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Azumamides A–E: Histone Deacetylase Inhibitory Cyclic Tetrapeptides from the Marine Sponge *Mycale izuensis***

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In memory of Takahiro Yamashita

Histone acetylation and deacetylation, which are catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively, play important roles in transcriptional regulation.[1] Inhibitors of these enzymes induce cell-cycle arrest,[2] p53-independent induction of the cyclin-dependent kinase inhibitor p21,[3] tumor-selective apoptosis,[4] and differentiation of normal and malignant cells.[5] Recently, HDAC inhibitors such as trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) were demonstrated to exert potent anti-angiogenic effects through the alteration of vascular endothelial growth factor signaling.[6] These direct and

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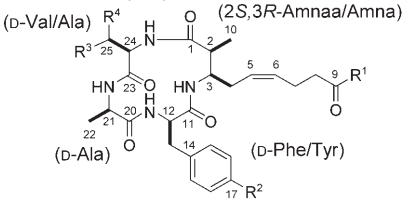
indirect effects on tumor growth and metastasis indicate that HDAC inhibitors are potential anticancer agents. In fact, HDAC inhibitors such as valproic acid, SAHA, and FR901228 (FK228) have entered clinical trials as anticancer drugs.[3]

In the course of our search for antitumor leads from Japanese marine invertebrates, we found two HDAC inhibitory sponges among 167 species of marine invertebrates tested. We reported the isolation of cyclostellettamines as active substances from *Xestospongia* sp.[7] Subsequently, we isolated five new cyclic tetrapeptides named azumamides A–E (**1**–**5**) from *Mycale izuensis* (Table 1). Herein, we report the isolation, structure elucidation, and biological activities of these compounds.

Table 1: Azumamides A–E (**1**–**5**).



Azumamide	R^1	R^2	$R^3 = R^4$	Yield[a] [mg]	Yield[a] [%]	IC_{50} (HDAC) [μM][b]
A (**1**)	NH_2	H	Me	2.7	1.2×10^{-4}[c]	0.045
B (**2**)	NH_2	OH	Me	1.6	7.3×10^{-5}	0.11
C (**3**)	OH	OH	Me	1.4	6.4×10^{-5}	0.11
D (**4**)	NH_2	H	H	0.6	2.7×10^{-5}	1.3
E (**5**)	OH	H	Me	0.9	4.1×10^{-5}	0.064

[a] Yield of azumamide isolated by extraction from marine sponge (see text for details). [b] Against the crude enzymes extracted from K562 cells. [c] Yield based on wet weight.

Frozen samples (2.2 kg) were exhaustively extracted with EtOH and MeOH. The combined extracts were partitioned between H_2O and Et_2O, and the aqueous layer was further extracted with *n*BuOH. The active *n*BuOH layer was successively subjected to ODS (octadecylsilane) flash chromatography, gel filtration, and ODS HPLC to afford azumamides A–E (Table 1).

Azumamide A (**1**) was obtained as an optically active colorless solid ($[\alpha]_D^{23} = +33°$) with a molecular formula of $C_{27}H_{39}N_5O_5$ as established by high-resolution fast atom bombardment mass spectrometry (HR-FABMS). The peptidic nature of **1** was readily inferred from exchangeable proton signals at $\delta = 7.63$, 7.85, 8.00, and 8.15 ppm, and methine proton signals at $\delta = 3.60$, 4.13, 4.17, and 4.29 ppm in the ^{1}H NMR spectrum. Two-dimensional NMR analysis including COSY, HOHAHA,[8] HMQC,[9] and HMBC[10] led to identification of three usual amino acids, namely alanine (Ala), valine (Val), and phenylalanine (Phe), as well as an unusual β-amino acid residue (see the Supporting Information).

The β-amino acid was assigned as 3-amino-2-methyl-5-nonenedioic acid, 9-amide (Amnaa) on the basis of the following observations: The spin system from H-2 to H_2-8 through two olefinic protons at $\delta = 5.38$ (H-5) and 5.50 ppm (H-6) was readily obtained by COSY analysis; the *Z* geometry of the double bond was deduced from the coupling constant of 11 Hz. COSY cross-peaks CH_3-10/H-2 and NH-3/H-3 placed a CH_3 group and an NH group on C-2 and C-3, respectively. HMBC cross-peaks from the α- and β-methine (H-2 and H-3) and CH_3-10 protons to the carbonyl carbon atom at $\delta = 176.9$ ppm placed this carbonyl group at C-1. On the other hand, HMBC correlations from H_2-8 to the carbonyl carbon atom at $\delta = 178.4$ ppm could place an amide or a carboxylate group at the terminus of the side chain. The molecular formula and two broad proton signals at $\delta = 7.58$ and 6.76 ppm, which were correlated with each other in the HOHAHA spectrum, indicated the amidated terminus of the side chain (Figure 1).

