Three New Cytotoxic Sesterterpenes from a Marine Sponge *Spongia* sp.

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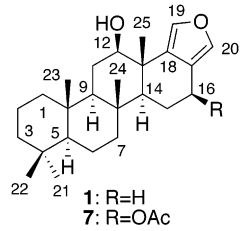
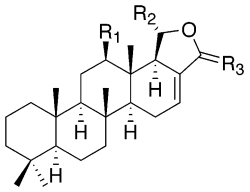
Three new sesterterpenes (**1**−**3**) were isolated from a marine sponge of the genus *Spongia*, and their structures were determined on the basis of spectroscopic analysis. The compounds **1**−**3** exhibited cytotoxicity against HeLa cells with IC_{50} values of 19.5, 15.0, and 5.3 μg/mL, respectively.

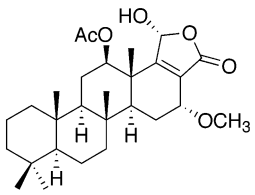
Many scalarane-type sesterterpenoids have been isolated from marine sponges belonging to the order Dictyoceratida,[1] and they showed a variety of biological activities such as antimicrobial,[2,3] cytotoxic,[4−6] antifeedant,[7] ichthyotoxic,[8] antiinflammatory,[9,10] platelet-aggregation inhibitory,[11] and nerve growth factor synthesis-stimulating.[12] As part of our studies of pharmacologically interesting metabolites of sponges,[13] we report here the bioassay-monitored isolation and structure elucidation of three new cytotoxic scalarane sesterterpenes, 12-*O*-deacetylscalarafuran (**1**), 12-*O*-deacetyl-12-*epi*-scalarin (**2**), and 12-*O*-acetyl-16-*O*-methylhyrtiolide (**3**), along with known compounds, 12-*epi*-scalarin (**4**),[14] 12-*epi*-deoxoscalarin (**5**),[14] and sesterterpene **6**,[15] from a marine sponge of the genus *Spongia*. In addition, we evaluated in vivo antitumor activity of three of the compounds on sarcoma-180-implanted mice.



1: R=H
7: R=OAc



2: R_1=OH; R_2=OH; R_3=O
4: R_1=OAc; R_2=OH; R_3=O
5: R_1=OAc; R_2=OH; R_3=H_2
6: R_1=OH; R_2=H; R_3=O
8: R_1=OAc; R_2=OAc; R_3=O



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Specimens of *Spongia* sp. (1.7 kg) were collected from Toyama Bay in the Japan Sea and kept frozen until they were extracted with MeOH. The active EtOAc-soluble portion of the extract was fractionated by a combination of silica gel and ODS chromatography followed by HPLC on ODS to furnish three new sesterterpenes, **1** (1.9 mg), **2** (8.2 mg), and **3** (7.8 mg), together with the known metabolites **4** (3.9 mg), **5** (33.0 mg), and **6** (3.6 mg).

Table 1. ^{1}H and ^{13}C NMR Data of **1** ($CDCl_3$)

no.	δ_H mult, *J* (Hz)	δ_C mult	COSY	HMBC
1	0.80 m	39.9 t	H-1′, H-2, H-2′	C-2
	1.69 m		H-1	
2	1.42 m	18.6 t	H-1, H-2′	
	1.59 m		H-1, H-2, H-3	
3	1.14 m	42.0 t	H-2′, H-3′	
	1.35 br d 13.2		H-3	C-1, C-5
4		33.3 s		
5	0.80 m	56.7 d	H-6, H-6′	C-4
6	1.44 m	18.2 t	H-5, H-7	
	1.60 m		H-5	
7	0.93 m	41.7 t	H-6, H-7′	C-6, C-9
	1.86 dt 13.2, 4.0		H-7	C-9, C-14, C-24
8		37.5 s		
9	0.92 dd 13.2, 2.0	58.8 d	H-11	
10		37.5 s		
11	1.52 m	28.0 t	H-9, H-11′, H-12	C-10, C-12
	1.78 m		H-11, H-12	C-12
12	3.64 dd 11.2, 4.4	80.0 d	H-11, H-11′	C-14, C-25
13		40.4 s		
14	1.13 m	55.8 d	H-15	C-7, C-8
15	1.64 m	17.7 t	H-14, H-15′, H-16′	
	1.80 m		H-15, H-16	C-13, C-17
16	2.41 ddd 16.1, 5.9, 1.5	21.0 t	H-15, H-15′, H-16′, H-20	C-15, C-17, C-18
	2.75 dd 16.1, 5.9		H-15, H-16, H-20	C-14, C-15, C-17, C-18
17		119.9 s		
18		134.6 s		
19	7.53 br s	137.4 d	H-20	C-17, C-18, C-20
20	7.04 br s	136.2 d	H-16, H-16′, H-19	C-17, C-18, C-19
21	0.84 (3H) s	33.3 q		C-3, C-4, C-22
22	0.82 (3H) s	21.3 q		C-3, C-4, C-5, C-21
23	0.85 (3H) s	16.2 q		C-1, C-5, C-9, C-10
24	0.89 (3H) s	17.6 q		C-7, C-8, C-14
25	1.20 (3H) s	19.2 q		C-12, C-13, C-14, C-18

