

**Aptamine, an alkaloid from the sponge *Aaptos suberitoides*, functions as a
proteasome inhibitor**

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Abstract

Aaptamine (1), iso-aaptamine (2), and demethylaaptamine (3) were isolated from the marine sponge *Aaptos suberitoides* collected in Indonesia as inhibitors of the proteasome. They inhibited the chymotrypsin-like and caspase-like activities of the proteasome with IC_{50} values of 1.6-4.6 $\mu\text{g/mL}$, while they showed less inhibition of the trypsin-like activity of the proteasome. The three compounds showed cytotoxic activities against HeLa cells, but their cytotoxicity did not correlate with their potency as proteasome inhibitors, strongly suggesting that their proteasomal inhibitory activity is dispensable to their cytotoxicity.

Selective protein degradation by the ubiquitin-proteasome system is an essential aspect of cell signaling pathways, functioning from cell-cycle control and transcription to development.¹⁻³ In the ubiquitin system, ubiquitin, composed of 76 amino acids, attaches to a target protein prior to degradation. The ubiquitination requires the sequential actions of three enzymes, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-protein ligase (E3), which results in the formation of the polyubiquitin chain.⁴ The polyubiquitin chain, attached to the target protein, is recognized by the 26S proteasome, an intracellular high-molecular weight protease subunit complex. The 26S proteasome consists of two subcomplexes, the 20S core particle (the 20S proteasome) and the 19S regulatory particle, and the protein portion of the polyubiquitinated target protein is degraded by the proteolytic active sites in a cavity of the 20S proteasome.^{5,6} The 20S proteasome is classified as a threonine protease that contains two pairs of three different sites, which catalyze chymotrypsin-like (CT-L), trypsin-like (T-L), and caspase-like (C-L) activities. Since the level of the proteasome is increased especially in tumor cells, it is reasonable to develop specific compounds targeting the proteasome-mediated proteolytic pathway for cancer treatment. The approval of bortezomib (PS-341, Velcade[®]), a synthetic proteasome inhibitor, for the treatment

of relapsed multiple myeloma in the United States in 2003 has opened the way to the discovery of drugs targeting the proteasome.^{7,8} Bortezomib shows antitumor activity against various tumor cells that are resistant to conventional chemotherapeutic agents.⁹ In addition, three irreversible proteasome inhibitors, salinosporamide A (NPI-0052),¹⁰⁻¹² carfilzomib (PR-171),¹³⁻¹⁵ and CEP-18770,¹⁶⁻¹⁸ are currently undergoing phase I and II clinical trials. To date, structurally-diverse proteasome inhibitors have been developed by chemical synthesis and also by searching natural sources and chemical libraries as drugs for the clinical treatment of cancer and also as molecular tools for the investigation of cellular events.¹⁹ We have been screening extracts of marine invertebrates and cultures of marine-derived fungi based on the inhibition of the CT-L activity of the proteasome and found agosterols,²⁰ mycalolides,²¹ and *cis*-hinokiresiol²² as proteasome inhibitors. In this study, we isolated aaptamine (**1**),²³ iso-aaptamine (**2**),²⁴ and demethylaaptamine (**3**)²⁵ (Fig. 1) from the marine sponge *Aaptos suberitoides* collected in Indonesia as proteasome inhibitors.

Fig. 1

Specimens of *Aaptos suberitoides*²⁶ (900 g, wet weight) were collected in North Sulawesi, Indonesia, in December 2006. The sponge was extracted with EtOH immediately after collection. The extract was evaporated, and the aqueous residue was extracted with EtOAc and then *n*-BuOH. The *n*-BuOH fraction (5.1 g) and the H₂O fraction showed inhibitory activity against the CT-L activity of the proteasome. The fractions were subjected to ODS column chromatography (CH₃CN/H₂O/TFA) and ODS HPLC (CH₃CN/H₂O/TFA) to afford **1-3**, which were identified on the basis of their spectroscopic data.²³⁻²⁵

The inhibition of the CT-L, T-L, and C-L activities of the proteasome was tested using a partially-purified proteasome preparation from rat liver and a purified 20S proteasome preparation

from human erythrocytes (Table 1).²⁷ With regard to the CT-L and C-L activities, **1-3** showed almost the same levels of inhibition (IC₅₀ 1.6-4.6 µg/mL) against the two proteasomes. On the other hand, they showed less inhibition of the T-L activity of the rat proteasome. It should be noted that the inhibition of the T-L activity of the human 20S proteasome could not be tested because this preparation showed little T-L activity. Compounds **1-3** were cytotoxic to HeLa cells with IC₅₀ values of 15, 3.1, and 1.4 µg/mL, respectively, but their cytotoxicity did not correlate with their potency as inhibitors (Table 1).²⁸ These results strongly suggest that their proteasomal inhibitory activity is dispensable to their cytotoxicity.

