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Article

Scalarane-type Sesterterpenes from the Philippines Sponge *Hyrtios* sp.

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Abstract : The marine sponge *Hyrtios* sp. collected in the Philippines was extracted and partitioned. The resulting organic layer was purified by C_{18} reversed-phase column chromatography and HPLC to achieve the separation of nine scalarane-type sesterterpenes, including one new compound with eight known scalarane analogs. The chemical structures of the isolated compounds **1**–**9** were elucidated by 1D and 2D NMR and MS data analysis. All nine compounds were evaluated for their antibacterial activities against three Gram-positive and three Gram-negative bacteria. The compound **3** exhibited potent antibacterial activities against *Bacillus subtilis* and *Micrococcus luteus*. The compounds **7** and **9** displayed considerable activities against *Bacillus subtilis* and the others had moderate results.

Key words : marine sponge, scalarane, sesterterpene, *Hyrtios* sp., antibacterial activities

1. Introduction

Marine organisms have received growing attention as potential sources for bioactive secondary metabolites exhibiting novel chemical structures. In particular, marine sponges are known to contain various classes of bioactive compounds, such as alkaloids, terpenoids, peptides, glycosides, and macrolides (Amina and Al Musayeib 2018). For example, spongistatin isolated from *Hyrtios* sp. and halichondrin isolated from *Halichondria okadai* were previously reported as microtubule-destabilizing agents (Miller et al. 2010).

Scalarane-type sesterterpenoids are compounds possessing a 6/6/6/6-tetracyclic or 6/6/6/6/5-pentacyclic fused ring system. Since scalarin, the first scalarane-type sesterterpene, was isolated from the sponge *Cacospongia scalaris* (Fattorusso et al. 1972), diverse subgroups of these skeletal structures have been discovered, including homoscalaranes (Fontana et al. 2000; Jaspars et al. 1997; Roy et al. 2002) and isoscalaranes (Davis and Capon 1993; Song et al. 2008; De Rosa et al. 1994). Moreover, further research into the synthesis (Ungur and Kulciţki 2004) or semi-synthesis (Kamel et al. 2009) of the scaffolds of scalarane derivatives has been conducted. Scalarane-type sesterterpenoids are commonly found in marine sponges belonging to the order Dictyoceratida (Liu et al. 2007; Gonzalez 2010), and are occasionally found in shell-less molluscs, such as nudibranchs (Gavagnin et al. 2004), likely due to their dietary source of sponges. For this reason, scalarane-type sesterterpenoids have been regarded as a useful chemotaxonomical marker (Jaspars et al. 1997).

Scalarane-type sesterterpenoids exhibit an extensive spectrum of bioactivities, including anticancer, antibacterial, anti-inflammatory, and anti-fouling activities. In particular, the majority of scalaranic sesterterpenoids have a broad range of cytotoxicities against many cancer cell lines, and so play the key role as eco-physiological mediators in the chemical defensive mechanism of marine invertebrates (Gonzalez 2010).

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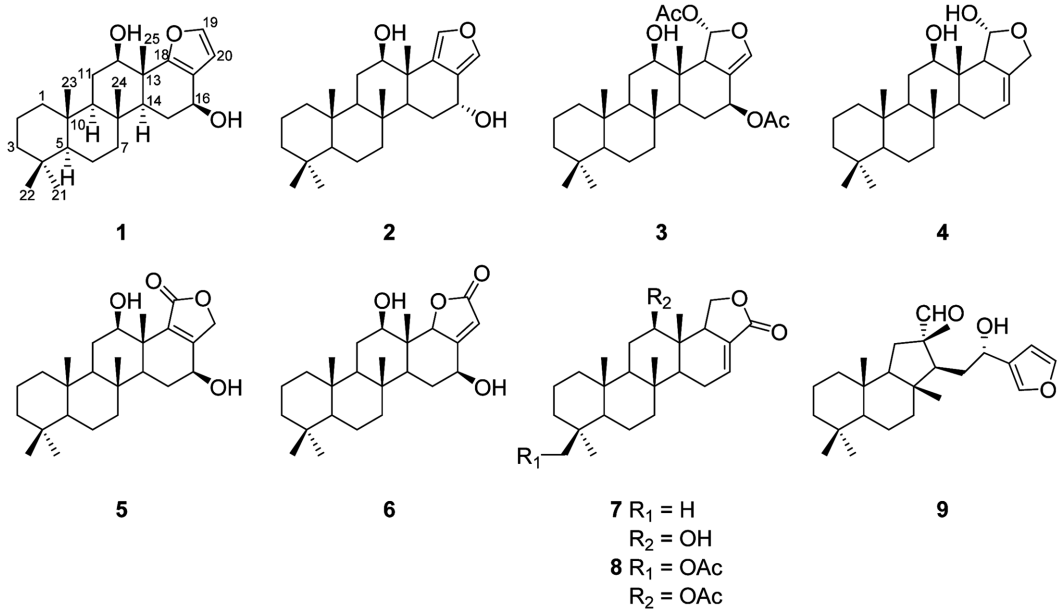