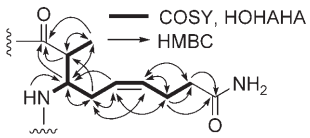


Figure 1. 3-Amino-2-methyl-5-nonenedioic acid, 9-amide (Amnaa). HOHAHA = Homonuclear Hartman–Hahn; HMBC = heteronuclear multiple bond coherence.

The connectivities of these four amino acids were established by analysis of the HMBC spectrum (Figure 2).

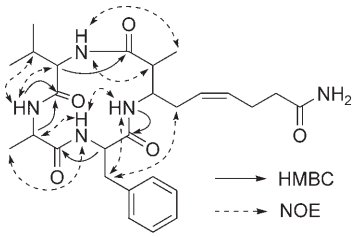


Figure 2. Interresidual HMBC and ROESY correlations for **1**.

An HMBC correlation between NH-3 ($\delta = 7.63$ ppm) and C-11 ($\delta = 174.0$ ppm) connected Amnaa to Phe, while that between H-24 ($\delta = 3.60$ ppm) and C-1 ($\delta = 176.9$ ppm) established the linkage between Amnaa and Val. HMBC cross-peaks H-12/C-20, H-21/C-23, and NH-21/C-23 placed the Ala residue between Val and Phe, thus completing the overall structure, which was also confirmed by ROESY correlations NH-3/H-13a and NH-12, NH-12/H-21 and H-22, NH-21/H-24 and H-25, and NH-24/H-2 and H-10 (Figure 2).

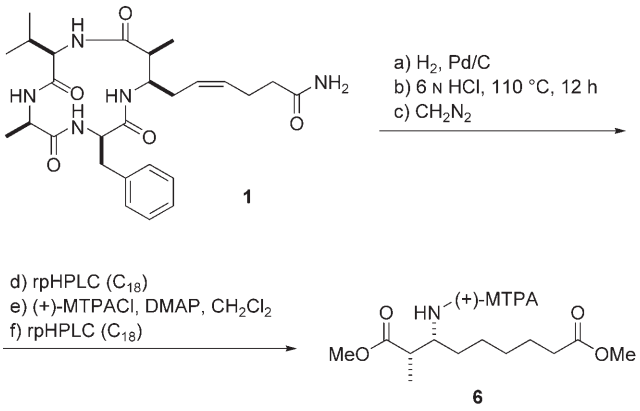
Azumamide B (**2**) has a molecular formula of $C_{27}H_{39}N_5O_6$ as established by HR-FABMS. The ^{1}H NMR spectrum of **2** was almost superimposable on that of **1**, except for signals in the aromatic region. Two doublet signals ($\delta = 7.02$ and 6.66 ppm), each of which was integrated as two protons, implied that the Phe residue in **1** was replaced by tyrosine (Tyr) in **2**.

In the ^{1}H NMR spectrum of azumamide C (**3**), the mutually coupled amide protons of Amnaa in **2** were not observed, suggesting that the terminal amide group of Amnaa in **2** was the free carboxylic acid, hence the relevant amino acid being 3-amino-2-methyl-5-nonenoic-1,9-diacid (Amnda)

in **3**. The molecular formula of **3** was established as $C_{27}H_{38}N_4O_7$ on the basis of HR-FABMS ($[M+H]^+$ m/z 531.2811; $\Delta = -0.7$ mmu) in accordance with the proposed structure.

Azumamide D (**4**; $C_{25}H_{35}N_5O_5$) was smaller than **1** by a C_2H_4 unit. The presence of Ala in **4** was established in straightforward manner by the presence of a doublet methyl group at $\delta = 1.49$ ppm (H-25) in the ^{1}H NMR spectrum instead of the two doublet methyl signals around $\delta = 0.95$ ppm in **1**, thus suggesting that Val residue in **1** was replaced by Ala in **4**. As in the case of **2** and **3**, azumamide E (**5**; $C_{27}H_{38}N_4O_6$) was related to **1** as its free acid form.

