

Secomycalolide A: A New Proteasome Inhibitor Isolated from a Marine Sponge of the Genus *Mycale*

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Abstract: A new oxazole-containing proteasome inhibitor, secomycalolide A, together with known mycalolide A and 30-hydroxymycalolide A, was isolated from a marine sponge of the genus *Mycale*. They showed proteasome inhibitory activities with IC₅₀ values of 11-45 µg/mL.

Keywords: proteasome inhibitor, secomycalolide A, mycalolide A, marine sponge, *Mycale*.

Introduction

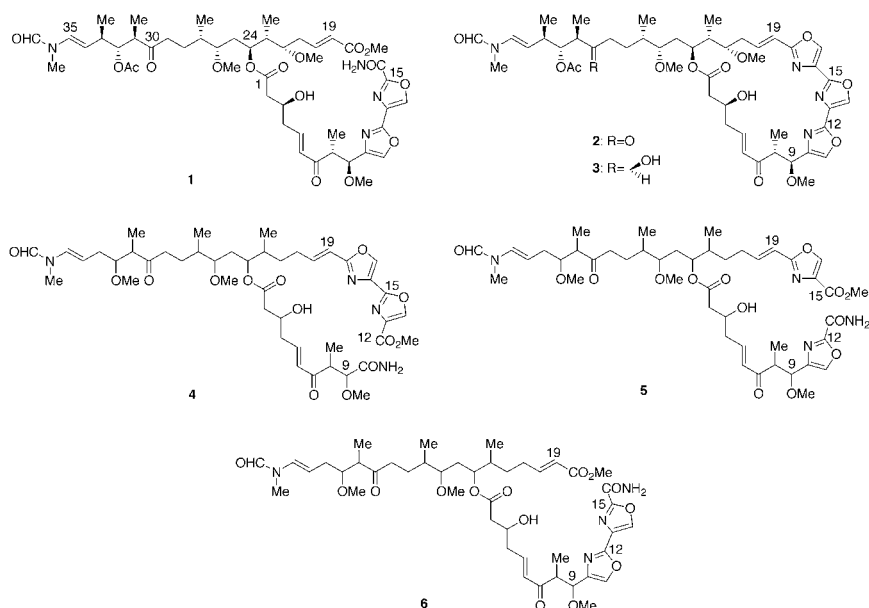
Mycalolides have been isolated from marine sponges of the genus of *Mycale* [1-3] and the hard coral *Tubastrea faulkneri* [4], and their structures are elucidated to be macrocyclic lactones characterized with an unusual tris-oxazole moiety and *N*-methylformyl terminus. So far, kabiramides [5], ulapualides [6], halichondramides [7], and jaspamides [8] are known as structurally related metabolites. Mycalolides A-C exhibited significant cytotoxicity against B-16 melanoma cells [1] and potent actin depolymerizing activity [9]. The ubiquitin-proteasome proteolytic pathway plays a major role in selective protein degradation and regulates various cellular events including cell growth and apoptosis [10-13]. Ubiquitin, composed of 76 amino acids, attaches to a target protein prior to

degradation. The polyubiquitin chains are recognized by the 26S proteasome, an intracellular high-molecular mass protease subunit complex, and the protein portion is degraded by the proteolytic active sites in a cavity in the 26S proteasome [10, 11]. Several proteasome inhibitors show anti-tumor activity against various tumor cells that are resistant to conventional chemotherapeutic agents. Proteasome inhibitors have been reported to inhibit the degradation of I κ -B followed by suppression of NF- κ B transcriptional activity to induce apoptosis [14]. To date, synthetic peptides, such as MG132 [15] and PS-341 (bortezomib, Velcade[®]) [16], and natural products, including lactacystin [17–19], epoxomicin [20, 21], and salinosporamide A [22], have been reported to inhibit proteasome activity. Among them, PS-341 has been recently approved by FDA for multiple myeloma treatment [23]. Interestingly, different classes of proteasome inhibitors can differentially affect the degradation of various proteasome substrates, resulting in diverse cellular responses [14]. Proteasome inhibitors are now under intensive investigation not only for anti-cancer drugs but also for drugs to treat inflammatory and immune deficiency diseases [24]. In the course of our search for inhibitors against the ubiquitin-proteasome pathway, we succeeded in isolating new agosterol derivatives [25] and a pyrone derivative named himeic acid A [26] as proteasome inhibitors and a ubiquitin-activating enzyme (E1) inhibitor, respectively. In addition, we found that girolline, an anti-cancer compound, is the first agent inhibiting the recruitment of polyubiquitinated p53 to the proteasome [27]. In this paper, we describe the isolation, structure elucidation, and proteasome inhibitory activity of three mycalolides.