Compound **1** had a molecular formula of $C_{25}H_{38}O_2$ as established by HREIMS and ^{13}C NMR data. In the IR spectrum the presence of a hydroxyl group was suggested by a broad band at 3300 cm^{-1}. The ^{13}C NMR spectrum (Table 1) displayed four olefin signals, and the ^{1}H NMR spectrum (Table 1) revealed five methyl signals that are consistent with a pentacyclic sesterterpene ring system.[14,15] The two mutually long-range-coupled olefinic hydrogens at

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Table 2. ^{1}H and ^{13}C NMR Data of **2** (DMSO-d_6) and **3** ($CDCl_3$)

	2		**3**	
no.	δ_H mult, J (Hz)	δ_C mult	δ_H mult, J (Hz)	δ_C mult
1	0.80 m	40.2 t	0.87 m	40.1 t
	1.62 m		1.64 m	
2	1.57 m	18.2 t	1.42 m	18.7 t
	1.63 m		1.60 m	
3	1.10 m	41.3 t	1.13 m	42.5 t
	1.33 m		1.35 m	
4		33.5 s		33.7 s
5	0.79 m	56.5 d	0.86 m	57.8 d
6	1.38 m	18.7 t	1.42 m	18.9 t
	1.46 m		1.60 m	
7	0.88 m	42.2 t	0.95 m	41.9 t
	1.63 m		1.78 m	
8		37.5 s		37.8 s
9	0.80 m	58.7 d	1.02 m	58.2 d
10		37.4 d		37.3 s
11	1.30 m	27.4 t	1.55 m	25.2 t
	1.55 m		1.80 m	
12	3.39 dt 12.0, 4.4	78.6 d	4.93 dd 11.0, 4.9	76.0 d
13		37.4 s		41.2 s
14	1.20 m	52.9 d	1.55 m	50.6 d
15	2.12 m	24.1 t	1.56 m	33.7 t
	2.25 m		2.11 m	
16	6.67 q 2.9	135.7 d	4.02 dd 3.1, 0.5	70.0 d
17		128.9 s		140.9 s
18	2.40 br s	58.1 d		157.4 s
19	5.72 t 5.9	100.5 d	6.03 s	94.9 d
20		167.5 s		168.4 s
21	0.63 (3H) s	33.7 q	0.81 (3H) s	33.8 q
22	0.77 (3H) s	21.7 q	0.83 (3H) s	21.8 q
23	0.80 (3H) s	16.4 q	0.85 (3H) s	16.4 q
24	0.81 (3H) s	16.8 q	0.92 (3H) s	17.8 q
25	0.83 (3H) s	9.8 q	1.21 (3H) s	15.7 q
12-OH	4.28 d 4.4			
19-OH	7.73 d 5.9			
12-$OCOCH_3$			2.12 (3H) s	21.8 q
				171.9 s
16-OCH_3			3.40 (3H) s	56.9 q