Fig. 1. Structures of compounds 1–9 isolated from *Hyrtios* sp.

Herein, we report the isolation of a new compound with eight known scalarane-type sesterterpene derivatives (Fig. 1) that generally exhibit antibacterial activities against Gram-positive bacteria.

2. Materials and Methods

General experimental procedure

HPLC was performed using the YMC-Pack C_{18} column and a Shodex RI-101 detector. The optical rotations were obtained on a JASCO digital polarimeter. ^{1}H and ^{13}C NMR spectra were recorded at 600 and 150 MHz on a Bruker spectrometer, respectively. UV spectra were measured using a Shimadzu UV-1650PC spectrophotometer. IR spectra were recorded using a JASCO FT/IR-4100 spectrophotometer. High-resolution mass spectra (HRMS) were obtained with a Shimadzu LC/MS spectrometer at the Korea Basic Science Institute.

Biological material

The marine sponge of the genus *Hyrtios* (sample number 163PIL-229) was collected by hand using Scuba at a depth of 20–30 m in April 2016, along the shore of Anda (N 9°43' E 124°31'), Bohol Island, the Philippines. The sponge was identified as *Hyrtios* sp. (order Dictyoceratida, family Thorectidae) by Dr. Young-A Kim. The specimen is stored in the Marine Biotechnology Research Center, Korea Institute of Ocean Science and Technology.

Extraction and isolation

The collected sponge *Hyrtios* sp. (1.0 kg, wet weight) was transported frozen and maintained at -20°C until required for chemical analysis. The sponge was extracted twice with MeOH (2 L), and cut into pieces prior to extraction with DCM (2 L) and MeOH : DCM = 1 : 1 (2 L). The extracts were then combined and the solvent was evaporated *in vacuo* to afford the crude extract (81.0 g), which was partitioned between n-BuOH and 3rd distilled water to remove salts and water-soluble components. The n-BuOH layer (19.4 g) was further partitioned between n-hexane and 15% aqueous MeOH to give an aqueous MeOH layer (8.9 g). The 15% aqueous MeOH layer was separated into eight fractions by C_{18} reversed-phase flash column chromatography using a step gradient of MeOH/H_2O (4:6, 5:5, 6:4, 7:3, 85:15, 1:0 v/v), then acetone and EtOAc. The 100% MeOH fraction was subjected to C_{18} reversed-phase flash column chromatography once again to give fractions mp 1–24. Fraction mp 9 (75.0 mg) was purified by reversed-phase HPLC (YMC-Pack C_{18}, 250 × 4.6 mm, 55% MeCN in H_2O) to yield compounds **1** (4.7 mg), **2** (3.9 mg), and **5** (2.6 mg). Fraction mp 8 was purified using the same column (65% MeCN in H_2O) to give compounds **1** (6.4 mg), **2** (7.9 mg), and **8** (1.8 mg). The combined fraction of mp 5–7 due to their similar ^{1}H NMR spectra was purified under the same conditions employed for mp 8 to afford compounds **6** (10.0 mg) and **8** (1.8 mg). Compound **7** (26.3 mg) was obtained from mp

11 using semi-preparative reversed-phase HPLC (YMC-Pack C_{18}, 250 × 10 mm, 65% MeCN in H_2O). Compounds **3** (8.0 mg) and **9** (5.5 mg) were purified from mp 14 by analytical reversed-phase HPLC (YMC-Pack C_{18}, 250 × 4.6 mm, 62% MeCN in H_2O). The combined fraction of mp 16−17 was purified by analytical reversed-phase HPLC (YMC-Pack C_{18}, 250 × 4.6 mm, 65% MeCN in H_2O) to give compound **4 (**7.0 mg).

