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A new sesterterpenoid showing anti-inflammatory effect from the Marine Sponge *Haliclona* species

Kyung Lee and Jung-Rae Rho*

Department of Marine Biotechnology, Kunsan National University, Kunsan 573-701, Korea

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Abstract Four spiroketal sestertepenoids (1 ~ 4) were isolated from the marine sponge Haliclona species. Their planar structures were completely determined from a combination of extensive 1D and 2D NMR experiments, and also the relative stereochemistry on the chiral centers were established by the ROESY experiment. Compounds 1 ~ 3 were determined as the same planar structures with different stereochemistry on the chiral centers C-11 and C-13. Of these, 1 was identified as a new stereoisomer. Four compounds showed the inhibition effect of nitric oxide (NO) production in LPS-stimulated RAW 264.7 cells (0.7 ~ 2.0 g/ml).

Keywords NMR, sestertepenoid, sponge *Haliclona sp*., NO inhibition

Introduction

Sesterterpenoids have largely been found in marine sponges[1-3] and displayed a wide range of biological activities such as antifungal effect,[4] cytotoxicity,[5] and induction of osteoblast differentiation.[6]

During the course of our search for bioactive compounds from Korean marine sponges, we recently isolated four sesterterpenoids from the marine sponge Genus *Haliclona* collected at Gageo Island, Korea. Followed by an activity-guided fractionation, the extract was separated to afford four sesterterpenoids, which showed the inhibition effect of nitric oxide (NO) production in LPS-stimulated RAW 264.7 cells. On the basis of 1D and 2D NMR analyses, three of them were determined as the same planar structures, but the stereochemistry on the chiral centers C-11 and C-13 was different. Two stereoisomers were recognized as gombaspiroketals A and B reported recently.[7] Compound **1** was identified as a new stereoisomer of gombaspiroketal sesterterpenoids. All compounds exhibited strong inhibition effect of NO production in LPS-stimulated RAW 264.7 cells. In this paper we describe the isolation and the structure determination of the compounds **1** ~ **4**.

Experimental Method

Animal Sample- The specimen of *Haliclona* sp. (Sample No. 10G-19) was collected by hand using SCUBA 25 m depth in June 2010 at Gageo Island, Korea. Texture is very soft and fragile and color is soft violet in life, gradually changed to ivory color in alcohol. Oscules are scattered on surface. This specimen was identified by Prof. Chung Ja Sim.

* Address correspondence to: **Jung-Rae Rho**, Department of Marine Biotechnology, Kunsan National University, K unsan 573-701, Korea; E-mail: jrrho@kunsan.ac.kr

Extraction and Isolation- The marine sponge was twice extracted with MeOH for 2 days at room temperature. The methanolic extract was partitioned between methylene chloride (CH_2Cl_2) and H_2O solvents, and then the organic layer repartitioned between *n*-hexane and 15% aqueous MeOH for defatting. The MeOH fraction (3.6 g) was subjected on the vacuum column chromatography eluting with seven different solvent mixtures of MeOH and water. Of these, the 10% aqueous MeOH fraction had well splitting NMR signals in the ^{1}H NMR spectrum and the effect of NO inhibition on RAW264.7 cells. This fraction (330 mg) was subjected on a Sephadex LH20 to yield seven fractions (M1 ~ M7). An activity-guided fraction, M3, was further separated by normal phased HPLC (YMC SIL column, 250mm × 10mm, Varian RI detector) using a solvent system (EtOAc / Hexane = 40 / 60) to give a fraction in the retention time of around 20 min and compounds **1** ~ **3** in the retention time of 30 ~ 40 min. More polar fraction in the earlier time was purified by reversed phased HPLC (YMC Pro C18, 250mm × 10mm, Varian RI detector) with a solvent system (Acetonitrile / water = 65 / 35) to yield compound **4**.

