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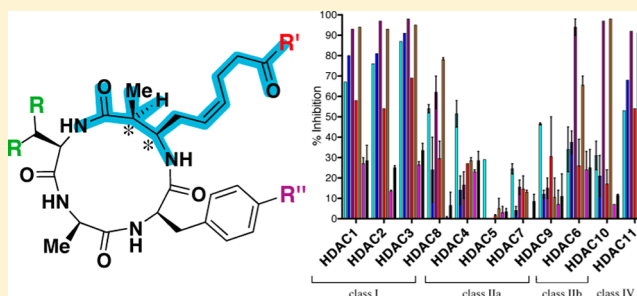
Total Synthesis and Full Histone Deacetylase Inhibitory Profiling of Azumamides A–E as Well as β^2 -*epi*-Azumamide E and β^3 -*epi*-Azumamide E

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S Supporting Information

ABSTRACT: Cyclic tetrapeptide and depsipeptide natural products have proven useful as biological probes and drug candidates due to their potent activities as histone deacetylase (HDAC) inhibitors. Here, we present the syntheses of a class of cyclic tetrapeptide HDAC inhibitors, the azumamides, by a concise route in which the key step in preparation of the noncanonical disubstituted β -amino acid building block was an Ellman-type Mannich reaction. By tweaking the reaction conditions during this transformation, we gained access to the natural products as well as two epimeric homologues. Thus, the first total syntheses of azumamides B–D corroborated the originally assigned structures, and the synthetic efforts enabled the first full profiling of HDAC inhibitory properties of the entire selection of azumamides A–E. This revealed unexpected differences in the relative potencies within the class and showed that azumamides C and E are both potent inhibitors of HDAC10 and HDAC11.



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INTRODUCTION

Macrocyclic peptides have played important roles in the field of epigenetics due to their potent activities as inhibitors of histone deacetylase (HDAC) enzymes. One of the two HDAC targeting drugs (**1**¹ and **3**) that are approved by the U.S. Food and Drug Administration (FDA) for clinical treatment of cutaneous T-cell lymphoma is the macrocyclic natural product romidepsin (**3**).² Furthermore, a cyclic tetrapeptide, trapoxin,³ played an instrumental role in the first isolation of a mammalian HDAC enzyme.^{4,5} Thus, this class of inhibitors holds promise as tool compounds as well as potential drug candidates targeting HDACs.^{6–9}

Though clearly bearing an overall resemblance to the classical cyclic tetrapeptide HDAC inhibitors [including, for example, apicidin (**4**)],¹⁰ the azumamides (**5**–**9**) are structurally unique in that their extended Zn²⁺-coordinating amino acid (shown in yellow in Figure 1) is a disubstituted β -amino acid.¹¹ Furthermore, we found the azumamides interesting due to the relatively strong potencies reported for azumamide E against class I HDACs¹² in spite of its weak Zn²⁺-coordinating carboxylic acid functionality.¹³ Previously, azumamide A^{14,15} and azumamide E^{12,14–16} have been prepared by multistep chemical syntheses, but only azumamide E was tested against recombinant HDAC isoforms 1–9.¹² Furthermore, *in vitro* profiling with recombinant HDACs has witnessed important new developments since the publication of those results.^{17,18} We therefore found it relevant to explore the properties of these macrocycles in more detail by preparing the complete selection of natural products (**5**–**9**), and profiling their activities against the full panel of recombinant human Zn²⁺-dependent HDAC enzymes, HDAC1–11.

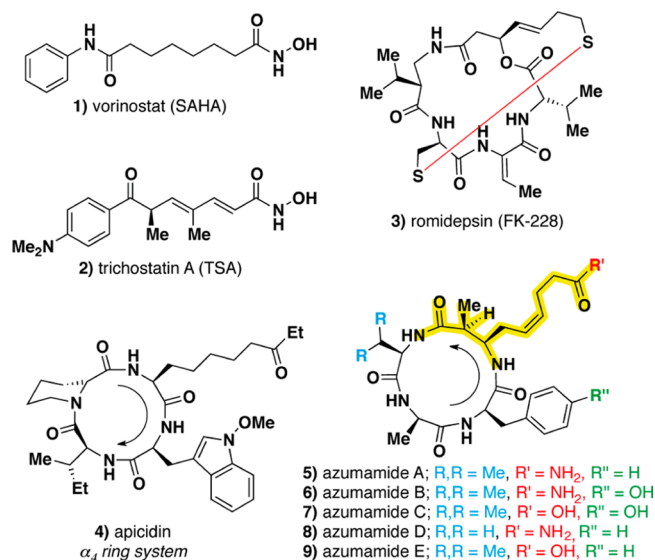


Figure 1. Structures of archetypical HDAC inhibitors (**1**–**4**) and target azumamides **5**–**9**.

As total syntheses of azumamides B–D had not been reported previously, this work would also allow unequivocal validation of the proposed structures.¹¹

For syntheses of the azumamides, we envisaged two significant challenges: first, efficient stereoselective synthesis

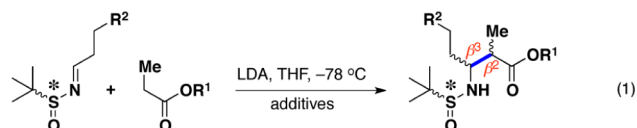
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of the disubstituted β -amino acid, and second, the macrocyclization step, which is known to be difficult for small cyclic peptides in general¹⁹ and furthermore proved challenging in previously reported syntheses of azumamide analogues.¹²

RESULTS AND DISCUSSION

Building Block Synthesis. For our synthesis of the β -amino acid building block, we chose a diastereoselective Ellman-type Mannich reaction to set the stereochemistry, as also previously reported by Ganesan and co-workers.¹⁵ However, to avoid having this important transformation at a late stage in our synthetic route, we decided to optimize this reaction between a propionate ester and a simple imine as shown in eq 1.



R¹ and R² are defined in Table 1.

This should give an intermediate with the correct stereochemistry (2*S*,3*R*), which could be readily elaborated to give the desired β -amino acid by robust organic synthetic transformations (*vide infra*). Mannich reactions between ester enolates and chiral sulfonimines have been studied extensively,^{20,21} and using previously reported conditions as our starting point we conducted an optimization study as outlined in Table 1. The *tert*-butyl ester showed superior selectivity (entry 5) compared to the less bulky methyl, ethyl, allyl, and PMB esters (entries 1–4), and furthermore, the methyl ester did not proceed to completion in our hands. Somewhat surprisingly, however, the major diastereoisomer in entry 5 proved to have (2*S*,3*S*) configuration as determined by X-ray crystallography upon desilylation (Figure 2).

This indicates that the pathway leading to our major isomer did not proceed through the six-membered Zimmerman–Traxler-type transition state,²² which has been proposed to be responsible for the diastereoselectivity with similar substrates.^{20,23} By using HMPA as an additive instead of a Lewis acid, this reaction has previously been shown to proceed through a different transition state,²⁰ and indeed we saw the same product distribution when using HMPA and TiCl(O^{*i*}Pr)₃ as additives with our substrates (entries 5 and 6). This indicates that the six-membered transition state, where coordination of

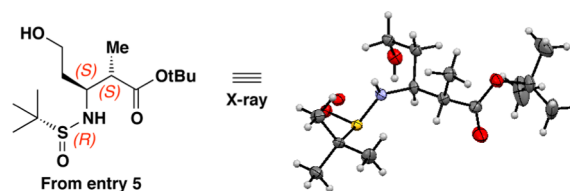


Figure 2. X-ray crystal structure of the (2*S*,3*S*) precursor obtained by desilylation of the major product in entry 5 of Table 1.