Compound **1** : White amorphous solid; $[\alpha]_D^{25}$ +10 (c 0.1, MeOH); IR ν_{max} 3407, 2929, 1742, 1654, 1554, 1456, 1389, 1318, 1251, 1042 cm^{-1}; UV(MeOH) λ_{max} (log ε) 214 (3.68) nm; HRESIMS *m/z* 409.2716 $[M + Na]^+$ (calcd for 409.2713, $C_{25}H_{38}O_3Na^+$); ^1H NMR (CD_3OD and C_5D_5N, 600 MHz) and ^{13}C NMR (CD_3OD and C_5D_5N, 150 MHz) see Table 1.

Minimum Inhibitory Concentration (MIC) assay

The MIC assay is a useful method to evaluate the lowest concentration of antibacterial sources, as it is based on an antibacterial susceptibility test for bioactive natural products that cause inhibited bacterial growth or death.

The assay was carried out against six bacteria. The three Gram-positive bacteria, namely *Bacillus subtilis* (KCTC-1021), *Micrococcus luteus* (KCTC-1915), and *Staphylococcus aureus* (KCTC-1927), and three Gram-negative bacteria, namely *Escherichia coli* (KCTC-2441), *Salmonella typhimurium* (KCTC-2515), and *Klebsiella pneumonia* subsp. (KCTC-2690) were purchased from the Korean Collection for Type Cultures (KCTC). All six bacteria were streaked onto Mueller-Hinton agar (MHA) plates and cultured in a standing incubator for 24 h at 37°C. A separated single colony was transferred from the MHA plates to Muller-Hinton broth, and incubated for 24 h at 37°C and 170 rpm in a shaking incubator. In brief, the six bacteria were inoculated into 96-well plates and then treated with compounds **1–9** diluted in DMSO (100 μL) in accordance with concentrations ranging from 128 to 0.25 μg/mL (i.e., 128, 64, 32, 16, 8, 4, 2, 1, 0.5, and 0.25 μg/mL). Subsequently, these mixtures were cultivated in a standing incubator for 24 h at 37°C.

3. Results and Discussion

Compound **1** has a molecular formula of $C_{25}H_{38}O_3$, as deduced from its high-resolution electrospray ionization mass spectrometry (HRESIMS) data. The ^1H NMR spectrum of **1** displayed resonance for two olefinic protons, seven methylene protons, five methine protons, and five methyl groups (Table 1). The planar structure of **1** was determined by combined investigation of the COSY and HMBC correlations (Fig. 2). The COSY correlations of H_2-1/H_2-2/H_2-3, H_2-6/H_2-7, H-9/H_2-11/H-12, and H-14/H_2-15/H_2-16 and the HMBC correlations from H_3-21 and H_3-22 to C-3, C-4 and C-5, from H_3-23 to C-1, C-5, C-9 and C-10, from H_3-24 to C-7, C-8, C-9 and C-14, and from H_3-25 to C-12, C-13, C-14 and C-18 confirmed the frame of a 6/6/6/6-membered ring system. The presence of a five-membered ring containing one oxygen atom was also elucidated by the HMBC correlations from H-19 to C-17, C-18 and C-20, from H-20 to C-17, C-18 and C-19, and from H-16 to C-17, C-18 and C-20. Furthermore, the HMBC correlations from H_3-21 and H_3-22 to C-3, C-4 and C-5 suggested the presence of a gem-dimethyl group on quaternary carbon C-4, thereby defining a 6/6/6/6/5-pentacyclic carbon skeleton. The structure of compound **1** shared the same carbon and heteroatom framework with 12-*O*-desacetylfuroscalarol, with the exception of differences in the stereochemistry at H-12. The unsaturated degree of compound **1** also indicated the presence of a pentacyclic ring system with a furan ring attached at C-17 and C-18.