δ 7.04 (1H, br s, H-20) and 7.53 (1H, br s, H-19) were directly bonded to carbons at δ 136.2 and 137.4 as shown by the HMQC spectrum. The presence of a furan ring was suggested by HMBC cross-peaks: H-19/C-17 (δ 119.9), C-18 (δ 134.6), C-20 (δ 136.2) and H-20/C-17, C-18, C-19 (δ 137.4). The olefinic signal at δ 7.04 was coupled to methylene signals at δ 2.41 (1H, ddd, J = 16.1, 5.9, 1.5 Hz, H-16) and 2.75 (1H, dd, J = 16.1, 5.9 Hz, H-16), which were further coupled to other methylene signals at δ 1.64 (1H, m, H-15) and 1.80 (1H, m, H-15) and then to a methine signal at δ 1.13 (1H, m, H-14). The HMBC correlations H-16/C-17 and C-18; H-15/C-17; and H_3-25/C-13, C-14, C-18, suggested **1** was a sesterterpene including a furan ring. A hydrogen [δ 3.64 (1H, dd, J = 11.2, 4.4 Hz)] on an oxygen-bearing carbon [δ 80.0 (d)] was coupled to methylene signals at δ 1.52 (1H, m, H-11) and 1.78 (1H, m, H-11) and then to a methine signal at δ 0.92 (1H, dd, J = 13.2, 2.0 Hz, H-9). The HMBC cross-peaks, δ_H 3.64/δ_C 19.2 (C-25) and 55.8 (C-14), suggested that the hydrogen signal should be assigned to H-12. The coupling constant of 11.2 Hz between H-11 and H-12 defined H-12 as axial (12β-OH). Interpretation of the NMR data including HMQC and HMBC experiments resulted in the complete assignment of 37 hydrogen and 25 carbon signals (Table 1). Thus, the structure of **1** was determined as 12-*O*-deacetylscalarafuran. It was previously prepared from scalarafuran (**7**) by hydrogenolysis,[7] but this is the first report of its isolation from nature.

The FAB mass spectrum of compound **2** exhibited an ion peak at m/z 403 $[M + H]^+$, which matched a formula of $C_{25}H_{38}O_4$. The ^{1}H and ^{13}C NMR spectra measured in DMSO-d_6 (Table 2) suggested **2** was also a scaralane-type sesterterpenoid. The COSY spectrum showed that a signal at δ 5.72 (t, J = 5.9 Hz, H-19) was coupled with a methine signal at δ 2.40 (br s, H-18) and a hydroxyl signal at δ 7.73 (d, J = 5.9 Hz, 19-OH) and that H-18 was further coupled with an olefin hydrogen at δ 6.67 (q, J = 2.9 Hz, H-16). The carbon signals in the low-field region at δ 100.5 (d, C-19), 128.9 (s, C-17), 135.7 (d, C-16), and 167.5 (s, C-20) were reminiscent of those of scalarin.[3] The signal at δ 3.39 (td, J = 4.4, 12.0 Hz, H-12) was coupled with a hydroxyl signal at δ 4.28 (d, J = 4.4 Hz, 12-OH) and methylene hydrogens at δ 1.30 (m, H-11) and 1.55 (m, H-11). H-12 was assigned as axial on the basis of the coupling constant of 12.0 Hz between H-11 and H-12. The NOE difference spectrum of **2** revealed a correlation between H-12 and H-18, suggesting that H-18 is in the α-orientation. Although the stereochemistry of C-19 remained to be resolved, the NMR data of **2** were similar to those of 12-*epi*-scalarin (**4**)[14] and suggested that **2** was a deacetyl derivative of **4**. Acetylation of **2** gave a diacetyl derivative (**8**), whose spectral data, ^{1}H NMR and EIMS, were all identical with those of 12-*epi*-19-*O*-acetylscalarin (**8**) derived from **4**. Hence, **2** is 12-*O*-deacetyl-12-*epi*-scalarin.

Compound **3** possessed the molecular formula $C_{28}H_{42}O_6$ on the basis of HRFABMS. The presence of a γ-hydroxybutenolide ring was suggested by the deshielded methine signals [δ_H 6.03 (s, H-19)/δ_C 94.9 (d, C-19)], two quaternary olefinic carbons [δ 140.9 (s, C-17), 157.4 (s, C-18)], and a carbonyl carbon [δ 168.4 (s, C-20)]. The ^{1}H and ^{13}C NMR data (Table 2) were compatible with hyrtiolide[16] except for the presence of *O*-acetyl [δ_H 2.12 (3H, s, CH_3CO); δ_C 21.8 (q, CH_3CO), 171.9 (s, CH_3CO)] and *O*-methyl groups [δ_H 3.40 (3H, s, CH_3O); δ_C 56.9 (q, CH_3O)]. The HMBC cross-peaks H-12/CH_3CO and H-16/CH_3O indicated that the acetoxy and methoxy groups were attached to C-12 and C-16, respectively. The coupling constants suggested that H-12 [δ 4.93 (dd, J = 11.0, 4.9 Hz)] and H-16 [δ 4.02 (dd, J = 3.1, 0.5 Hz)] must be in axial and equatorial orientation, respectively. The NOE correlation between H-19 and H_3-25 revealed the β-orientation of H-19. Therefore, the structure of **3** was established as 12-*O*-acetyl-16-*O*-methylhyrtiolide.