Results and Discussion

The MeOH extract of a sponge collected from shallow waters off Sugashima Island 130 km southeast of Osaka was subjected to solvent partitioning between EtOAc and water. The active EtOAc fraction was further partitioned between *n*-hexane and 90% MeOH/H₂O, and the latter fraction was purified by ODS chromatography and ODS HPLC to afford a new mycalolide derivative, secomycalolide A (**1**) together with known mycalolide A (**2**) [1] and 30-hydroxymycalolide A (**3**) [2].

The ¹H NMR spectrum of **1** in CDCl₃ (Table 1) showed two low-field heteroaromatic protons (δ 7.77 and 8.50), a pair of formamides for the major and minor rotational isomers (δ 8.30 and 8.08, intensity 5:3), three *E*-olefines (δ 5.98/6.84, 6.25/7.00, and 6.50/4.97), six oxygene-bearing protons δ 2.99, 3.58, 4.22, 4.34, 5.16, and 5.22), four *O*-methyls (δ 3.19, 3.30, 3.45, and 4.05), a pair of *N*-methyls (δ 3.03 and 3.08), an acetoxy methyl (δ 2.01), and five doublet methyls (δ 0.84, 0.93, 0.94, 1.05, and 1.07). These data readily suggested that **1** was a congener of mycalolide A (**2**). Comparison of the ¹H NMR spectrum of **1** with that of **2** showed the absence of an oxazole proton in the region of δ 7.5–8.6 instead of the presence of an additional *O*-methyl at δ 4.05. These data indicated that **1** was a seco-oxazole derivative of **2**, which was supported by the molecular formula of **1**, C₄₇H₆₈N₄O₁₆ established by HRFABMS, a H₄O₂ unit more than **2**. So far three seco-oxazole halichondramides were isolated and are known as halishigamides C (**4**) and D (**5**) [28] and halichondramide ester (**6**) [7]. By comparison of their ¹H NMR data, the signals for the olefin group at δ 5.98/6.84 (H-19/H-20) in **1** were matched with those in **6** (δ 5.89/6.85). On the contrary, the olefin protons in **4** and **5** were



observed at δ 6.37/6.84 (H-19/H-20) and δ 6.31/6.76 (H-19/H-20), respectively. Since H-19 in **1** and **6** corresponded to the α -position in an α,β -unsaturated methyl ester system, they revealed the up-field shift compared to those of **4** and **5**, in which H-19 was bonded to an oxazole ring. Thus, the structure of **1** was defined to be a seco-oxazole derivative of mycalolide A as shown [29].

The proteasome displays multicatalytic activities, e.g. chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolyzing activities. Inhibitory activities of three mycalolides were tested using a chymotrypsin-like substrate, and the IC_{50} values of **1**, **2**, and **3** were found to be 11, 30, and 45 $\mu\text{g/mL}$, respectively.

Table 1. ^1H NMR Data of Secomycalolide A (**1**) in CDCl_3

position	δ_{H}	position	δ_{H}
2	2.60 m, 2.70 m	24	5.22 m
3	4.22 m	25	1.55m, 1.60 m
4	2.50 m, 2.50 m	26	2.99 m
5	7.00 dt (16.8, 6.6)	26-OMe	3.30 s
6	6.25 d (16.8)	27	1.74 m
8	3.50 m	27-Me	0.84 d (6.6)
8-Me	0.93 d (6.6)	28	1.25 m, 1.74 m
9	4.34 d (9.0)	29	2.47 m, 2.47 m
9-OMe	3.19 s	31	2.77 m
11	7.77 s	31-Me	1.07 d (6.6)
14	8.50 s	32	5.16 dd (9.7, 2.9)
18-OMe	4.05 s	32-OAc	2.01 s
19	5.98 d (16.2)	33	2.50 m

20	6.84 ddd (16.2, 7.5, 6.0)	33-Me	1.05 d (6.6)
21	2.50 m, 2.75 m	34	4.97 dd (14.1, 9.4) [5.00 dd (14.1, 9.4)] ^a
22	3.58 m	35	6.50 d (14.1) [7.16 d (14.1)] ^a
22-OMe	3.45 s	35-NMe	3.03 s [3.08 s] ^a
23	1.92 m	35-NCHO	8.30 s [8.08 s] ^a
23-Me	0.94 d (6.6)		

^a Chemical shifts for the minor conformer are bracketed.