Compound **1** has not been reported, but its 12-epimer has been already isolated from *Hyrtios* sp. and *Hyrtios* cf. *erectus* (Cimino et al. 1978; Doi et al. 1993). The previously reported ^1H and ^{13}C NMR chemical shifts of the C-12 position in C_5D_5N (δ_C 71.4 and δ_H 4.62) were somewhat different from those of compound **1** (δ_C 77.2 and δ_H 3.90) in C_5D_5N. The stereochemistry of H-12 was therefore further investigated by the analysis of NOESY correlations (Fig. 3) and through comparison of the proton-proton coupling constants.

More specifically, the NOESY correlations of H-2/H-11/H-22/H-23/H-25 and H-6/H-24/H-25 suggested that H-22, H-23, H-24, and H-25 are on the *β*-face, while the strong NOESY correlations of H-9/H-12/H-14 suggested that H-9 and H-14 are on the *α*-face and H-12 is an axial proton. In addition, the stereochemistry of H-12 (δ_H 3.90) was confirmed as the down orientation by study of the coupling constant, which showed *J* value of 11.04 Hz due to the trans-diaxial coupling between the axial proton on H-12 (δ_H 3.90) and the axial proton on H-11 (δ_H 1.77); such a coupling was not observed in the previously reported 12-epimer (Lee et al. 2014). Furthermore, the strong NOESY signals of H-14/H-15/H-16 indicated that H-14, H-15, and H-16 present the same orientation as H-12. A strong NOESY correlation was also observed between H-12 and pseudoaxial proton H-16 due to the D ring being a cyclohexene ring.

Table 1. ^{1}H (600 MHz) NMR and ^{13}C (150 MHz) NMR data for 1 (δ in ppm, J in Hz)

Position	^{13}C NMR[a]	^{1}H NMR[a]	^{13}C NMR[b]	^{1}H NMR[b]
1	41.0	0.87, m	40.4	0.56, brd (12.5)
		1.73, brd (13.7)		1.58, m
2	19.7	1.69, dd (10.3, 2.3)	19.3	1.33, m
		1.47, m		1.53, m
3	43.3	1.18, ddd (13.5, 13.5, 4.3)	42.7	1.09, m
		1.38, brdd (12.9)		1.32, m
4	34.2		33.8	
5	58.0	0.88, m	57.1	0.65 m
6	19.3	1.61, m	18.9	1.29, m
		1.47, m		1.46, m
7	42.6	1.03, m	42.1	1.07, m
		1.81, m		1.82, m
8	38.7		38.0	
9	60.3	1.02, m	59.5	0.82, m
10	38.0		37.6	
11	27.4	1.81, m	27.7	1.77, m
		1.58, m		1.96, m
12	78.0	3.71, dd (11.4, 4.2)	77.2	3.90, dd (11.0, 3.2)
13	44.1		44.2	
14	50.6	1.69, m	50.2	2.03, m
15	29.0	1.88, dd (14.5, 3.9)	29.4	2.15, d (13.3)
				1.90, m
16	63.4	4.62, m	62.8	5.05, brs
17	118.5		119.7	
18	162.1		162.1	
19	142.2	7.30, d (1.9)	141.6	7.48, brd (0.8)
20	110.5	6.32, d (1.9)	111.0	6.63. brs
21	21.7	0.85, s	21.9	0.80, s
22	33.8	0.87, s	33.8	0.85, s
23	16.7	0.91, s	16.7	0.81, s
24	18.2	0.95, s	18.4	0.94, s
25	15.9	1.14, s	16.6	1.45, s

[a] ^{1}H and ^{13}C NMR spectra were measured in CD_3OD
[b] ^{1}H and ^{13}C NMR spectra were measured in C_5D_5N

