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Microsporins A and B: new histone deacetylase inhibitors from the marine-derived fungus *Microsporum* cf. *gypseum* and the solid-phase synthesis of microsporin A

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Abstract—Two new cyclic peptides, microsporins A and B (**7** and **8**), were isolated from culture extracts of the marine-derived fungus *Microsporum* cf. *gypseum* obtained from a sample of the bryozoan *Bugula* sp. collected in the U.S. Virgin Islands. The structures of the new compounds were determined by extensive interpretation of 2D NMR data and by chemical methods. Microsporins A and B are potent inhibitors of histone deacetylase and demonstrate cytotoxic activity against human colon adenocarcinoma (HCT-116), as well as against the National Cancer Institute 60 cancer cell panel. The total synthesis of microsporin A on solid-phase is also reported.

1. Introduction

The nucleosome is comprised of DNA and core histones, which are proteins that are subjected to reversible acetylation at the ε-amino group of specific lysine residues.[1] Histone acetylation reduces the net positive charge of the histone by converting the ammonium functionalities of lysine residues to amides. Acetylation plays a crucial role in modulating interactions between histones (positively charged proteins) and DNA (negatively charged polynucleotides) and is a post-translational modification that is correlated with transcription, chromatin assembly, DNA repair, and recombinational activities.[2] Histone deacetylases (HDACs) comprise a family of enzymes that catalyze the removal of acetyl groups from lysine residues of histones and mediate chromatin remodeling and gene expression.[3] Three families of HDACs have been identified in eukaryotes, termed Class I, II, and III. Class I (HDAC 1, 2, 3, and 8) and Class II (HDAC 4, 5, 6, 7, 9, and 10) (HDAC 11 is at the boundary between Class I and II) are Zn^{2+}-dependent enzymes that are important therapeutic targets for the treatment of cancer.[4] HDAC inhibitors, potent inducers of growth arrest, differentiation, and apoptotic cell death, suppress cell proliferation in a variety of transformed cells in culture and in tumor bearing animals, have great promise as new cancer drugs,[5] and are well tolerated by cancer patients.[6]

The principal classes of HDAC inhibitors include hydroxamates,[7] short-chain fatty acids, cyclic tetrapeptides containing the 6-oxo-7,8-epoxyoctyl side chain [such as chlamydocin (**1**)[8] and trapoxin (**2**)[9]], cyclic tetrapeptides without the epoxy side chain [such as apicidin A (**3**)[10]], benzamides, and electrophilic ketones.[11] Crystal structures of a hyperthermophilic bacterium-derived HDAC[12] and of human HDAC8,[13] with the benzamide hydroxamate, suberoylanilide hydroxamic acid (**4**, SAHA), bound shows that the hydroxamate part, which corresponds to the carbonyl or epoxide in the side chain of **1**–**3**, extends into a deep cleft and coordinates to the active-site zinc ion.

Very few marine natural products have been identified as HDAC inhibitors. These include several bromotyrosine analogues of the psammaplin A (**5**) family of marine natural products, which were isolated from various marine sponges.[14] Recently, azumamides A–E (**6** shows the structure of azumamide A), the first example of cyclic tetrapeptides isolated from a marine source (the marine sponge *Mycale izuensis*), were reported.[15] These natural products contain an unusual β-amino acid [(2*S*,3*R*)-3-amino-2-methyl-5-nonenedioic

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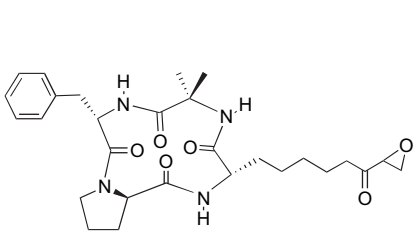

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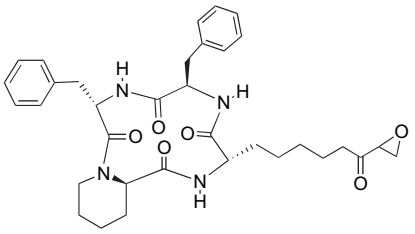

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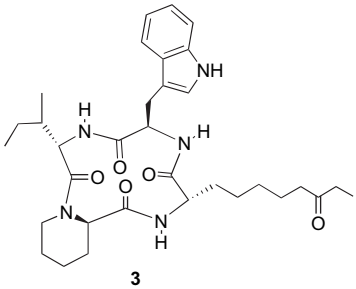