NMR experiment- The 1D and 2D NMR spectra were obtained on a Varian VNMRS system working at 500MHz for proton and 125MHz for carbon. The ^{1}H and ^{13}C NMR chemical shifts refer to CD_3OD at 3.30 and 49.0ppm, respectively. For all experiments, the temperature was stabilized at 297 K. The parameters used for 2D NMR spectra were as follows; gCOSY spectrum were collected with a spectral width 4000 Hz in a 512 (t1) × 1024 (t2) matrix applying the pulse gradient of 1 *ms* duration with a strength of 10 G/m and processed with a sinebell function. The gHSQC and gHMBC spectra were measured with J_{CH} = 140 Hz and $^nJ_{CH}$ = 8 Hz, respectively, and processed in a

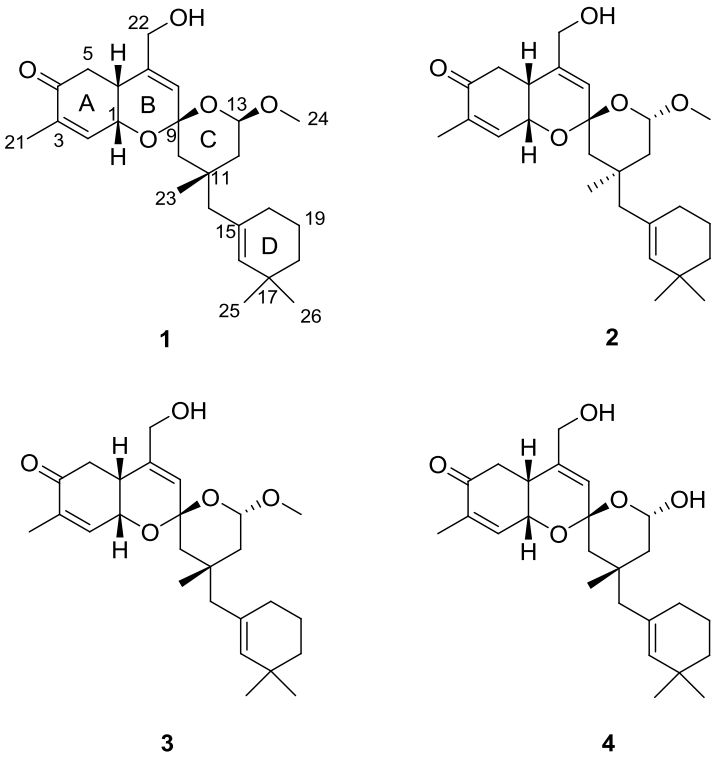

Figure 1. Four compounds **1** ~ **4** isolated from the marine sponge *Haliclona* sp.

256 (t1) × 1024 (t2) matrix by a linear prediction method for a higher resolution. The ROESY spectrum was measured with a spin- locking time of 350 *ms* and a scan number of 32.

Results and Discussion

The 10% aqueous MeOH fraction of the extract was separated by HPLC to afford four sesterterpenoids (compounds **1** ~ **4**): three stereoisomers and one hydroxyl derivative. Compounds **1** ~ **3** possess the identical planar structure with the different stereochemistry on C-11 and C-13, and **4** has a very similar structure as **3**, except for the conversion of methoxy to hydroxyl group.

Compound **1** was isolated as a yellowish oil and its molecular formula was determined as $C_{26}H_{38}O_5$ from HRESIMS analysis, consistent with eight degrees of unsaturation. Its optical rotation was measured as $[\alpha]^{25}$ = -18.3 (*c* = 0.13, MeOH) and the IR spectrum showed strong absorption bands at 3445 cm^{-1}(hydroxyl) and 1682 cm^{-1}(carbonyl group). The ^{13}C NMR spectrum indicated the presence of an α, β-unsaturated ketone group from the carbon signal at δ_C 200.9 and the absorption band at 228 nm in the IR spectrum. Additional downfield shifted resonances (δ_C 143.2, 142.1, 139.1, 138.1, 133.8, and 126.9) and two carbon resonances at δ_C 101.0 and 98.2 in the ^{13}C NMR spectrum suggested the presence of three double bonds and ketal groups. Together with this information and the molecular formula, compound **1** was deduced to possess four cyclic rings. Furthermore, the edited HSQC spectrum revealed that **1** was composed of five methyls, eight methylenes, six methines and seven quaternary carbons.

