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

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Received: 28 April 2005 / Accepted: 25 May 2005 / Published: 1 June 2005

**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 IC\textsubscript{50} 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 IκB followed by suppression of NF-κB 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], epoxomycin [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 oxygen-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 IC50 values of 1, 2, and 3 were found to be 11, 30, and 45 µg/mL, respectively.

| Table 1. 1H NMR Data of Secomycalolide A (1) in CDCl3 |
|----------------|----------------|
| position | δH | position | δH |
| 2 | 2.60 m, 2.70 m | 24 | 5.22 m |
| 3 | 4.22 m | 25 | 1.55 m, 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 |
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 (δH 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 × 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 × 10^-5%, wet weight) and 30-hydroxymycalolide A (3, 0.54 mg, 1.3 × 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 × 10^-5%).
Secomycalolide A (1): $^1$H NMR (CDCl$_3$), see Table 1; FABMS $m/z$ 967 [M + Na]$^+$; HRFABMS $m/z$ 967.4553 (calcd for C$_{47}$H$_{68}$N$_4$O$_{16}$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 × g for 20 min, and the resultant supernatant was further centrifuged at 300,000 × 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.

Acknowledgments

We thank Prof. H. Yokosawa of the Graduate School of Pharmaceutical Sciences, Hokkaido University, for his valuable advice on assay for proteasome activity. Thanks are also due to Prof. H. Sawada of the Sugashima Marine Biological Laboratories of the Graduate School of Science, Nagoya University, for his help in collection of the sponge. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, Culture, and Technology of Japan and the Ichiro Kanehara Foundation.

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

*Samples Availability:* Not available.

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