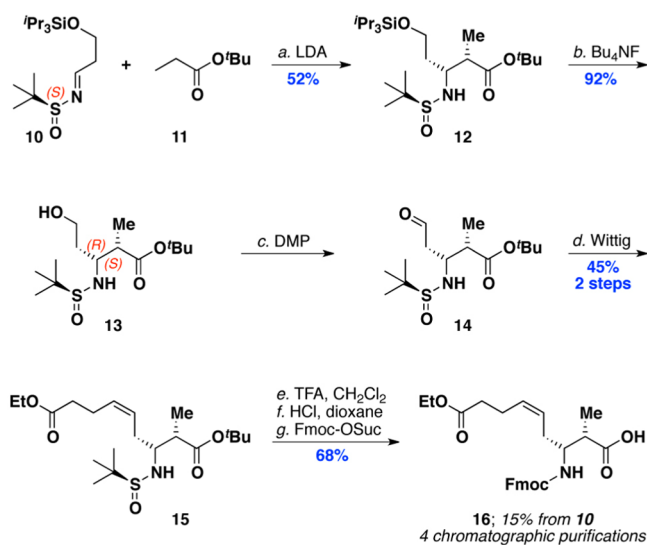
titanium is crucial, is highly unlikely to play a significant role in the formation of our major isomer. This is not in agreement with the diastereoselectivities observed with the substrates reported by Ganesan and co-workers.¹⁵ Thus, to address whether the steric bulk of the triisopropylsilyl ether was responsible for interrupting the six-membered transition state, we performed the reaction with different means of protecting the alcohol (entries 7–9). No significant effect was observed, however, indicating instead that the steric bulk of the *tert*-butyl ester caused the predominance of a different transition state when using our substrates. This is also in agreement with the original study by Tang and Ellman²⁰ where the level of selectivity decreased for 2,3-disubstituted β -amino acids when the bulk of the ester increased from methyl to *tert*-butyl.

Because we were interested in taking advantage of solid-phase synthesis methods to prepare the linear tetrapeptide azumamide precursors with a minimum of chromatographic purification steps, we were keen on keeping the acid-labile *tert*-butyl ester protecting group, which would allow easy protecting group manipulation to give an Fmoc-protected β -amino acid building block. Hence, instead of substituting this protecting group, we decided to optimize the Mannich reaction conditions to deliver the desired stereochemistry. First, we changed the stereochemistry of the sulfonimine to the *R*-enantiomer, which expectedly furnished the enantiomer of entries 5–9 (2*R*,3*R*) as the major isomer (entry 10). We then hypothesized that the configuration of the 2-position would be sensitive to the *E/Z* configuration of the enolate. Using Ireland's conditions for forming the enolate in the presence of HMPA,^{24,25} we achieved >80% *Z*-isomer, which gratifyingly afforded the (2*S*,3*R*) product as major isomer (entry 11). Under the developed conditions, we prepared compound 12, which was further elaborated to give Fmoc-protected β -amino acid 16 in 15% overall yield with just four column chromatographic purification steps from compound 10 (Scheme 1).

Table 1. Optimization of Stereochemical Outcome of the Mannich Reaction Shown in Equation 1

entry	auxiliary*	R ¹	R ²	additive	enolate ^a	dr ^b	major isomer
1	<i>R</i>	Me	OSi(^{<i>i</i>} Pr) ₃	TiCl(O ^{<i>i</i>} Pr) ₃	<i>E</i>	47:39:10:4	ND ^c
2	<i>R</i>	Et	OSi(^{<i>i</i>} Pr) ₃	TiCl(O ^{<i>i</i>} Pr) ₃	<i>E</i>	49:29:11:11	ND
3	<i>R</i>	allyl	OSi(^{<i>i</i>} Pr) ₃	TiCl(O ^{<i>i</i>} Pr) ₃	<i>E</i>	46:34:10:10	ND
4	<i>R</i>	PMB	OSi(^{<i>i</i>} Pr) ₃	TiCl(O ^{<i>i</i>} Pr) ₃	<i>E</i>	46:33:11:10	ND
5	<i>R</i>	^{<i>t</i>} Bu	OSi(^{<i>i</i>} Pr) ₃	TiCl(O ^{<i>i</i>} Pr) ₃	<i>E</i>	60:26:8:6	(2 <i>S</i> ,3 <i>S</i>) ^d
6	<i>R</i>	^{<i>t</i>} Bu	OSi(^{<i>i</i>} Pr) ₃	HMPA	<i>E</i>	71:15:14:0	(2 <i>S</i> ,3 <i>S</i>)
7	<i>R</i>	^{<i>t</i>} Bu	OBn	TiCl(O ^{<i>i</i>} Pr) ₃	<i>E</i>	70:18:12:0	ND
8	<i>R</i>	^{<i>t</i>} Bu	OPMB	TiCl(O ^{<i>i</i>} Pr) ₃	<i>E</i>	77:13:10:0	(2 <i>S</i> ,3 <i>S</i>)
9	<i>R</i>	^{<i>t</i>} Bu	OSi(Et) ₃	TiCl(O ^{<i>i</i>} Pr) ₃	<i>E</i>	75:21:4:0	(2 <i>S</i> ,3 <i>S</i>) ^d
10	<i>S</i>	^{<i>t</i>} Bu	OSi(^{<i>i</i>} Pr) ₃	HMPA	<i>E</i>	77:18:5:0	(2 <i>R</i> ,3 <i>R</i>) ^e
11	<i>S</i>	^{<i>t</i>} Bu	OSi(^{<i>i</i>} Pr) ₃	HMPA ^f	<i>Z</i>	64:25:8:2	(2 <i>S</i> ,3 <i>R</i>) ^g

^aMajor configuration of the enolate as determined by NMR and by trapping with ^{*t*}BuMe₂SiCl. ^bDiastereomeric ratio determined by ¹H NMR. ^cND = not determined. ^dDetermined by X-ray crystallography on its desilylated homologue. ^eDetermined spectroscopically by comparison with its enantiomer from entries 5 and 9. ^fHMPA (5.4 equiv) was added prior to the substrate to obtain the (*Z*)-enolate (>80%). ^gDetermined by comparison of spectroscopic data of the fully elaborated Boc-protected β -amino acid with previously reported data.¹²

Scheme 1. Synthesis of β -Amino Acid Building Block **16**.^a

^aReagents and conditions: (a) HMPA (6.4 equiv), LDA (2.6 equiv), **11** (2.5 equiv), THF, -78°C , 30 min; then **10**, -78°C , 30 min. (b) AcOH (1.0 equiv), Bu_4NF (2.0 equiv), THF, $0^{\circ}\text{C} \rightarrow \text{rt}$, 1.5 h. (c) NaHCO_3 (1.5 equiv), Dess–Martin periodinane (1.4 equiv), dry CH_2Cl_2 , $0^{\circ}\text{C} \rightarrow \text{rt}$, 1.5 h. (d) KHMDs (1.9 equiv), $\text{Ph}_3\text{PBr}(\text{CH}_2)_3\text{COOEt}$ (2.0 equiv), THF, $-78^{\circ}\text{C} \rightarrow \text{rt}$, 18 h. (e) TFA– CH_2Cl_2 (1:1, 10 mL, 80 equiv), $0^{\circ}\text{C} \rightarrow \text{rt}$, 3 h. (f) HCl (4.0 M in dioxane, 3.0 equiv), dioxane, 3 h. (g) Na_2CO_3 (4.0 equiv), Fmoc-OSuc (1.2 equiv), dioxane– H_2O , $0^{\circ}\text{C} \rightarrow \text{rt}$, 2 h.