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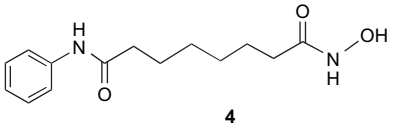

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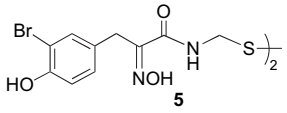
acid or -amide], resulting in 13-membered cyclic tetrapeptide rings; all of the other amino acids have D configurations. Azumamide A was reported to exhibit moderate cytostatic activity on a human colon cancer (IC_{50} 5.8 μM) and a human leukemia (IC_{50} 4.5 μM).



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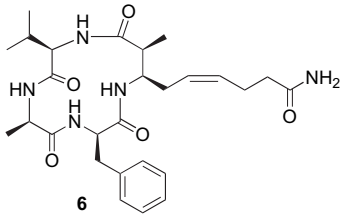

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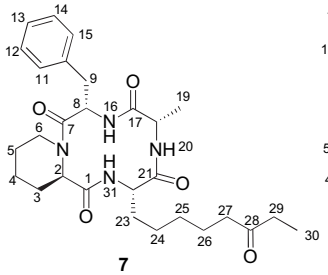
Here we report the isolation and characterization of microsporin A (**7**) and microsporin B (**8**), the first cyclic tetrapeptide HDAC inhibitors with cytotoxic activity isolated from a marine microorganism (a cultured marine-derived fungus identified as *Microsporum* cf. *gypseum*). Unlike the azumamides, these cyclic tetrapeptides contain all α-amino acids and, therefore, are comprised of 12-membered cyclic peptide rings. Also, three of the amino acids have L configurations, while the pipecolic acid has a D configuration.



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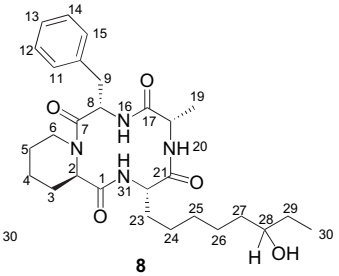

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2. Results and discussion

2.1. Natural products isolation

Continuing with our program to search for new metabolites that inhibit cancer cell proliferation from cultured marine fungi, we have isolated microsporin A (**7**) and microsporin B (**8**), cyclic tetrapeptides containing the unusual amino acid Aoda ((*S*)-2-amino-8-oxodecanoic acid) and its hydroxyl derivative ((2*S*)-2-amino-8-hydroxydecanoic acid), respectively. Aoda has been found in other cyclic tetrapeptides including the potent protozoan HDAC inhibitor apicidin (**3**) and 9,10-deepoxy-chlamydocin.[16] In addition to Aoda, the closely related amino acid Aeo (2-amino-9,10-epoxi-8-oxodecanoic acid) also contains the aminodecanoic acid backbone. Cyclic tetrapeptides containing Aeo include fungal metabolites such as chlamydocin (**1**)[8] and others[9,17–20] that have Aeo–Pro or Aeo–Pip residues. These compounds are known to possess significant and broad-ranging biological activities.

Microsporins A (**7**) and B (**8**) were isolated from the culture extract of fungal strain CNL-629 by bioassay-guided fractionation. The producing fungus was obtained from a sample of the bryozoan *Bugula* sp. collected in the U.S. Virgin Islands, and identified as *M.* cf. *gypseum* by fatty acid methyl ester (FAME) analysis.[21]

2.2. Structure elucidation

Microsporin A (**7**) analyzed form the molecular formula $C_{28}H_{40}N_4O_5$ by high-resolution FAB mass spectrometry ([M+Na]$^+$ *m/z* 535.2881; calcd 535.2896). The ^{1}H and ^{13}C NMR spectra indicated resonances typical of a peptide (Table 1). The presence of four carbonyl resonances (171.8; 173.9; 174.6; and 175.8 Hz) suggested that the compound