Table 3. Cytotoxicity of Sesterterpenes **1**−**6** against Tumor Cell Lines

	IC_{50} (μg/mL)			
compd	L1210	HeLa	A549	KB
1	>50	19.5	>50	>50
2	2.3	15.0	14.8	14.3
3	2.2	5.3	5.3	15.6
4	13.2	26.0	23.7	18.5
5	2.1	22.5	29.4	16.2
6	1.6	16.5	16.5	17.1
mitomycin C[a]	0.020	0.015	0.020	0.015

[a] Positive control.

Cytotoxicity of **1**−**6** was tested against murine leukemia (L1210), human cervix epithelioid carcinoma (HeLa), human lung adenocarcinoma (A549), and human oral epidermoid carcinoma (KB) cell lines (Table 3). Although sesterterpenes **2**−**6** exhibited cytotoxicity against L1210, A549, and KB cells, **1** was inactive to all of the cell lines at 50 μg/mL. However, **1** was cytotoxic against HeLa cells with an IC_{50} value of 19.5 μg/mL. Compounds **2**, **5**, and **6** were also tested for in vivo mean survival times (MST) and increases of life spans (ILS) in sarcoma-180-implanted mice. Among these three compounds, **6** showed significant ILS: 50.3% of ILS at 5 mg/kg intraperitoneal administration, and this is more potent than a positive control, 5-fluorouracil (5-FU; 32.9%), at the same dose. Compound

5 also showed comparable ILS (28.0%) to 5-FU at 10 mg/kg, and **2** was inactive at 5 mg/kg.

Experimental Section

General Experimental Procedures. Optical rotations were determined with a Horiba SEPA-300 high sensitive polarimeter. UV spectra were measured on a Shimadzu UV-1600 UV−visible spectrophotometer. IR spectra were recorded on a Shimadzu IR-460 infrared spectrophotometer. NMR spectra were recorded on a JEOL GSX500 NMR spectrometer in $CDCl_3$ or DMSO-d_6. All chemical shifts were reported with respect to $CDCl_3$ (δ_H 7.26, δ_C 77.0) or DMSO-d_6 (δ_H 2.49, δ_C 39.5). Mass spectra were measured on a JEOL GCmate or SX-102 mass spectrometer.

Animal Material. The marine sponge was collected at a depth of 5 m in Toyama Bay in the Japan Sea, frozen immediately, and kept frozen until processed. The sponge was identified as *Spongia* sp. (class Demospongiae, order Dictyoceratida, family Spongiidae). The sponge is an unusual *Spongia* for having a yellow rather than more typical brown or blackish color, but the color is known to be variable depending on exposure to light. The shape is compact and rounded, with one larger elevated oscula of 8 mm diameter. The surface is microconulose, and consistency is compressible. Deeper inside the sponge body the tissue is clathrous, but at the periphery it is compact. The skeleton consists of a tightly meshed system of amber-colored spongin fibers making polygonal meshes of variable diameter. Primary fibers are recognizable only at the surface, where they are lightly cored with broken spicules, and they are of the same thickness, 28−40 μm, as the secondary fibers. The species name is not known. A voucher specimen (ZMA POR. 17000) was deposited at the Zoological Museum, University of Amsterdam, The Netherlands.

Extraction and Isolation. The frozen sponge (1.7 kg, wet wt) was extracted with MeOH. The extract was concentrated under reduced pressure and extracted with EtOAc. The EtOAc layer (4.14 g) was subjected to silica gel chromatography with a stepwise gradient of $CHCl_3$−MeOH. The first fraction (1.73 g) eluted with $CHCl_3$ was purified by a combination of silica gel and ODS chromatography followed by reversed-phase HPLC to afford sesterterpene **6** (3.6 mg, 2.1×10^{-4}%, wet wt). The second fraction (488.0 mg) eluted with 5% MeOH−$CHCl_3$ was purified by ODS chromatography with CH_3CN followed by reversed-phase HPLC to furnish 12-*epi*-deoxoscalarin (**5**, 33.0 mg, 1.9×10^{-3}%, wet wt), 12-*O*-deacetylscalarafuran (**1**, 1.9 mg, 1.1×10^{-4}%), and 12-*O*-deacetyl-12-*epi*-scalarin (**2**, 8.2 mg, 4.8×10^{-4}%). The third fraction (462.0 mg) eluted with 10% MeOH−$CHCl_3$ was purified by ODS chromatography with 90% MeOH−H_2O followed by reversed-phase HPLC to afford 12-*O*-acetyl-16-*O*-methylhyrtiolide (**3**, 7.8 mg, 4.6×10^{-4}%) and 12-*epi*-scalarin (**4**, 3.9 mg, 2.3×10^{-4}%).