The Boc-protected homologue of **16** was also prepared to confirm the (2*S*,3*R*) stereochemistry by comparison of spectroscopic data (optical rotation and NMR) with those previously reported (Figure S1 in Supporting Information).¹² Furthermore, the β^2 - and β^3 -epimeric building blocks were prepared by elaboration of the major isomers from entries 10 and 5, respectively (see Supporting Information for details). Although the achieved diastereomeric ratios were not particularly impressive, this strategy very nicely provided the correct stereochemistry along with two novel β -amino acids, enabling investigation of the biochemical effect of stereochemical configuration at these two chiral centers.

Cyclic Peptide Synthesis. Because three different points of cyclization had been reported for azumamide E and since these were all performed with different coupling reagents,^{12,14,15} we performed cyclization experiments using a simplified model peptide to address the issue. Not too surprisingly, this showed that macrolactamizations with the most sterically hindered amino acids at the C-terminal were particularly poor, resulting in significant amounts of N-terminal guanidinylation, incomplete cyclization, epimerization, and/or dimerization (Table S1 in Supporting Information). Thus, we prepared the linear tetrapeptides **17**, **19**, and **21** on solid support by standard Fmoc solid-phase synthesis using β -amino acid **16** and commercially available Fmoc-D-amino acids.

In Scheme 2A, the cyclization was then performed at the β -amino acid position and in Scheme 2B at the alanine residue, whereas the preparation of azumamide D (**8**) was achieved by cyclization between the two least sterically challenging alanine residues (Scheme 2C). After cleavage from the 2-chlorotrityl polystyrene resin with dilute TFA, the linear tetramers were ring-closed by use of HATU under dilute conditions (0.4–0.5 mM),^{26–29} and furthermore slow addition of the linear

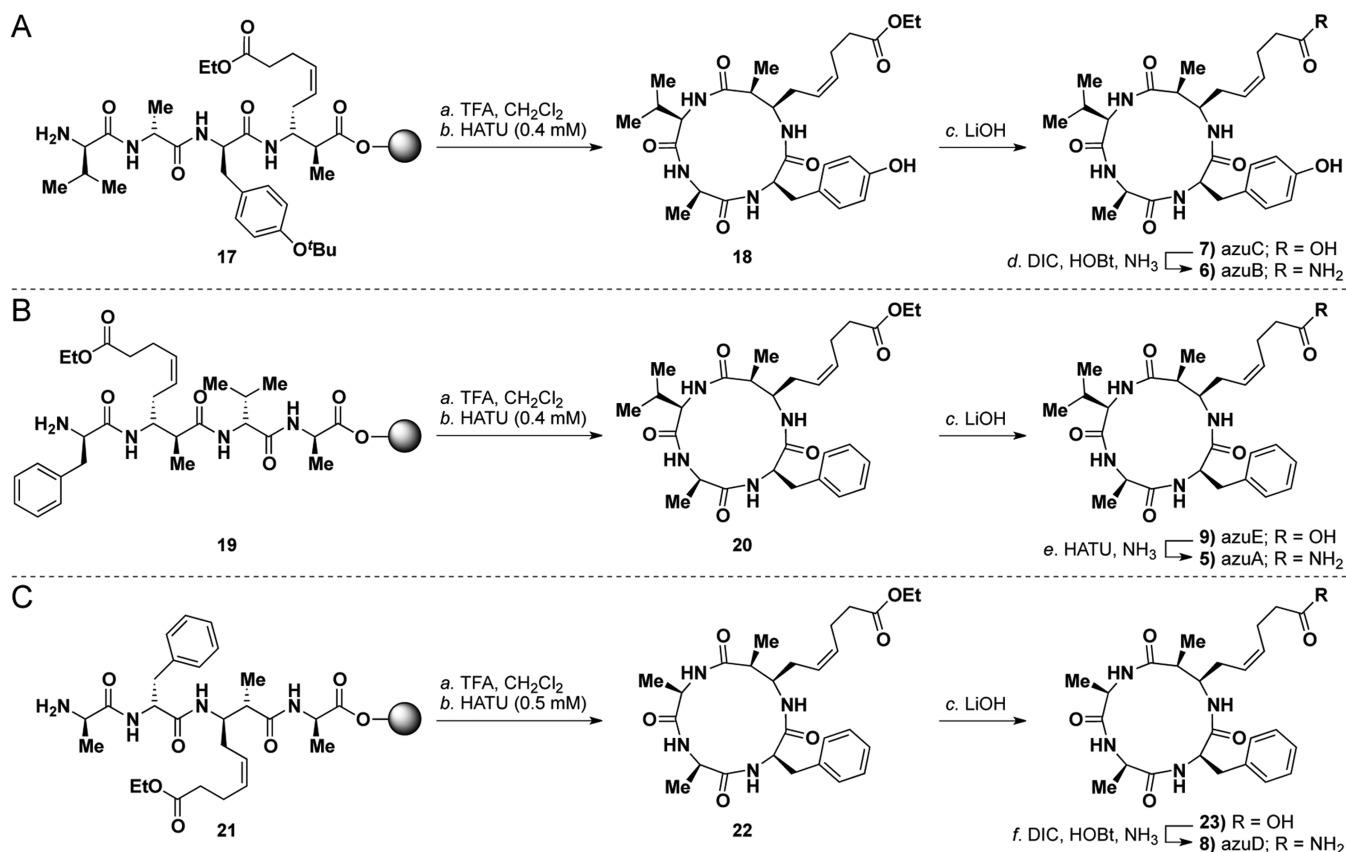
peptide by syringe pump to a solution of Hünig's base and HATU, as described by Ganesan and co-workers,¹⁵ was tested. Judging from LC–MS analyses of the reaction mixtures, we could not observe any significant differences between the cyclization yields obtained with the different methods. Although all the couplings proceeded satisfactorily, with full conversion of linear peptides and minor amounts of the corresponding dimers as the only observed byproducts, the resulting overall isolated yields were relatively low ($\sim 10\%$). We ascribe this to difficulties during purification of the macrocyclic products by preparative reversed-phase HPLC caused by poor water solubility, as we were able to recover more material by purifying the macrocycles by column chromatography. Unfortunately, however, this did not provide the final compounds in satisfyingly high purity for the bioassays, and thus the final compounds were all subjected to preparative reversed-phase HPLC purification although this resulted in a loss of material. Carbodiimide-mediated amidation of the side chain was attempted for conversion of **7** to **6** and **23** to **8**, but the reaction was slow and gave varying yields (**6** vs **8**, Scheme 2). Instead, HATU-mediated coupling was attempted for conversion of **9** to **5**, and this proved faster and gave an acceptable yield (**5**). Spectral data of all the natural products **5**–**9** were in excellent agreement with those originally reported for the azumamides isolated from natural sources,⁵ thus corroborating the original structural assignment (Figures S2–S6 in Supporting Information). Finally, the two epimeric β -amino acid building blocks were applied in analogous syntheses of β^3 -*epi*-azumamide E (**26**) and β^2 -*epi*-azumamide E (**29**) as shown in Scheme 3.