The absolute configuration of the α-amino acids in **1**–**5** was determined by Marfey analysis[11] to be the all-D form, while the stereochemistry of the β-amino acid (Amnaa) in **1** was determined as follows (Scheme 1): To prevent side



Scheme 1. Degradation and derivatization of **1**. rp = reversed phase; MTPA = α-methoxy-α-(trifluoromethyl)phenylacetic acid; DMAP = 4-(dimethylamino)pyridine.

reactions during acid hydrolysis, **1** was hydrogenated over Pd/C, followed by acid hydrolysis. The hydrolyzed residues were esterified with CH_2N_2 and separated by reversed-phase HPLC. Then, the fraction containing the desired methyl ester was derivatized with (+)-MTPACl in the presence of DMAP in CH_2Cl_2, followed by reversed-phase HPLC to obtain the desired MTPA ester **6** (m/z 484 $[M+Na]^+$). Comparison of the spectrum of this MTPA ester **6** with those of the model compounds derived from four stereoisomers of 3-amino-2-methylhexanoic acids suggested its 2*S*,3*R* configuration (see the Supporting Information). Although the limited amounts of samples prevented us from applying the same strategy for **2**–**5**, the similar chemical shifts and coupling constant values for these units allowed us to infer the same 2*S*,3*R* configuration for **1**–**5**.

Azumamides A–E (**1**–**5**) showed potent HDAC inhibitory activity with IC_{50} values of 0.045 to 1.3 μM in an assay using enzymes prepared from K562 human leukemia cells (Table 1). The effect of azumamide A (**1**) on histone deacetylation at the cell level was also evaluated. After incubation during 6 h of K562 cells with **1**, acetylation of histones H3 and H4 was detected by western blot analysis, which clearly showed a dose-dependent inhibition of the deacetylation of Ac-H3 (Lys9 and Lys14) and Ac-H4 (Lys8) by **1** in the concentration range 0.19–19 μM (Figure 3). In accordance with the cell-based assay, azumamide A showed moderate cytostatic effects on WiDr (human colon cancer) cells and K562 (human leukemia) cells with IC_{50} values of 5.8 and 4.5 μM, respectively.

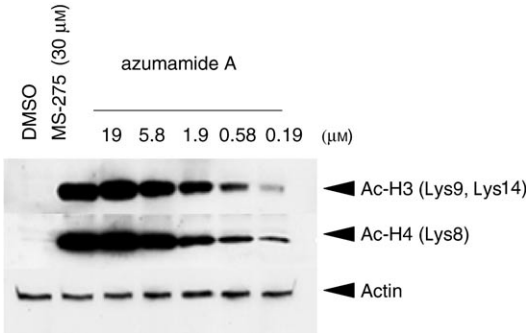


Figure 3. Western blot analysis following the treatment of azumamide A on K562 cells (MS-275: positive control; DMSO = dimethyl sulfoxide).

Furthermore, the anti-angiogenic effect of azumamide A was tested by the in vitro vascular organization model using mouse ES cells.[12] Azumamide A (**1**) at 19 μM significantly inhibited the vascular formation from aggregates of vascular progenitor cells in three-dimensional culture using type-I collagen gel (Figure 4).

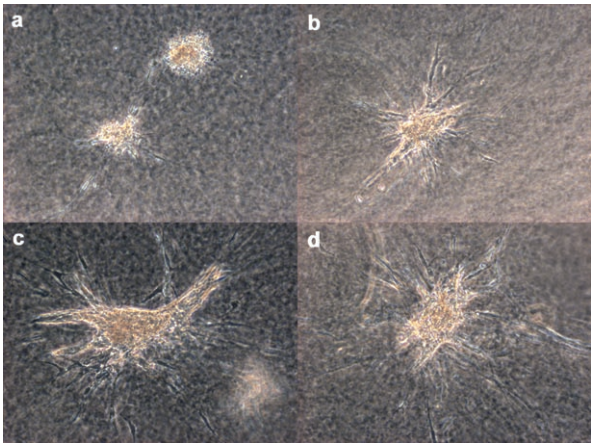


Figure 4. Anti-angiogenic effects of azumamide A (**1**) at a) 19 μM, b) 1.9 μM, and c) 0.19 μM. d) Control (MeOH only).

