.5)		2.45 (1H, dd, 19.5, 5.5)		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)
19-OH						4.36 (1H, s)
20	15.6	0.66 (3H, s)	62.4	3.19 (1H, dd, 10.5, 5.0)	16.9	0.65 (3H, s)
				3.46 (1H, dd, 10.5, 5.0)		
20-OH				4.11 (1H, t, 5.0)		
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)	72.4	4.81 (1H, d, 18.0)	72.5	4.81 (1H, d, 18.0)
		4.86 (1H, d, 18.0)		4.86 (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 μg/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*-scalarobutenolide[7] 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; 3.0×10^{-7}%; ED_{50} 0.46 μg/mL; mp 297−298 °C; $[\alpha]^{22}_D$ + 16.3° (*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 $C_{25}H_{38}O_4$; and refer to Table 1 and Figure 1 (NOE results) for the NMR data.

Sesterstatin 2 (3): 1.5 mg; 3.0×10^{-7}%; ED_{50} 4.2 μg/mL; mp 295−296 °C; $[\alpha]^{22}_D$ + 13.8° (*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 $C_{25}H_{38}O_4$; and see Table 1 for the NMR results.

Sesterstatin 3 (4): 2.7 mg; 5.4×10^{-7}%; ED_{50} 4.3 μg/mL; mp 293−294 °C; $[\alpha]^{22}_D$ + 27.2° (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 $C_{25}H_{38}O_4$; 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 μg/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*.

Acknowledgment. With appreciation we acknowledge the following quite necessary financial support provided by Outstanding Investigator Grant CA44344-01-08 awarded by the Division of Cancer Treatment Diagnosis and Treatment, NCI, DHHS, the Arizona Disease Control Research Commission, the Fannie E. Rippel Foundation, Virginia Piper, the Robert B. Dalton Endowment Fund, Gary L. and Diane Tooker, Diane and Bruce Halle, John and Edith Reyno, the Fraternal Order of Eagles Art Ehrmann Cancer Fund, and the Ladies Auxiliary to the Veterans of Foreign Wars. Other helpful assistance was provided by the Government of the Republic of Maldives (Maizan H. Maniku, A. Naseer, and M. Shiham), Drs. Michael D. Williams, Fiona Hogan, Jean M. Schmidt, Jean-Charles Chapuis, Daniel Brune, and Ron Nieman, Mr. Larry Tackett, Ms. Denise Nielsen-Tackett, Mr. David Carnell, Mr. Lee Williams,

Ms. Deanna Hansen, the U.S. National Science Foundation (Grants BBS 88-04992, CHE-8409644), and the NSF Regional Instrumentation Facility in Nebraska (Grant CHE-8620177).

References and Notes

(1) For part 385, refer to Pettit, G. R.; Cichacz, Z. A.; Tan, R.; Herald, D. L.; Melody, N.; Hoard, M. S.; Doubek, D. L.; Hooper, J. N. A. *J. Chem. Soc., Perkin Trans. 1*, submitted.
(2) Pettit, G. R. *J. Nat. Prod.* **1996**, *59*, 812−821.
(3) (a) Pettit, G. R.; Cichacz, Z. A.; Gao, F.; Herald, C. L.; Boyd, M. R.; Schmidt, J. M.; Hooper, J. N. A. *J. Org. Chem*. **1993**, *58*, 1302−1304. (b) Pettit, G. R.; Cichacz, Z. A.; Herald, C. L.; Gao, F.; Boyd, M. R.; Schmidt, J. M.; Hamel, E.; Bai, R. *J. Chem. Soc., Chem. Commun*. **1994**, 1605−1606.
(4) Nasu, S. S.; Yeung, B. K. S.; Hamann, M. T.; Scheuer, P. J.; Kelley-Borges, M.; Goines, K. *J. Org. Chem*. **1995**, *60*, 7290−7292.
(5) Bourguet-Kondracki, M. L.; Debitus, C.; Guyot, M. *Tetrahedron Lett*. **1996**, *37*, 3861−3864.
(6) (a) Ravi, B. N.; Perzanowski, H. P.; Ross, R. A.; Erdman, T. R.; Scheuer, P. J. *Pure and Applied Chem*. **1979**, *51*, 1893−1900. (b) Hamann, M. T.; Scheuer, P. J.; Kelley-Borges, M. *J. Org. Chem*. **1993**, *58*, 6565−6569.
(7) (a) Bourguet-Kondracki, M. L.; Martin, M. T.; Debitus, C.; Guyot, M. *Tetrahedron Lett*. **1994**, *35*, 109−110. (b) Ryu, G.; Matsunaga, S.; Fusetani, N. *J. Nat. Prod*. **1996**, *59*, 515−517.
(8) Schmidt, E. W.; Faulkner, D. J. *Tetrahedron Lett*. **1996**, *37*, 3951−3954.
(9) Pettit, G. R.; Butler, M. S.; Bass, C. G.; Doubek, D. L.; Williams, M. D.; Schmidt, J. M.; Pettit, R. K.; Hooper, J. N. A.; Tackett, L. P.; Filiatrault, M. J. *J. Nat. Prod*. **1995**, *58*, 680−688.
(10) National Committee for Clinical Laboratory Standards. *Performance Standards for Antimicrobial Disk Susceptibility Tests−6th Edition: Approved Standard M2-A6*. NCCLS: Villanova, PA, 1997.

NP970203+