HDAC Screening. As an initial test of the HDAC inhibitory potency of all seven compounds, we first screened against the full panel of recombinant human HDACs at two compound concentrations (50 μM and 5 μM). Protocols for HDAC1–9 were adapted from Bradner et al.,¹⁸ using the fluorogenic Ac-LeuGlyLys(Ac)-AMC substrate for HDAC1–3 and 6 while using the Ac-LeuGlyLys(tfa)-AMC substrate for HDAC4, 5, and 7–9. For HDAC10 we used the tetrapeptide Ac-ArgThr-Lys(Ac)Lys(Ac)-AMC,³⁰ which was recently reported to perform well with this enzyme.³¹ Finally, for HDAC11, we also used Ac-LeuGlyLys(Ac)-AMC as substrate.³²

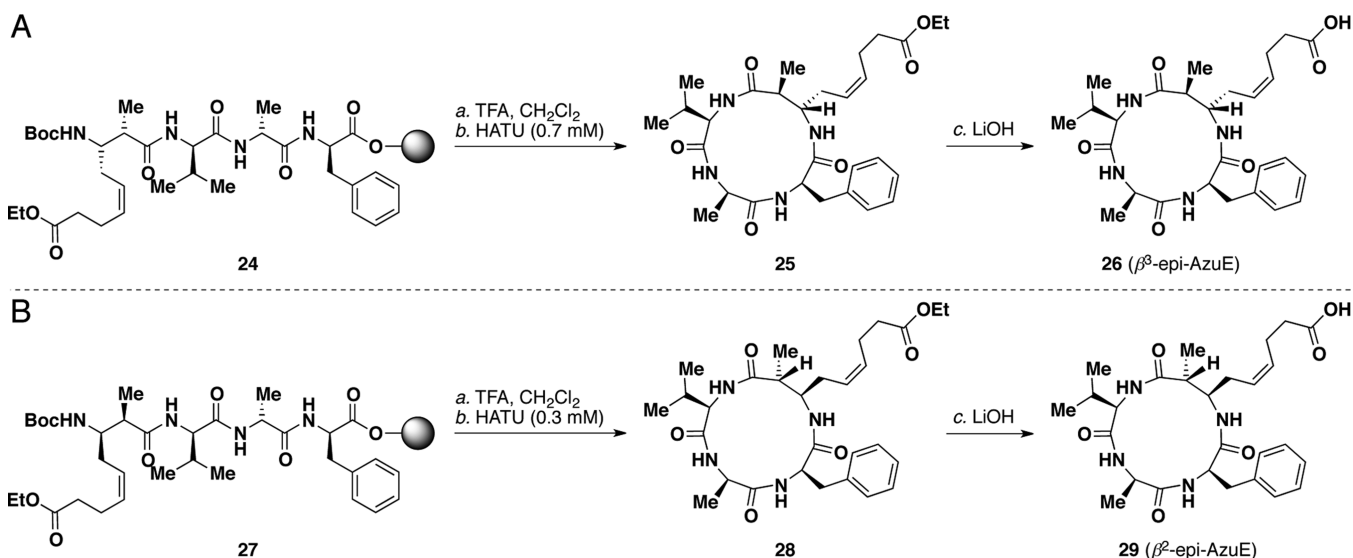
The site-specifically epimerized compounds exhibited no activity as previously reported for an analogue having both stereocenters inverted.⁷ It was not surprising that **26** was inactive, but it is noteworthy that the subtle change of inverting the stereochemistry of a single methyl group in **29** had such a detrimental effect across the entire selection of enzymes (Figure 3). Furthermore, none of the compounds **5**–**9** were able to inhibit class IIa HDAC activity against a trifluoroacetylated substrate (Figure 3).

Inhibitor K_i Values. Next, we performed dose–response experiments for all compound–HDAC combinations that gave above 50% inhibition in the initial assay (Figure S7 and Table S2 in Supporting Information). The obtained IC_{50} values were converted to K_i values by use of the Cheng–Prusoff equation [$K_i = \text{IC}_{50}/(1 + [\text{S}]/K_m)$] with the assumption of a standard fast-on–fast-off mechanism of inhibition. Reported K_m values were applied for the calculations except HDAC10, where we determined the K_m for the used substrate to be $1.5 \pm 0.2 \mu\text{M}$ (Figure 4).

Low potencies were recorded against HDACs **6** and **8**, which is in accordance with previous data for azumamide E (Table 2);⁶ however, compounds **7** and **9** were both potent inhibitors of HDACs **10** and **11**. Although they are classified together

Scheme 2. Synthesis of Azumamides A–E by Solid-Phase Synthesis Followed by Head-to-Tail Macrolactamization in Solution^a

^aReagents and conditions: (a) TFA–CH₂Cl₂ (1:1), 2 × 30 min. (b) HATU, ⁱPr₂NEt (8.0 equiv), DMF (0.4–0.5 mM peptide concn), 17–21 h; then HATU (0.5 equiv), 1–3 h [A, 11% **18** after preparative HPLC; B, 25% **20** after column chromatography; C, 19% **22** after column chromatography]. (c) LiOH, THF–H₂O (1:1). (d) DIC (11 equiv), HOBT (3.0 equiv), ⁱPr₂NEt (4.0 equiv), NH₃–dioxane (25 equiv), DMF–CH₂Cl₂ (2:1), 5 days, 67%. (e) HATU (2.0 equiv), ⁱPr₂NEt (5.5 equiv), NH₃–dioxane (25 equiv), DMF, 5.5 h, 40% (for steps c and e). (f) DIC (6.0 equiv), HOBT (3.0 equiv), ⁱPr₂NEt (4.0 equiv), NH₃–dioxane and NH₃–MeOH (30 equiv), CH₂Cl₂–DMF (2:1), 13 days, 11% (for steps c and f).

Scheme 3. Synthesis of the Two Epimers **26** and **29** of Azumamide E by Solid-Phase Synthesis Followed by Head-to-Tail Macrolactamization in Solution^a

^aReagents and conditions: (a) TFA–CH₂Cl₂ (1:1), 2 × 30 min. (b) HATU, ⁱPr₂NEt (8.0 equiv), DMF (0.3–0.7 mM peptide concn), 17–21 h. (c) LiOH, THF–H₂O (1:1).

(class Iib), HDACs **6** and **10** clearly interact very differently with these inhibitors.

Generally, we found the compounds with a carboxylic acid Zn²⁺-binding group (**7** and **9**) to be more potent than the

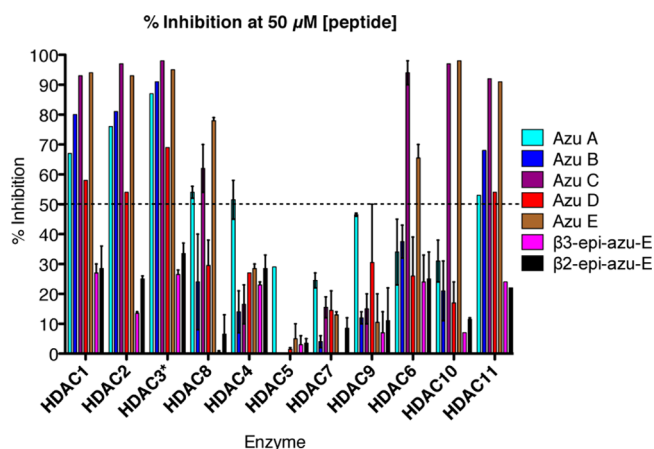


Figure 3. Single-dose HDAC inhibitory screening. Assays were performed at 50 μM (shown) and 5 μM (not shown) peptide concentrations. We chose <50% inhibition at 50 μM as our cutoff to sort away inactive compounds before performing full dose–response experiments. All compound–enzyme combinations that were discarded at this stage were tested in at least two individual assays performed in duplicate. Error bars represent the standard deviation. (*) Fusion protein of GST-tagged HDAC3 with the deacetylase activation domain (DAD) of nuclear receptor corepressor (NCoR1).

carboxamides (5, 6, and 8), which is in contrast to the originally reported HDAC inhibition data obtained for the natural products against an HDAC-containing cell extract.⁵ However, the data presented herein agree with subsequent work from

Ganesan and co-workers¹⁵ on azumamide A (5) and azumamide E (9). We thus show that this applies to all the azumamides, which also confirms that a carboxylate Zn^{2+} -binding group renders HDAC inhibitors significantly more potent than a corresponding carboxamide, as would be expected from literature precedents.^{19,26,27} Furthermore, compound 7 was more potent than 9 against HDACs 1–3, 6, 10, and 11, which is also in contrast to the original evaluation that found azuE (9) more potent than azuC (7) against crude enzymes from K562 cell extract.⁵ The tyrosine-containing compound (7) exhibited ~2-fold higher potency against HDACs 1, 3, 6, 10, and 11, whereas the phenylalanine-containing azumamide E (9) was only more potent against HDAC8, albeit at micromolar K_i values.