Only a few examples of marine HDAC inhibitors are known to date; psammaplin obtained from marine sponge *Pseudoceratina purpurea* inhibited HDAC with IC_{50} values of 2.1–327 nM,[13] while azumamides A–E (**1**–**5**) are the first examples of cyclic peptides with HDAC inhibitory activity isolated from marine organisms. Recently, the crystal structure of the complex between a histone deacetylase like protein (HDLP) and trichostatin A (TSA)[14] was solved.[15] The crystal structure provides interesting insights into the binding modes of HDAC inhibitors. According to this

structure, TSA binds to the enzyme by inserting its long aliphatic chain into the hydrophobic pocket. The hydroxamic acid group at one end of the aliphatic chain reaches the polar bottom of the pocket, where it coordinates with a zinc ion in a bidentate fashion. The aromatic dimethylaminophenyl portion at the other end of the TSA chain makes contacts with the pocket entrance and in an adjacent surface groove, thereby capping the pocket (Figure 5).

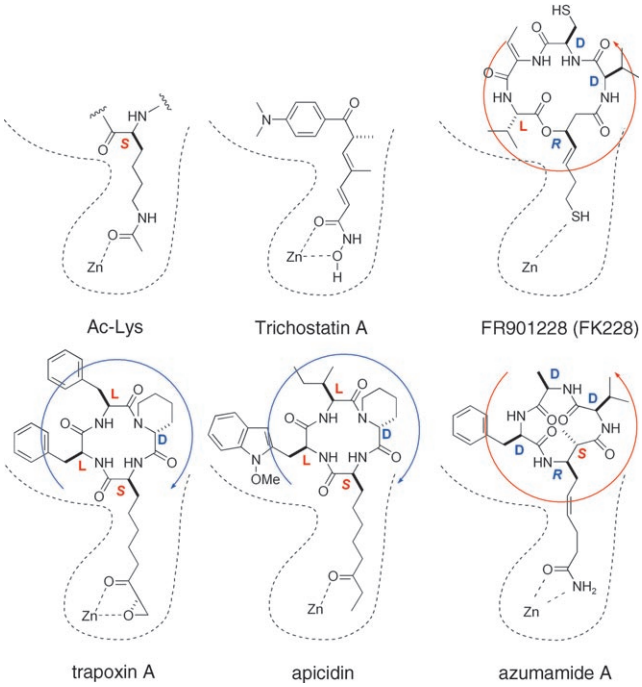


Figure 5. Comparison of plausible binding modes.

Trapoxins (Tpx)[16] and apicidins,[17] two classes of HDAC inhibitory cyclic tetrapeptides, contain groups that may be analogous to the aliphatic chain, the active-site/zinc-binding group, and the cap of TSA. Trapoxins contain a 2-amino-8-oxo-9,10-epoxy-decanoic acid (Aoeda), in which the epoxide group is thought to irreversibly bind to the enzyme,[14b] while the apicidins comprise a 2-amino-8-oxo-decanoic acid (Aoda), with the ketone group being responsible for zinc chelation.[18] The corresponding terminal functions for azumamides are the amide or carboxylate group. Generally, the affinity of an amide group to zinc is much weaker than that of a carboxylic acid. Therefore, it is quite interesting that azumamides A (**1**) and C (**3**) with an amide end showed the equivalent level of HDAC inhibitory activity as azumamides B (**2**) and E (**5**), which have a carboxylate moiety.

Hydrophobic amino acids in Tpx and apicidins may play a role of the dimethylaminophenyl group in trichostatin A. The reversed absolute stereochemistry of Phe/Tyr, Ala, and β-carbon atoms of Amnaa/Amnda in azumamides to that of the corresponding residues of Phe, Phe/Ile, Aoeda/Aoda in trapoxin A/apicidin suggest the possibility that azumamides are retro-enantio types of HDAC inhibitors of trapoxins or apicidins (Figure 5).

Azumamides are the first cyclic peptides from marine invertebrates that exhibit anti-HDAC inhibitory activity. Potent inhibitory activity against HDACs prepared from K562 cells was observed for azumamide A (**1**), while the activity for induction of histone acetylation in K562 cells of **1** remained moderate. The reason for the difference between these results is not yet clear.[19]

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[19] The potency of azumamides varied with HDACs of different origins. These results will be published elsewhere.