Table 1. NMR spectral assignments for microsporins A and B in $CDCl_3$

#	Microsporin A (**7**)		Microsporin B (**8**)	
	^1H[a] J (Hz)	^{13}C[b]	^1H[a] J (Hz)	^{13}C[b]
1	—	171.8	—	171.8
2	5.02, (d, 4.8)	51.1	5.04 (d, 4.8)	51.1
3	2.04 (m), 1.57 (m)	24.2	2.04 (m), 1.48 (m)	24.2
4	2.10 (m), 1.50 (m)	19.2	1.50 (m), 1.48 (m)	19.5
5	1.76 (m), 1.30 (m)	25.5	1.71 (m), 1.27 (m)	25.3
6	3.91 (d, 12.0), 3.02 (m)	44.2	3.91 (d, 12.6), 3.01 (m)	44.2
7	—	173.9	—	173.9
8	5.3 (q, 10, 15.5)	50.1	5.31 (dd, 9.3, 15.0)	50.1
9	3.24 (dd, 7.2, 14.5), 3.01 (m)	36.7	3.25 (dd, 7.2, 13.7), 3.02 (m)	36.9
10	—	137.1	7.10–7.30 (m)	137.1
11	7.10 (m)	129.2	7.10–7.30 (m)	129.3
12	7.25 (m)	128.7	7.10–7.30 (m)	128.7
13	7.20 (m)	126.9	7.10–7.30 (m)	126.9
14	7.25 (m)	128.7	7.10–7.30 (m)	128.7
15	7.10 (m)	129.2	7.10–7.30 (m)	129.3
16	7.27 (d, 11.4)	—	7.30 (d, 11.4)	—
17	—	174.6	—	174.6
18	3.73 (m)	56.4	3.76 (m)	56.1
19	1.69 (d, 7.5)	16.2	1.68 (d, 7.5)	16.2
20	6.61 (d, 6.0)	—	6.76 (d, 5.4)	—
21	—	175.8	—	175.6
22	4.27 (m)	54.0	4.30 (m)	54.2
23	1.76 (m)	25.3	1.60 (m)	25.6
24	1.30 (m)	29.2	1.30 (m)	29.4
25	1.50 (m)	28.9c	1.74 (m)	29.4
26	1.50 (m)	23.7c	1.30 (m)	25.3
27	2.39 (m)	42.3	1.24 (m)	30.5
28	—	211.7	3.91 (d, 12.6)	73.5
29	2.41 (q, 7.5, 15.3)	36.2	1.42 (m)	36.8
30	1.05 (t, 7.2)	8.1	0.94 (t, 7.2)	10.2
31	6.42 (d, 10.2)	—	6.44 (d, 10.2)	—

[a] Recorded at 300 MHz. Assignments are by interpretation of COSY, 1D-TOCSY, HMQC, and HMBC spectral data.
[b] Recorded at 100 MHz. Assignments are by interpretation of DEPT, HMQC, and HMBC spectral data.

was a cyclic tetrapepetide. The peptide nature of the molecule was further supported by the presence of three NH proton resonances in the ^1H NMR spectrum at δ 6.42 (d, 10.2), 6.61 (d, 6.0), and 7.27 (d, 11.4). The presence of a carbon resonance at δ 211.7 Hz suggested the presence of a ketone in one of the amino acids. Subsequently, the planar structure of microsporin A was fully assigned using a combination of 1- and 2D NMR spectral methods (Table 1). Analysis of 1D-TOCSY spectral data indicated the presence of one unit of alanine, while analysis of combined 1D-TOCSY and HMBC data established the presence of one unit each of phenylalanine, pipecolic acid, and the unusual amino acid, 2-amino-8-oxo-decanoic acid (Aoda). The connectivities within each amino acid were established by HMBC experiments, which showed correlations of the carbonyl carbons of each amino acid with the corresponding NH proton of the adjacent amino acid, except for the carbonyl at δ 173.9 Hz, which showed a correlation with the α-proton of Pip.