Finally, the inhibition of HDAC11 by azumamides C (7) and E (9) is, to the best of our knowledge, the first demonstration of potent cyclic peptide inhibitors of this isozyme.³³ Notably, these binding affinities were achieved without the presence of a strong Zn^{2+} chelator, such as hydroxamic acid.

CONCLUSIONS

In summary, we report total syntheses of all five azumamides, including for the first time azumamides B–D, which corroborate the originally proposed structures. Our synthetic route furthermore enabled preparation of site-specifically edited analogues for exploration of structure–activity relationships (SAR).^{34–36} The HDAC profiling results show that the β -amino acid residue, present in all the azumamides, is sensitive to even slight modifications. In addition, the original HDAC testing using cell extract indicated that azumamide E was the most

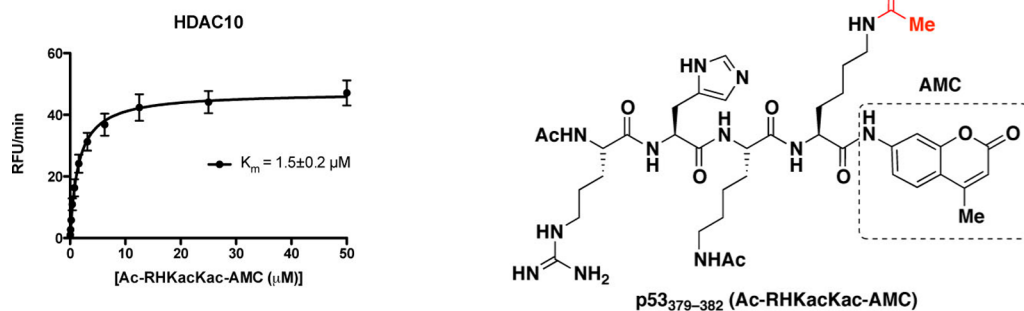


Figure 4. Michaelis–Menten plot for HDAC10.

Table 2. Potencies of Azumamides against Zn^{2+} -Dependent Histone Deacetylases^a

compd	K_i values (nM)										
	class I				class IIa				class IIb		class IV
	HDAC1	HDAC2	HDAC3 ^b	HDAC8	HDAC4	HDAC5	HDAC7	HDAC9	HDAC6	HDAC10	HDAC11
5 (azuA)	>5000	>5000	3200	>5000	52% ^c	IA ^d	IA	IA	IA	IA	>5000
6 (azuB)	5000	3000	3000	IA	IA	IA	IA	IA	IA	IA	>5000
7 (azuC)	32 ± 1	40 ± 20	14 ± 1	>5000	IA	IA	IA	IA	2000	10 ± 4	35 ± 3
8 (azuD)	>5000	>5000	3700	IA	IA	IA	IA	IA	IA	IA	>5000
9 (azuE)	67 ± 7	50 ± 30	25 ± 5	4400	IA	IA	IA	IA	>5000	20 ± 12	60 ± 16
26 (β^3 -epi-azuE)	IA	IA	IA	IA	IA	IA	IA	IA	IA	IA	IA
29 (β^2 -epi-azuE)	IA	IA	IA	IA	IA	IA	IA	IA	IA	IA	IA
1 (SAHA)	8 ± 1.5	7 ± 1.5	12 ± 4	700 ± 20	IA	IA	IA	IA	22 ± 9	NT ^e	13 ± 2
3 (FK-228) ^f	0.002	0.038	0.15	0.15	20.5	550	1250	1100	10	NT	NT

^a IC_{50} values were determined from at least two individual dose–response experiments performed in duplicate (Figure S7 in Supporting Information), and K_i values were calculated from the Cheng–Prusoff equation. ^bFusion protein of GST-tagged HDAC3 with deacetylase activation domain NCoR1. ^cPercent inhibition at 50 μM inhibitor concentration. ^dIA = inactive (<50% inhibition at 50 μM [inhibitor], Figure 3). ^eNT = not tested. ^fData from Bradner et al.¹⁸

potent of the series, but the comprehensive profiling presented herein shows that azumamide C is in fact ~2-fold more potent than azumamide E against the majority of the isozymes.

By taking advantage of the modular methodologies described in this article and building on the gained SAR information, we are currently investigating collections of azumamide analogues in search of more potent and selective ligands based on this promising scaffold.

EXPERIMENTAL SECTION

General. All chemicals and solvents were analytical-grade and were used without further purification. Vacuum liquid chromatography (VLC) was performed on silica gel 60 (particle size 0.015–0.040 μm). UPLC–MS analyses were performed on a Phenomenex Kinetex column (1.7 μm , 50 \times 2.10 mm) by use of a Waters Acquity ultra-high-performance liquid chromatography system. A gradient with eluent I (0.1% HCOOH in water) and eluent II (0.1% HCOOH in acetonitrile) rising linearly from 0% to 95% II during $t = 0.00$ –2.50 min was applied at a flow rate of 1 mL/min (gradient A) or during $t = 0.00$ –5.20 min (gradient B). Analytical HPLC was performed on a Phenomenex Luna column [150 mm \times 4.6 mm, C₁₈ (3 μm)] by use of an Agilent 1100 LC system equipped with a UV detector. Gradient C, with eluent III (0.1% TFA in water) and eluent IV (0.1% TFA in acetonitrile) rising linearly from 0% to 95% IV during $t = 2$ –20 min, was applied at a flow rate of 1 mL/min. Preparative reversed-phase HPLC was performed on a Phenomenex Luna column [250 mm \times 20 mm, C₁₈ (5 μm , 100 \AA)] by use of an Agilent 1260 LC system equipped with a diode-array UV detector and an evaporative light scattering detector (ELSD). A gradient, with eluent V (95:5:0.1, water–MeCN–TFA) and eluent VI (0.1% TFA in acetonitrile) rising linearly from 0% to 95% IV during $t = 5$ –45 min, was applied at a flow rate of 20 mL/min. All tested compounds were purified to homogeneity and shown by both analytical HPLC (gradient C) and LC–MS (gradient A) to be of more than 95% purity. One- and two-dimensional NMR spectra were recorded on a Varian Mercury 300 instrument or a Varian INOVA 500 MHz instrument. All spectra were recorded at 298 K. Correlation spectroscopy (COSY) spectra were recorded with a relaxation delay of 1.5 s before each scan, a spectral width of 6k \times 6k, and eight FIDs and 1k \times 512 data points collected. Heteronuclear single quantum coherence (HSQC) spectra were recorded with a relaxation delay of 1.5 s before each scan, a spectral width of 6k \times 25k, and 16 FIDs and 1k \times 128 data points collected. Heteronuclear two-bond correlation (H2BC) spectra were recorded with a relaxation delay of 1.5 s before each scan, a spectral width of 4k \times 35k, and 16 FIDs at 295 K and 1k \times 256 data points collected. Heteronuclear multiple-bond correlation (HMBC) spectra were recorded with a relaxation delay of 1.5 s before each scan, a spectral width of 6k \times 35k, and 32 FIDs and 1k \times 256 data points collected. Chemical shifts are reported in parts per million (ppm) relative to deuterated solvent peaks as internal standards (δH , DMSO- d_6 2.50 ppm; δC , DMSO- d_6 39.52 ppm, δH , CD₃OH 3.30 ppm; δH , CDCl₃ 7.26 ppm; δC , CDCl₃ 77.16 ppm). Coupling constants (J) are given in hertz (Hz). Multiplicities of ¹H NMR signals are reported as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet.