Conclusion

We have isolated a new mycalolide derivative secomycalolide A (**1**) together with known mycalolide A (**2**) and 30-hydroxymycalolide A (**3**). They showed moderate inhibitory activities against chymotrypsin-like activity of the proteasome. Among three mycalolides, seco-oxyazole derivative **1** showed the most potent inhibitory activity. So far, cytotoxicity [1] and potent actin depolymerizing activity [9] of mycalolides have been reported; however, this is the first report of proteasome inhibitory activity of mycalolides.

Experimental

General

NMR spectra were recorded on a JEOL GSX500 in CDCl₃. All chemical shifts were reported with respect to the residual solvent peaks (δ_{H} 7.26). Mass spectra were measured on a JEOL SX-102 mass spectrometer. The fluorescence intensity (excitation, 360 nm; emission, 460 nm) was measured using a BIO-RAD VersaFluor Fluorometer.

Extraction and Isolation

A marine sponge of the genus *Mycale* was collected from shallow waters off Sugashima Island 130 km southeast of Osaka. The frozen material (4.2 kg, wet wt) was extracted with MeOH (3 L $\times$ 3). The extract was concentrated under reduced pressure and extracted with EtOAc. The EtOAc layer was partitioned between *n*-hexane and 90% MeOH/H₂O. The aqueous MeOH fraction (2.1 g) was subjected to ODS chromatography with MeOH/H₂O. The fraction (450 mg) eluted with 80% MeOH/H₂O was purified by gel filtration on Sephadex LH-20 with CHCl₃/MeOH (1:1) followed by ODS HPLC with 70% MeOH/H₂O to afford two active fractions. The first fraction (7.8 mg) was purified by ODS HPLC with 70% CH₃CN/H₂O to afford mycalolide A (**2**, 1.51 mg, $3.6 \times 10^{-5}\%$, wet weight) and 30-hydroxymycalolide A (**3**, 0.54 mg, $1.3 \times 10^{-5}\%$). The second fraction (6.9 mg) was purified by ODS HPLC with 50% CH₃CN/H₂O to afford secomycalolide A (**1**, 1.45 mg, $3.5 \times 10^{-5}\%$).

Secomycalolide A (1): ^1H NMR (CDCl_3), see Table 1; FABMS m/z 967 $[\text{M} + \text{Na}]^+$; HRFABMS m/z 967.4553 (calcd for $\text{C}_{47}\text{H}_{68}\text{N}_4\text{O}_{16}\text{Na}$, 967.4528).

Preparation of Proteasome-enriched Fraction

Proteasome used in this study was partially purified from rat liver. The liver was dissected and homogenized in ice-cold lysis buffer consisting of 20 mM HEPES, pH 7.5, 5 mM KCl, 1.5 mM MgCl_2 , 1 mM dithiothreitol, 2 mM ATP, and 10% glycerol at 4 °C for 5 min. The extract was filtered through cheese cloth, and the filtrate was immediately centrifuged at 10,000 rpm for 5 min. The supernatant was centrifuged at $105,000 \times g$ for 20 min, and the resultant supernatant was further centrifuged at $300,000 \times g$ for 2 h. The precipitates thus obtained were suspended in lysis buffer containing 50% glycerol and used as the crude proteasome-enriched preparation.

Assay for Proteasome Activity

The fluorogenic substrate succinyl-leucyl-leucyl-valyl-tyrosine 4-methylcoumaryl-7-amide (MCA) (Peptide Institute, Inc., Osaka) was used as a substrate for chymotrypsin-like activity of the proteasome. The proteasome-enriched fraction in a mixture (0.1 mL) that contained 50 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol, and 5 mM EDTA was pre-incubated with each inhibitor at 30 °C for 10 min. Then, 0.05 mM substrate was added to the mixture and the mixture was further incubated at 30 °C for 1 h. The reaction was stopped by adding 0.1 mL of 10% SDS and the fluorescence intensity owing to 7-amino-4-methylcoumarin (AMC) was measured (excitation, 360 nm; emission, 460 nm). The value of IC_{50} , the concentration required for 50% inhibition of proteasome inhibitory activity, was calculated from the data of duplicate measurements.

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 29. Since secomycalolide A (**1**) decomposed during storage, ¹³C NMR spectra could not be measured.

Samples Availability: Not available.