12-*O*-Deacetylscalarafuran (1): $[\alpha]^{25}_D$ −9.1° (*c* 0.076, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 221.5 nm (3.4); IR ($CHCl_3$) ν_{max} 3300, 2990, 1450, 1381, 1033 cm^{-1}; NMR data, see Table 1; EIMS *m*/*z* 370 $[M]^+$; HREIMS *m*/*z* 370.2872 (calcd for $C_{25}H_{38}O_2$, 370.2872).

12-*O*-Deacetyl-12-*epi*-scalarin (2): $[\alpha]^{25}_D$ −17.3° (*c* 0.20, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 203.5 nm (3.8); IR (film) ν_{max} 3400, 3010, 1750, 1684, 1629, 1603, 1010 cm^{-1}; NMR data, see Table 2; FABMS (positive, glycerol matrix) *m*/*z* 403 $[M + H]^+$; HRFABMS (positive) *m*/*z* 403.2864 (calcd for $C_{25}H_{39}O_4$, 403.2849).

12-*O*-Acetyl-16-*O*-methylhyrtiolide (3): $[\alpha]^{24}_D$ −6.5° (*c* 0.12, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 204.0 nm (3.4); IR ($CHCl_3$) ν_{max} 3500, 3000, 1609, 1496, 1439, 1250, 1038 cm^{-1}; NMR data, see Table 2; FABMS (positive, glycerol matrix) *m*/*z* 475 $[M + H]^+$; HRFABMS (positive) *m*/*z* 475.3043 (calcd for $C_{28}H_{43}O_6$, 475.3059).

Acetylation of 12-*O*-Deacetyl-12-*epi*-scalarin (2) and 12-*epi*-Scalarin (4). To a solution of 12-*O*-deacetyl-12-*epi*-scalarin (**2**, 1.00 mg) in pyridine (100 μL) were added acetic anhydride (50 μL) and DMAP (0.1 mg), and the mixture was kept at 50 °C for 12 h. After evaporation, the residue was purified by silica gel column chromatography with $CHCl_3$ to afford diacetate (**8**, 1.08 mg, 89% yield) as a colorless solid: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.81 (3H, s), 0.84 (3H, s), 0.85 (3H, s), 0.91 (3H, s), 0.94 (3H, s), 1.65 (1H, m), 1.72 (1H, m), 1.88 (1H, m), 1.97 (3H, s, CH_3CO), 2.09 (3H, s, CH_3CO), 2.20 (1H, m, H-15), 2.43 (1H, m, H-15), 2.80 (1H, m, H-18), 4.69 (1H, dd, J = 11.2, 4.4 Hz, H-12), 6.50 (1H, d, J = 5.4 Hz, H-19), 6.93 (1H, q, J = 3.4 Hz, H-16); EIMS *m*/*z* 486 $[M]^+$. To a solution of 12-*epi*-scalarin (**4**, 1.00 mg) in pyridine (100 μL) was added acetic anhydride (50 μL), and the mixture was kept at room temperature for 13 h. After evaporation, the residue was purified by silica gel chromatography with $CHCl_3$ to afford 12-*epi*-19-*O*-acetylscalarin (**8**, 0.91 mg, 83% yield) as a colorless solid. ^{1}H NMR and EIMS spectral data of **8** were identical with those of **8** from **2**.

Life-Prolonging Effects on Mice with Sarcoma-180 Cells. Six ICR/kwl mice [Kiwa Laboratory Animals Co., LTD (Wakayama, Japan), female, 7 weeks old, weighing 19−21 g] per one group were used for this test. Sarcoma-180 cells were suspended in saline solution at a concentration of 1×10^7 cells/mL. The cell suspension (0.1 mL) was intraperitoneally injected to a mouse using a syringe with a 26-gauge needle. Test compounds were dissolved in a small volume of dimethylacetamide and suspended in 10% Tween 80−saline at a concentration of 1 mg/mL. The solution was intraperitoneally injected into mice on days 1, 5, and 9 after tumor inoculation. The survival days were observed, and antitumor activity was estimated by the increase in lifespan, ILS (%), of mice calculated from the equation ILS = $(A/B - 1) \times 100$, where A is the weighed median of survival days in the treated group and B is the weighed median of survival days in the untreated control group.[17]

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Supporting Information Available: Color photograph of a marine sponge of the genus *Spongia*. This material is available free of charge via the Internet at http://pubs.acs.org.

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