General Procedure for Mannich Reactions. A solution of LDA (2.1 equiv) was added dropwise to a solution of the ester (2.0 equiv) in dry THF at -78 °C. After the mixture was stirred for 30 min, Ti(*O*-*i*Pr)₃Cl (4.2 equiv) in dry THF was added dropwise. The orange solution was stirred for 30 min and the imine (1.0 equiv) in dry THF was added dropwise. The mixture was stirred for 3 h or until thin-layer chromatography (TLC) showed full conversion of the imine. The mixture was quenched with saturated aqueous NH₄Cl and allowed to reach room temperature. Water was added and the mixture was decanted into a separatory funnel. EtOAc–water (1:1) was added to the remaining Ti precipitate, and the mixture was stirred vigorously for 5 min before being added to the separatory funnel. The aqueous phase was extracted with EtOAc and the combined organic phases were washed again with water, dried (MgSO₄), filtered, and concentrated in vacuo.

Azumamide A, (Z)-6-[(2R,5R,8R,11R,12S)-8-Benzyl-2-isopropyl-5,12-dimethyl-3,6,9,13-tetraoxo-1,4,7,10-tetraazacyclotridecan-11-yl]hex-4-enamide (5). LiOH (89 mg, 3.72 mmol, 85 equiv) in water (4.0 mL) was added to a stirred solution of the impure cyclic peptide **20** (24.2 mg, approximately 0.045 mmol) in THF (4 mL). After 2.5 h of stirring, the organic solvent was removed in vacuo. The aqueous phase was acidified with 1 M HCl to pH 2 and extracted with EtOAc (4 \times 30 mL) and CH₂Cl₂ (40 mL). The organic phases were dried (Na₂SO₄), filtered, and concentrated to afford crude azumamide E, which was used without further purification. Analytical UPLC–MS gradient A, $t_{\text{R}} = 1.47$ min. To a solution of the above crude azumamide E (≈ 0.045 mmol) in DMF (3.0 mL) were added HATU (34 mg, 0.09 mmol, 2 equiv), *i*Pr₂NEt (43 μL , 0.25 mmol, 5.5 equiv), and, after 5 min, NH₃–dioxane (0.9 mL, 0.45 mmol, 10 equiv). After 1 h, NH₃–dioxane (0.45 mL, 0.23 mmol, 5 equiv) was added. UPLC–MS analysis showed 50% conversion after 3 h, and HATU (34 mg, 0.09 mmol, 2 equiv) and NH₃–dioxane (0.45 mL, 0.23 mmol, 5 equiv) were added. After an additional 1 h, DMF (1.0 mL) followed by HATU (17 mg, 0.045 mmol, 1 equiv) and NH₃–dioxane (0.45 mL, 0.23 mmol, 5 equiv) were added, and stirring was continued for 1 h before concentration in vacuo. The residue was dissolved in MeCN–H₂O and purified by preparative HPLC to give azumamide A (**5**) (4.8 mg, 12% overall). $[\alpha]_{\text{D}}^{25} +56^{\circ}$ ($c = 0.2$, MeOH); previously reported¹¹ $[\alpha]_{\text{D}}^{25} +33^{\circ}$ ($c = 0.1$, MeOH). ¹H NMR (500 MHz, CD₃OH) δ 8.18 (d, $J = 8.6$ Hz, 1H), 8.16 (d, $J = 9.0$ Hz, 1H), 7.72 (d, $J = 8.2$ Hz, 1H), 7.59 (m, 2H), 7.20 (m, 5H), 6.74 (br s, 1H), 5.48 (m, 1H), 5.37 (m, 1H), 4.33 (dt, $J = 9.0, 7.0$ Hz, 1H), 4.24 (m, 2H), 3.81 (dd, $J = 10.4$ Hz and 8.4 Hz, 1H), 3.10 (m, 2H), 2.72 (m, 1H), 2.57 (dt, $J = 14.1, 6.9$ Hz, 1H), 2.41 (m, 2H), 2.27 (m, 4H), 1.30 (d, $J = 7.5$ Hz, 3H), 1.23 (d, $J = 7.3$ Hz, 3H), 0.96 (d, $J = 6.5$ Hz, 3H), 0.94 (d, $J = 6.7$ Hz, 3H). HRMS (ESI-TOF) m/z calcd for C₂₇H₃₈N₄O₇H⁺ 514.3029; found 514.3032 [M + H]⁺. HPLC gradient C, $t_{\text{R}} = 11.62$ min (>95%).

Azumamide B, (Z)-6-[(2R,5R,8R,11R,12S)-8-(4-Hydroxybenzyl)-2-isopropyl-5,12-dimethyl-3,6,9,13-tetraoxo-1,4,7,10-tetraazacyclotridecan-11-yl]hex-4-enamide (6). An aqueous solution of LiOH (0.5 M, 55 μL , 2.0 mmol, 2.5 equiv) was added to the cyclic peptide **18** (6.1 mg) in THF–H₂O (1:1, 2 mL) at 0 °C. After 30 min the ice bath was removed. Additional portions of LiOH solution (55 μL , 2.0 mmol, 2.5 equiv) were added after 2, 4, and 6 h, and stirring was continued for an additional 19 h to ensure full conversion. Then water (0.5 mL) was added and the organic solvent was removed in vacuo. The aqueous phase was acidified with 1 M HCl and extracted with EtOAc (5 \times 20 mL). The organic phase was dried (Na₂SO₄), filtered, and concentrated in vacuo to afford the crude azumamide C, which was used without further purification. To a solution of crude azumamide C (5.8 mg, 10.9 μmol) in DMF (2 mL) were added HOBt (4.4 mg, 33 μmol , 3 equiv), DIC (5.1 μL , 34 μmol , 3 equiv), and *i*Pr₂NEt (7.6 μL , 44 μmol , 4 equiv). After 10 min, NH₃–dioxane (0.5 M, 0.11 mL, 55 μmol , 5 equiv) was added. After 1.5 h, DIC (5 μL , 34 μmol , 3 equiv) was added, followed by NH₃–dioxane (0.5 M, 0.11 mL, 55 μmol , 5 equiv). After the mixture was stirred for 16 h, additional DIC (2 equiv) and NH₃–dioxane (5 equiv) were added, and this procedure was repeated once more after 18 h. Finally, CH₂Cl₂ (1 mL) was added, followed by DIC (3 equiv) and NH₃–dioxane (10 equiv), and after 2 days of stirring at room temperature, the reaction mixture was concentrated, dissolved in MeCN–H₂O (2:1), and purified by preparative HPLC to give azumamide B (**6**) (3.6 mg, 62%, two steps) as a white solid. $[\alpha]_{\text{D}}^{25} +65^{\circ}$ ($c = 0.15$, MeOH); previously reported¹¹ $[\alpha]_{\text{D}}^{25} +45^{\circ}$ ($c = 0.1$, MeOH). ¹H NMR (500 MHz, CD₃OH) δ 8.13 (d, $J = 7.9$ Hz, 1H), 8.00 (d, $J = 8.9$ Hz, 1H), 7.85 (d, $J = 7.2$ Hz, 1H), 7.61 (d, $J = 8.2$ Hz, 1H), 7.01 (d, $J = 8.4$ Hz, 3H), 6.67 (d, $J = 8.4$ Hz, 3H), 5.49 (m, 1H), 5.37 (dd, $J = 18.0$ and 7.3 Hz, 2H), 4.29 (pentet, $J = 7.2$ Hz, 1H), 4.15 (m, 1H), 4.05 (m, 1H), 3.60 (m, 1H), 3.13 (dd, $J = 13.7, 10.1$ Hz, 1H), 3.00 (dd, $J = 13.8, 6.3$ Hz, 1H), 2.70 (m, 2H), 2.36 (ddd, $J = 22.3, 21.5, 7.1$ Hz, 11H), 1.29 (d, $J = 7.2$ Hz, 3H), 1.27 (d, $J = 7.4$ Hz, 3H), 0.95 (d, $J = 5.7$ Hz, 3H), 0.93 (d, $J = 6.0$ Hz, 3H). HRMS (ESI-TOF) m/z calcd for C₂₇H₃₉N₅O₆H⁺ 530.2978; found 530.2973 [M + H]⁺. HPLC gradient C, $t_{\text{R}} = 10.31$ min (>95%).