In the COSY spectrum, the olefinic H-2 (δ_H 6.72) was sequentially coupled with an oxymethine H-1 (δ_H 4.54), H-1 with H-6 (δ_H 2.55), and H-6 with two H-5' s. The downfield shifted H-13 (δ_H 4.83) strongly coupled with two methylene protons at H-12 (δ_H 1.70 and 1.78). A linear connection of three methylenes from H-18 to H-20 was also revealed by COSY correlations. Above three substructures established by the COSY experiment were then connected by the HMBC experiment. First, the vinyl methyl protons at δ_H 1.81 showed HMBC cross peaks with the trisubstituted double bonded C-2 and C-3, and the ketone carbon (δ_C 200.9), of which the latter was correlated by H-5 to construct ring A unit. Moreover, the oxymethylene protons at δ_H 4.06 correlated with C-6 in ring A and another trisubstituted double bonded C-8 and C-9 in the HMBC spectrum. Second, the methyl singlet at δ_H 1.12 correlated with the three neighboring methylene carbons and one quaternary carbon. Among them, the methylene H-14 on the carbon at δ_C 53.2 showed HMBC cross peaks with two carbons in a new trisubstituted double bond. In succession, the methine carbon in the double bond was also correlated by two singlet methyl protons in the HMBC spectrum. Additional HMBC correlations (H-14/C20, H-25/C-16, H-25/C-17, C-25/C-18, H-25/C-16,

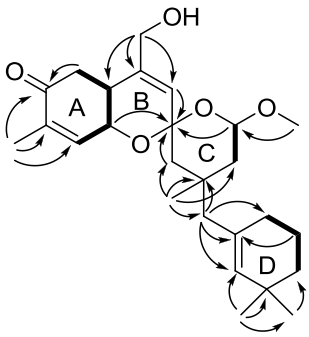

Figure 2. Key correlations of COSY (bold line) and HMBC (arrows) experiments for compound **1**

H-26/C-17, and H-26/C-18) led to build ring D substructure (Figure 2). Finally, a two ring system corresponding to the remaining degree of unsaturation was recognized as a spiro skeleton by extensive HMBC correlations of a ketal quaternary carbon δ_C 98.2 with four different protons: one olefinic proton (H-9), two methylene proton (H-12), and two oxymethine protons (H -10, and H-13). The

unassigned methoxy group was linked to C-13 by HMBC cross peak between C-13 and the methoxy singlet at δ_H 3.46, determining the planar structure of compound **1** which was identical to that of Gombaspiroketals A and B reported recently. Compound **1** was one of tetracyclic sesterterpenes which also had a resemblance to phorbaketals and alotaketals from the sponges *Phorbas* sp.[8], *Monanchora* sp.[9], and *Hamigera* sp.[3]

Interestingly, the planar structures of compounds **2** and **3** were also identical to that of **1** on the interpretation of their NMR spectra. Although the planar structures of the three compounds are the same, their ^{1}H NMR spectra were different as shown in Figure 3, which suggested the different stereochemistry of some chiral centers in the three compounds. Three isolated compounds **1** ~ **3** possess five chiral carbon centers: C-1, C-5, C-9, C-11, and C-13.

Their relative configurations for each compound were determined by ROESY experiments. In all three compounds, the strong ROE cross peak of H-1/H-6 indicated a *cis* configuration in the A/B ring junction.

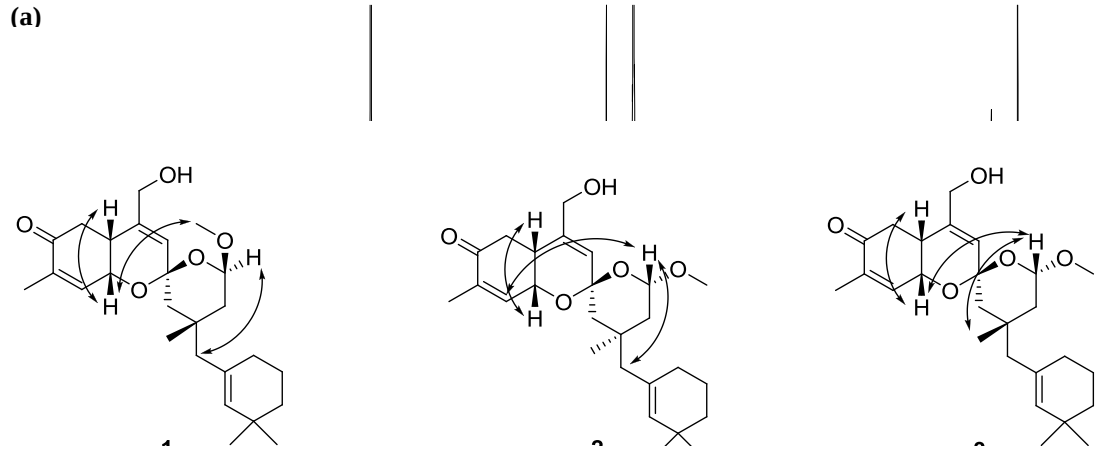

Table 1. NMR spectral data for compounds **1** ~ **4** in CD_3OD at 500MHz NMR.