Aptamine (**1**) was originally isolated from the marine sponge *Aaptos aaptos* as a factor showing α-adrenoceptor blocking activity.^{23,29} Subsequently, **1** and its derivatives were found to show antitumor, antimicrobial, and antiviral activities.³⁰⁻³⁶ Bowling et al. proposed that the compounds have potent cytotoxicity due to their ability to intercalate DNA.³⁶ In addition, a bisbenzyl derivative of **1**, hystatin 2, shows PKC inhibitory activity and blocks the S-phase of the cell cycle.³⁴ On the other hand, it has been reported that **1** and its derivatives have a variety of biological activities, including antioxidant activity in a chemical assay but not in a cellular-based assay,³⁷ antifouling activity against zebra mussel,³⁸ inhibitory activities against *Staphylococcus aureus* sortase A and adhesion of *S. aureus* to fibronectin,³⁹ and antidepressant activity in the forced swim test but not in the tail suspension test.⁴⁰

In this study, we first found that **1** and its derivatives **2** and **3** function as proteasome inhibitors, although **1** and its derivatives have been reported to show various biological activities. In connection with our findings, it should be noted that **1** is capable of promoting expression of the CDK inhibitor p21 in a p53-independent manner, leading to G2/M phase arrest.⁴¹ It has been reported that the p21 promoter is activated through Sp1 sites, to which transcription factors such as Sp1 and Smad3 are able to bind.⁴² There is much evidence that the proteasome inhibitors have antitumor activity and that the stability of transcription factors is regulated by the ubiquitin-

proteasome system. For example, bortezomib (PS-341, Velcade[®]) has been approved for myeloma therapy⁷⁻⁹ and the stability of Sp1 and Smad3 is regulated by the ubiquitin-proteasome system.⁴³⁻⁴⁵ Thus, it can be inferred that the accumulation of Sp1 and Smad3 caused by treating cells with proteasome inhibitors, including **1**, could result in the promotion of p21 expression, leading to cell cycle arrest. Further study is necessary to define the precise mechanism of the antitumor activity of **1**.

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26. The marine sponge was collected by scuba diving at a depth of 10 m in North Sulawesi, Indonesia in December 2006 and soaked in EtOH immediately. The sponge was identified as *Aaptos suberitoides*. A voucher specimen (RMNH POR. 4808) was deposited at the National Museum of Natural History, The Netherlands. The sponge (900 g) was extracted with EtOH three times. The extract was concentrated under reduced pressure, and the residual aqueous solution was extracted with EtOAc and then with *n*-BuOH. The activity-guided purification of a portion (1.5 g) of the *n*-BuOH fraction (5.1 g) by ODS column chromatography (CH₃CN/H₂O/TFA) and ODS HPLC (CH₃CN/H₂O/TFA) afforded aaptamine (**1**, 32.0 mg, 0.012%, wet weight), isoaptamine (**2**, 11.5 mg, 0.0043%), and demethylaaptamine (**3**, 2.1 mg, 0.00079%). Furthermore, a portion (60 mL out of 550 mL) of the H₂O fraction was subjected to purification with the same procedure to afford **1-3** (**1**, 45.1 mg, 0.046%; **2**, 100.7 mg, 0.10%; **3**, 28.7 mg, 0.029%).
27. The rat proteasome used in this study was partially purified from liver and the human 20S proteasome from erythrocytes was purchased from Enzo Life Sciences (PW8720). **Purification of the proteasome from rat liver:** Liver was dissected and homogenized in ice-cold lysis

buffer consisting of 20 mM HEPES, pH 7.5, 5 mM KCl, 1.5 mM MgCl₂, 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 proteasome-enriched preparation. **Proteasome inhibition assay:** The fluorogenic substrates Suc-Leu-Leu-Val-Tyr-MCA, Boc-Phe-Ser-Arg-MCA, and Z-Leu-Leu-Glu-MCA (Peptide Institute, Inc., Osaka) were used as substrates for the CT-L, T-L, and C-L activities of the proteasome, respectively. The proteasome in a mixture (100 μL) that contained 50 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol, 5 mM EDTA, and 0.02% SDS was pre-incubated with test compounds at various concentrations at 30 °C for 10 min. Then, the substrate (10 μM) was added to the mixture and the mixture was further incubated at 30 °C for 6 h. The reaction was stopped by adding 100 μL of 10% SDS and the fluorescence intensity owing to 7-amino-4-methylcoumarin (AMC) was measured (excitation, 360 nm; emission, 450 nm). The value of IC₅₀, the concentration required for 50% inhibition of proteasome inhibitory activity, was calculated from the data of duplicate measurements.

28. The cytotoxicity test was carried out with HeLa cells. HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (50 units/mL), and streptomycin (50 μg/mL) under a humidified atmosphere containing 5% CO₂ at 37 °C. The cells were seeded into 96-well microplates (3 × 10³ cells/well) and pre-cultured for a day. The medium was replaced with that containing test compounds at various concentrations and the cells were further cultured at 37 °C for 3 days. The medium was then replaced with 50 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (200 μg/mL in medium) and the cells were incubated under the same conditions for 3 h. After addition of 200

μL of DMSO, the optical density at 570 nm was measured with a microplate reader.

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Figure legend

Figure 1. Structures of aaptamine (1), iso-aaptamine (2), and demethyloaaptamine (3).

Table 1. Biological activities of **1-3**.

Compound	Proteasome inhibition, IC ₅₀ (μg/mL)					Cytotoxicity IC ₅₀ (μg/mL)
	A			B		
	CT-L	T-L	C-L	CT-L	C-L	
1	1.6	18	2.7	4.3	4.6	15
2	2.1	10	2.4	1.7	1.7	3.1
3	2.1	12	2.3	2.0	1.9	1.4

Proteasome inhibition was tested with a partially-purified proteasome preparation from rat liver (A) and a 20S proteasome preparation from human erythrocytes (Enzo Life Sciences PW8720) (B). Note that the trypsin-like (T-L) activity of the human 20S proteasome (B) could not be detected. Cytotoxicity was tested using HeLa cells. CT-L, chymotrypsin-like; C-L, caspase-like.

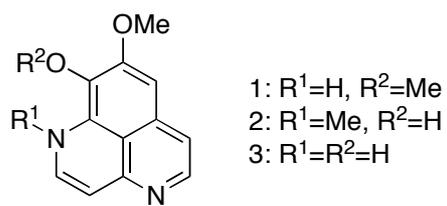


Fig. 1