Azumamide C, (Z)-6-[(2R,5R,8R,11R,12S)-8-(4-Hydroxybenzyl)-2-isopropyl-5,12-dimethyl-3,6,9,13-tetraoxo-1,4,7,10-tetraazacyclotridecan-11-yl]hex-4-enoic Acid (7). LiOH (49 mg, 2.0 mmol, 35 equiv) in water (5.0 mL) was added to the crude cyclic peptide **18** (61 mg) in THF (5.0 mL). The solution was stirred for 16 h and concentrated in vacuo. The resulting residue was dissolved in THF–H₂O (1:1, 10 mL) by adding a few drops of TFA, and then purification by preparative HPLC afforded azumamide C (**7**) (2.2 mg, 9% overall) as a white solid. $[\alpha]_D^{+49}$ ($c = 0.14$, MeOH); previously reported¹¹ $[\alpha]_D^{+21}$ ($c = 0.1$, MeOH). ¹H NMR (500 MHz, CD₃OH) δ 8.08 (d, $J = 7.7$ Hz, 1H), 7.99 (d, $J = 8.4$ Hz, 1H), 7.84 (s, 1H), 7.62 (d, $J = 8.1$ Hz, 1H), 7.01 (d, $J = 8.4$ Hz, 2H), 6.67 (d, $J = 8.4$ Hz, 2H), 5.48 (t, $J = 8.8$ Hz, 1H), 5.38 (dt, $J = 10.7, 7.0$ Hz, 1H), 4.29 (pentet, $J = 7.3$ Hz, 1H), 4.16 (m, 1H), 4.01 (m, 1H), 3.58 (m, 1H), 3.15 (dd, $J = 13.7, 10.2$ Hz, 1H), 3.00 (dd, $J = 13.7, 6.0$ Hz, 1H), 2.72 (m, 1H), 2.67 (m, 1H), 2.39 (m, 5H), 1.29 (d, $J = 7.2$ Hz, 3H), 1.27 (d, $J = 7.3$ Hz, 3H), 0.95 (d, $J = 6.0$ Hz, 3H), 0.93 (d, $J = 6.0$ Hz, 3H). HRMS (ESI-TOF) m/z calcd for C₂₇H₃₈N₄O₆H⁺ 531.2819; found 531.2815 [M + H]⁺. HPLC gradient C, $t_R = 11.04$ min (>95%).

Azumamide D, (Z)-6-[(2R,5R,8R,11R,12S)-8-Benzyl-2,5,12-trimethyl-3,6,9,13-tetraoxo-1,4,7,10-tetraazacyclotridecan-11-yl]hex-4-enamide (8). LiOH (53 mg, 2.21 mmol) in water (5.0 mL) was added to a stirred solution of the impure cyclic peptide **22** (22.7 mg, approximately 0.044 mmol) in THF (3 mL). After 4 h the organic solvent was removed in vacuo and the water (0.5 mL) was added to the aqueous phase, which was then acidified with 1 M HCl (2 mL) and extracted with EtOAc (4 × 20 mL). The organic phases were dried (MgSO₄), filtered, and concentrated to give the crude acid **23**, which was used without further purification. To a solution of the crude acid **23** (≈23 μmol) in CH₂Cl₂–DMF (8:1, 2.3 mL) were added HOBt (10 mg, 66 μmol, 3.0 equiv), DIC (10 μL, 66 μmol, 3 equiv), and *i*Pr₂NEt (15 μL, 88 μmol, 4 equiv). After 5 min, NH₃–dioxane (0.5 M, 0.22 mL, 110 μmol, 5 equiv) was added. After 1 h, NH₃–dioxane (0.5 M, 0.22 mL, 110 μmol, 5 equiv) was added. After the mixture was stirred for 18 h, additional DMF (0.5 mL) was added, followed by NH₃–MeOH (2.0 M, 0.11 mL, 230 μmol, 10 equiv). After an additional 5 h, DIC (7 μL, 46 μmol, 2 equiv) was added. The next day NH₃–MeOH (2.0 M, 0.06 mL, 111 μmol, 5 equiv) was added and the mixture was stirred for 10 days. Finally, DIC (3.4 μL, 23 μmol, 1 equiv) was added, followed by NH₃–MeOH (2.0 M, 0.055 mL, 210 μmol, 5 equiv), and after 2 days the mixture was concentrated, dissolved in MeCN–H₂O (2:1), and purified by preparative HPLC to afford azumamide D (**8**) (1.2 mg, 4% overall) as a white solid. $[\alpha]_D^{+32}$ ($c = 0.08$, MeOH); previously reported¹¹ $[\alpha]_D^{+25}$ ($c = 0.1$, MeOH). ¹H NMR (500 MHz, CD₃OH) δ 8.03 (d, $J = 7.8$ Hz, 1H), 8.01 (d, $J = 8.8$ Hz, 1H), 7.94 (d, $J = 6.8$ Hz, 1H), 7.58 (s, 1H), 7.32 (d, $J = 7.5$ Hz, 1H), 7.25–7.14 (m, 5H), 6.75 (s, 1H), 5.47 (m, 1H), 5.39 (m, 1H), 4.35 (m, 1H), 4.19 (m, 1H), 4.17–4.11 (m, 2H), 3.09 (m, 2H), 2.69 (m, 1H), 2.62 (m, 1H), 2.41 (m, 2H), 2.28 (m, 1H), 1.47 (d, $J = 7.4$ Hz, 3H), 1.28 (d, $J = 7.4$ Hz, 3H), 1.22 (d, $J = 7.3$ Hz, 3H). HRMS (ESI-TOF) m/z calcd for C₂₅H₃₅N₅O₅H⁺ 486.2716; found 486.2710 [M + H]⁺. HPLC gradient C, $t_R = 10.55$ min (>95%).