	1		**2**	**3**	**4**
	δ_H(*J* in Hz)	δ_C	δ_C	δ_C	δ_C
1	4.54, dd(5.8, 3.3)	64.5, CH	69.5	64.3	64.1
2	6.72, dd(5.8, 1.5)	142.1, CH	145.5	141.7	141.8
3		139.1, C	137.4	139.4	139.3
4		200.9, C	200.1	200.7	200.8
5	a 2.37, dd (16.5, 13.9); b 2.53, dd(16.5, 4.4)	38.9, CH_2	39.7	38.8	38.8
6	2.55, m	34.4, CH	35.8	34.4	34.4
7		143.2, C	140.8	143.7	143.5
8	5.59, dd(2.3, 1.5)	126.9, CH	127.2	126.2	126.4
9	1.57, m	98.2, C	99.9	99.3	99.5
10	a 1.56, d(14.2); b 1.72, d(14.2)	46.8, CH_2	45.1	46.0	45.8
11		32.4, C	34.7	35.2	35.4
12	a 1.70, d(13.9, 5.1); b 1.78, d(13.9, 5.4)	40.2, CH_2	42.3	43.3	45.1
13	4.83, dd(5.4, 5.1)	101.0, CH	98.2	98.2	90.4
14	1.89, d(13.5); 1.93, d(13.5)	53.2, CH_2	47.1	55.4	55.5
15		133.8, C	134.4	133.0	133.1
16	5.14, s	138.1, CH	138.0	138.2	138.2
17		33.0, C	32.9	32.9	32.9
18	1.39, m	38.2, CH_2	38.2	39.2	38.2
19	1.60, m	21.4, CH_2	21.4	21.3	21.4
20	1.94, m	33.0, CH_2	33.1	33.0	33.0
21	1.81, s	15.9, CH_3	15.6	15.9	15.9
22	4.06, d(1.5)	63.7, CH_2	63.6	63.7	63.7
23	1.12, s	29.0, CH_3	31.2	25.9	25.7
24	3.46, s	56.1, CH_3	56.6	56.6	-
25	0.96, s	30.7, CH_3	30.7	30.6	30.7
26	0.96, s	30.8, CH_3	30.8	30.7	30.9

Figure 3. ^{1}H NMR spectra for (a) compound 1, (b) compound 2, (c) compound 3, and (d) compound 4.

The configuration in the C/D spiro junction could be established by the NOE correlations between the olefinic H-10 of ring B and H-12 of ring C, and also between H-12 and H-5a. Unlike these common features, the configurations of C-11 and C-13 in the three compounds differed (Figure 4). Compound **1** showed the strong ROE correlations of H-1/H-24, and H13/H-14, allowing us to assign the orientations of C-11 and C-13 in ring C as *S** and *R**, respectively. On the other hand, the ROESY cross peaks of H-2/H-13 and H-13/H-14 in compound **2** placed the all protons to the same direction, resulting in orienting the methoxy and methyl groups to the opposite direction with those of **1**. In a similar way, the configurations of C-11 and C-13 in compound **3** were assigned as *S** and *S**, respectively, by the ROESY correlations of H-1/H-13 and H-13/H-23. Accordingly, while compounds **2** and **3** were revealed as gombaspiroketal B and A, together with their highly agreements of the proton and carbon chemical shifts, compound **1** was identified as a new stereoisomer of gombaspiroketal compounds.

Compound **4** has molecular formula $C_{25}H_{36}O_5$, short of CH_2. The ^1H spectrum of **4** was very closely similar to that of **3**, except for the loss of methoxy signal and the presence of a proton signal at δ_H 5.24. This could be explained by the conversion of methoxy to hydroxyl group.

Table 2. LC_{50} values of compound s **1** ~ **4** for the inhibition of NO production

compounds	IC $_{50}$ of NO inhibition (μg / ml)
1	2.00
2	0.71
3	0.84
4	2.00

In this study, we isolated four similar sesterterpenoids a sponge *Haliclona* species and their structures were completely determined by NMR experiments. Four compounds exhibited the inhibitory effect of NO production in LPS-stimulated RAW264.7 cells. Table 2 listed their LC_{50} values.

Acknowledgements

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