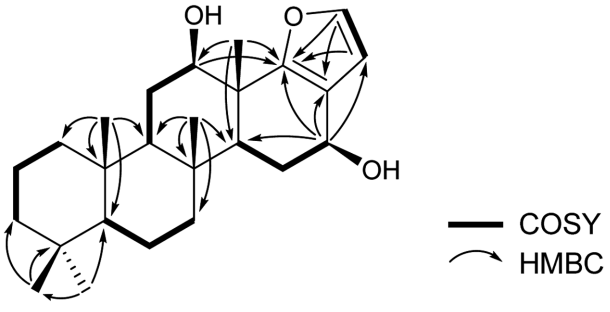

Fig. 2. Key COSY and HMBC correlations of 1

The other purified compounds, namely compounds **2**–**9**, were previously reported to be sesterstatin 4 (**2**) (Pettit et al. 1998), heteronemin (**3**) (Kashman and Rudi 1977), 12-deacetyl-12-*epi*-deoxoscalarin (**4**) (Fontana et al. 1999), 16-hydroxyscalarolide (**5**) (Miyaoka et al. 2000), 16-*O*-deacetyl-16-*epi*-scalarolbutenolide (**6**) (Ryu et al. 1996), 12-*O*-deacetyl-19-deoxyscalarin (**7**) (Pettit et al. 1998), hyrtiosin A (**8**) (Yu et al. 2005), and hyrtiosal (**9**) (Iguchi et al. 1991) (Fig. 1). These known compounds were confirmed through a comparison of the obtained ^{1}H NMR

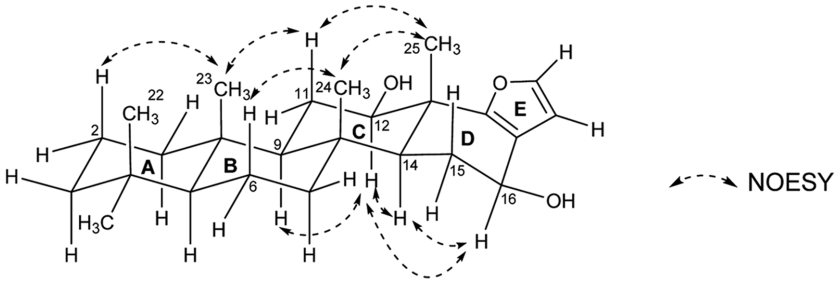

Fig. 3. Key NOESY correlations of 1

Table 2. MIC assay against Gram-positive bacteria

Strains		MIC values (μg/mL)								
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Gram-positive	*Bacillus subtilis*	32	32	2	-	32	-	16	128	16
	Micrococcus luteus	-	-	4	-	-	-	-	-	64
	Staphylococcus aureus	128	-	64	-	-	-	-	-	128

MIC values are the minimal inhibitory concentration inhibiting cell growth or inducing cell death

and ^{13}C NMR data with those reported in the literatures.

The minimum inhibitory concentration (MIC) assay method was then employed to test the antibacterial activities of nine compounds against three Gram-positive and three Gram-negative bacteria. The majority of compounds were highly active against *Bacillus subtilis* compared with the other bacteria examined. More specifically, compound **3** displayed the strongest activity against *Bacillus subtilis* and *Micrococcus luteus* with MIC values of 2 and 4 μg/mL, respectively. In addition, compounds **7** and **9** exhibited MIC values of 16 μg/mL against *Bacillus subtilis*. None of the nine compounds exhibited activity against the three Gram-negative bacteria.

4. Conclusions

Chemical analysis of the 1D and 2D NMR spectra recorded for nine scalarane-type sesterterpene derivatives isolated from the Philippines marine sponge *Hyrtios* sp. led to the determination of their chemical structures. The carbon framework was found to belong to the 6/6/6/6/5-pentacyclic ring system-scalarane-type sesterterpenoids class. In addition, compound **1** was identified as a novel 12-epimer exhibiting an *α*-axial orientation at H-12 position. Known compounds were also isolated, and these were confirmed as sesterstatin 4 (**2**), heteronemin (**3**), 12-deacetyl-12-*epi*-deoxoscalarin (**4**), 16-hydroxyscalarolide (**5**), 16-*O*-deacetyl-16-*epi*-scalarolbutenolide (**6**), 12-*O*-deacetyl-19-deoxyscalarin (**7**), hyrtiosin A (**8**), and hyrtiosal (**9**). The compounds **1**–**9** were then investigated for their antibacterial activities against three Gram-positive bacteria (*Bacillus subtilis*, *Micrococcus luteus*, and *Staphylococcus aureus*) and three Gram-negative bacteria (*Escherichia coli*, *Salmonella typhimurium*, and *Klebsiella pneumonia* subsp.) using the minimum inhibitory concentration assay. It was found that compound **3** showed potent activities against *Bacillus subtilis* and *Micrococcus luteus*, and the compounds **7** and **9** had moderate to good bioactivities against *Bacillus subtilis*. The other scalarane analogs displayed weak activities against the three Gram-positive bacteria, and none of the compounds exhibited activity against the three Gram-negative bacteria.

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