Azumamide E, (Z)-6-[(2R,5R,8R,11R,12S)-8-Benzyl-2-isopropyl-5,12-dimethyl-3,6,9,13-tetraoxo-1,4,7,10-tetraazacyclotridecan-11-yl]hex-4-enoic Acid (9). LiOH (18.5 mg, 0.77 mmol, 40 equiv) in water (4 mL) was added to a stirred solution of the impure cyclic peptide **20** (10.5 mg, approximately 0.02 mmol) in THF (4 mL). After 1 h, LiOH (10 mg, 0.42 mmol, 20 equiv) in water (1 mL) was added, and after 2 h, LiOH (5.0 mg, 0.21 mmol, 1 equiv) in water (0.5 mL) was added. The solution was stirred for 16 h and another portion of LiOH (6.0 mg, 0.25 mmol, 1.3 equiv) in water (0.5 mL) was added. After an additional 2.5 h of stirring, the organic solvent was removed in vacuo. The aqueous phase was acidified with 1 M HCl and extracted with EtOAc (4 × 25 mL). The combined organic phases were dried (Na₂SO₄), filtered, and concentrated. The resulting residue was dissolved in MeCN–water [(3:2), 2.5 mL] and purified by preparative HPLC to afford azumamide E (**9**) (4.3 mg, 15% overall) as a white solid. $[\alpha]_D^{+66}$ ($c = 0.2$, MeOH); previously reported¹¹

$[\alpha]_D^{+53}$ ($c = 0.1$, MeOH). ¹H NMR (500 MHz, CD₃OH) δ 8.10 (d, $J = 7.7$ Hz, 1H), 7.95 (d, $J = 8.8$ Hz, 1H), 7.84 (br s, 1H), 7.63 (d, $J = 8.4$ Hz, 1H), 7.28–7.16 (m, 5H), 5.48 (m, 1H), 5.37 (m, 1H), 4.28 (pentet, $J = 7.5$ Hz, 1H), 4.16 (m, 1H), 4.08 (m, 1H), 3.59 (m, 1H), 3.25 (dd, $J = 13.6, 10.4$ Hz, 1H), 3.11 (dd, $J = 13.6, 6.1$ Hz, 1H), 2.72 (m, 1H), 2.68 (m, 1H), 2.39 (d, $J = 1.7$ Hz, 6H), 2.39 (m, 6H), 1.28 (d, $J = 7.1$ Hz, 3H), 1.27 (d, $J = 7.4$ Hz, 3H), 0.94 (m, 6H). HRMS (ESI-TOF) m/z calcd for C₂₇H₃₈N₄O₆H⁺ 515.2869; found 515.2869 [M + H]⁺. HPLC gradient C, $t_R = 12.53$ min (>95%).

Assay Materials. HDAC1 (purity >45% by SDS–PAGE according to the supplier), HDAC4 (purity >90% by SDS–PAGE according to the supplier), and HDAC7 (purity >90% by SDS–PAGE according to the supplier) were purchased from Millipore (Temecula, CA). HDAC2 used for dose–response experiments (full length, purity ≥94% by SDS–PAGE according to the supplier), HDAC5 (full length, purity ≥4% by SDS–PAGE according to the supplier), and HDAC8 used for dose–response experiments (purity ≥90% by SDS–PAGE according to the supplier) were purchased from BPS Bioscience (San Diego, CA). HDAC2 used for initial screening experiments (full length, purity 50% by SDS–PAGE according to the supplier), HDAC3–“NCoR1” complex [(purity 90% by SDS–PAGE according to supplier; fusion protein of GST-tagged HDAC3 with the deacetylase activation domain (DAD) of NCoR1 (nuclear receptor corepressor)], HDAC6 (purity >90% by SDS–PAGE according to the supplier), HDAC8 for initial screening experiments (purity >50% by SDS–PAGE according to the supplier), HDAC10 (purity >50% by SDS–PAGE according to the supplier), and HDAC11 (purity >50% by SDS–PAGE according to the supplier) were purchased from Enzo Life Sciences (Postfach, Switzerland). HDAC9 (full length, purity 12% by SDS–PAGE according to the supplier) was purchased from Abnova (Taipei, Taiwan). The HDAC assay buffer consisted of 50 mM Tris–HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, and bovine serum albumin (0.5 mg/mL). Trypsin [10 000 units/mg, from bovine pancreas, treated with *l*-(tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK)] was from Sigma Aldrich (Steinheim, Germany). All peptides were purified to homogeneity (>95% purity by HPLC_{230nm} via reversed-phase preparative HPLC), and the white fluffy materials obtained by lyophilization were kept at –20 °C. For assaying, peptides were reconstituted in DMSO to give 5–10 mM stock solutions, the accurate concentrations of which were determined by coinjection on HPLC with a standard of known concentration.

In Vitro Histone Deacetylase Inhibition Assays. For inhibition of recombinant human HDACs, dose–response experiments with internal controls were performed in black low-binding Nunc 96-well microtiter plates. Dilution series (3-fold dilution, 10 concentrations) were prepared in HDAC assay buffer from 5–10 mM DMSO stock solutions. The appropriate dilution of inhibitor (10 μL of 5× the desired final concentration) was added to each well followed by HDAC assay buffer (25 μL) containing substrate [Ac-Leu-Gly-Lys(Ac)-AMC, 40 μM for HDAC1–3 and 80 μM for HDAC6 and 11; Ac-Leu-Gly-Lys(Tfa)-AMC, 40 μM for HDAC4, 240 μM for HDAC5, 80 μM for HDAC7, 400 μM for HDAC8, and 160 μM for HDAC9; Ac-Arg-His-Lys(Ac)-Lys(Ac)-AMC, 100 μM for HDAC10]. Finally, a solution of the appropriate HDAC (15 μL) was added and the plate was incubated at 37 °C for 30 min [HDAC1, 150 ng/well; HDAC2, 100 ng/well; HDAC3, 10 ng/well; HDAC4, 2 ng/well; HDAC5, 40 ng/well; HDAC6, 60 ng/well; HDAC7, 2 ng/well; HDAC8, 5 ng/well; HDAC9, 40 ng/well; HDAC10, 500 ng/well; HDAC11, 500 ng/well]. Then trypsin (50 μL, 0.4 mg/mL) was added and the assay development was allowed to proceed for 15–30 min at room temperature, before the plate was read on a Perkin-Elmer Enspire plate reader with excitation at 360 nm and detecting emission at 460 nm. Each assay was performed in duplicate. The data were analyzed by nonlinear regression with GraphPad Prism to afford IC₅₀ values from the dose–response experiments, and K_i values were determined from the Cheng–Prusoff equation [$K_i = IC_{50}/(1 + [S]/K_m)$] with the assumption of a standard fast-on–fast-off mechanism of inhibition.

■ ASSOCIATED CONTENT

■ Supporting Information

Two tables showing cyclization experiments performed on a simplified model peptide and IC₅₀ values from dose–response experiments; seven figures showing comparison of ¹H and ¹³C chemical shifts for **S18** with previously reported values, ¹H NMR data comparisons for azumamides A–E, and dose–response curves for determination of IC₅₀ values for “active” inhibitors; two schemes illustrating synthesis of β³-epi building block (**S6**) and β²-epi building block (**S11**); additional text with full experimental details and compound characterization data; and ¹H and ¹³C NMR spectra. A CIF file for the X-ray crystal structures is available (CCDC 933151). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AMC, 7-amino-4-methylcoumarin; Boc, *tert*-butoxycarbonyl; DAD, deacetylase activation domain; DIC, *N,N'*-diisopropylcarbodiimide; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; ESI, electrospray ionization; FID, free induction decay; Fmoc, fluorenylmethyloxycarbonyl; H3, histone 3 protein; H4, histone 4 protein; HATU, *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate; HDAC, histone deacetylase; HMPA, hexamethylphosphoramide; HOBT, hydroxybenzotriazole; HPLC, high-performance liquid chromatography; KHMDS, potassium hexamethyldisilazide; LDA, lithium diisopropylamide; MS, mass spectrometry; NCoR, nuclear receptor corepressor; NMR, nuclear magnetic resonance; PMB, *p*-methoxybenzyl; rt, room temperature; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TOF, time-of-flight; *t*_R, retention time; UPLC, ultra-high-performance liquid chromatography

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