Microsporin B (**8**), which had a very close HPLC retention time to microsporin A, was assigned the molecular formula $C_{28}H_{42}N_4O_5$ by analysis of HRFABMS data ($[M+Na]^+$ m/z 537.3039; calcd 537.3053). The ^1H and ^{13}C NMR spectra of these compounds were also very similar; the most significant difference was the presence in microsporin B of a methine carbon at δ 73.5 Hz instead of the ketone group in **1**. The structural difference between microsporins A and B was found to lie in the substitution of the Aoda amino acid-bearing ketone for a secondary hydroxyl group. The structure of microsporin B was confirmed by facile conversion to microsporin A (**7**) by oxidation with PDC.[22]

The absolute configurations of the amino acids in microsporins A and B were assigned by acid hydrolysis[23] followed by derivatization of the amino acids with Marfey's reagent[24] and HPLC analysis. This method was successful in determining the absolute configurations of Ala (L), Pip (D), and Phe (L), based on retention times and co-injection with the appropriate standards. Once the absolute configurations of these three amino acids have been established, that of the unusual amino acid, Aoda, was determined using NMR spectral methods, relying on NOESY correlations, as an authentic standard was unavailable. Because of demand for compound for screening purposes, the absolute configuration at C-8 (the hydroxyl-containing carbon), which in principle could have been defined in microsporin B by application of the Mosher method, could not be explored.

A preferred conformation for microsporin A was deduced by interpretation of NOE spectral data considering the absolute configurations of the amino acids Ala, Pip, and Phe. The phenylalanine NH showed NOE correlations with the phenylalanine methylene group and with the alanine methyl group. The phenylalanine α-H showed an NOE correlation with one proton of Pip, and another correlation was observed between the Pip α-H and the Aoda NH. These data permitted us to establish the solution conformation of **1** shown in Figure 1.

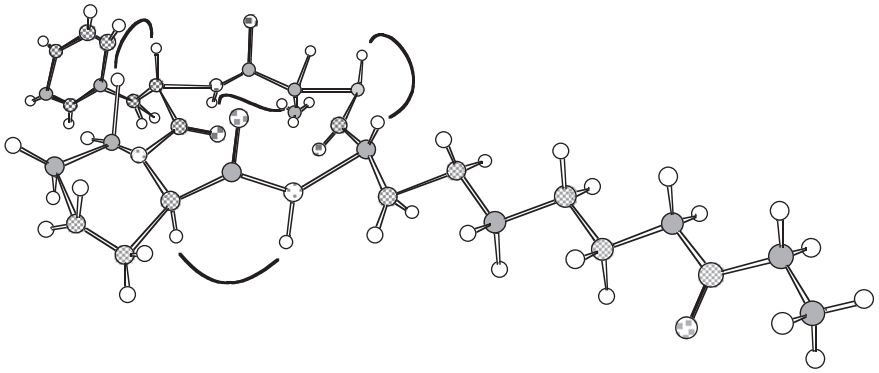
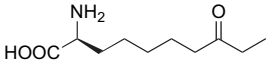


Figure 1. Selected NOESY correlations of microsporin A (**7**). The curved lines represent the NOESY correlations.

At the same time, NOE correlations were observed between the alanine α-H and the Aoda α-H with alanine NH. Thus, the Aoda functionality was assigned an L configuration (*S*), which also agrees with the coupling constant of the NH protons, indicating that, with the exception of the alanine residue, all of the NH groups have an *anti*-relationship with their respective α-protons.

2.3. Synthesis of microsporin A

The total synthesis of microsporin A (**7**) was achieved using solid-phase methods. The unusual keto amino acid, (*S*)-2-amino-8-oxodecanoic acid (Aoda, **9**), is the key amino acid in microsporin A. The synthesis of **9** was reported in 2001,[25] however a shorter synthetic route to **9** published in 2003,[26,27] was the synthetic route that we employed to prepare Boc-**9** for incorporation into the cyclic tetrapeptide microsporin A.



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As shown in Scheme 1, the safety-catch sulfonamide linker resin **10** of Ellman and co-workers,[28] which has already been used as a linker resin in cyclic peptide synthesis,[29] was selected as the linker resin for the solid-phase synthesis of microsporin A. Fmoc–D-Pip–OH was selected as the first amino acid residue to attach to the resin because of its D configuration.[30] Then Fmoc–Phe–OH, Fmoc–Ala–OH, and Boc–Aoda–OH were coupled to the growing peptide chain on the resin to generate linear tetrapeptide resin **14**. Activation of **14** by treatment with ICH_2CN under basic conditions gave activated resin **15**. Boc deprotection of **15** followed by cyclization and cleavage completed the synthesis of microsporin A.

2.4. Bioactivity

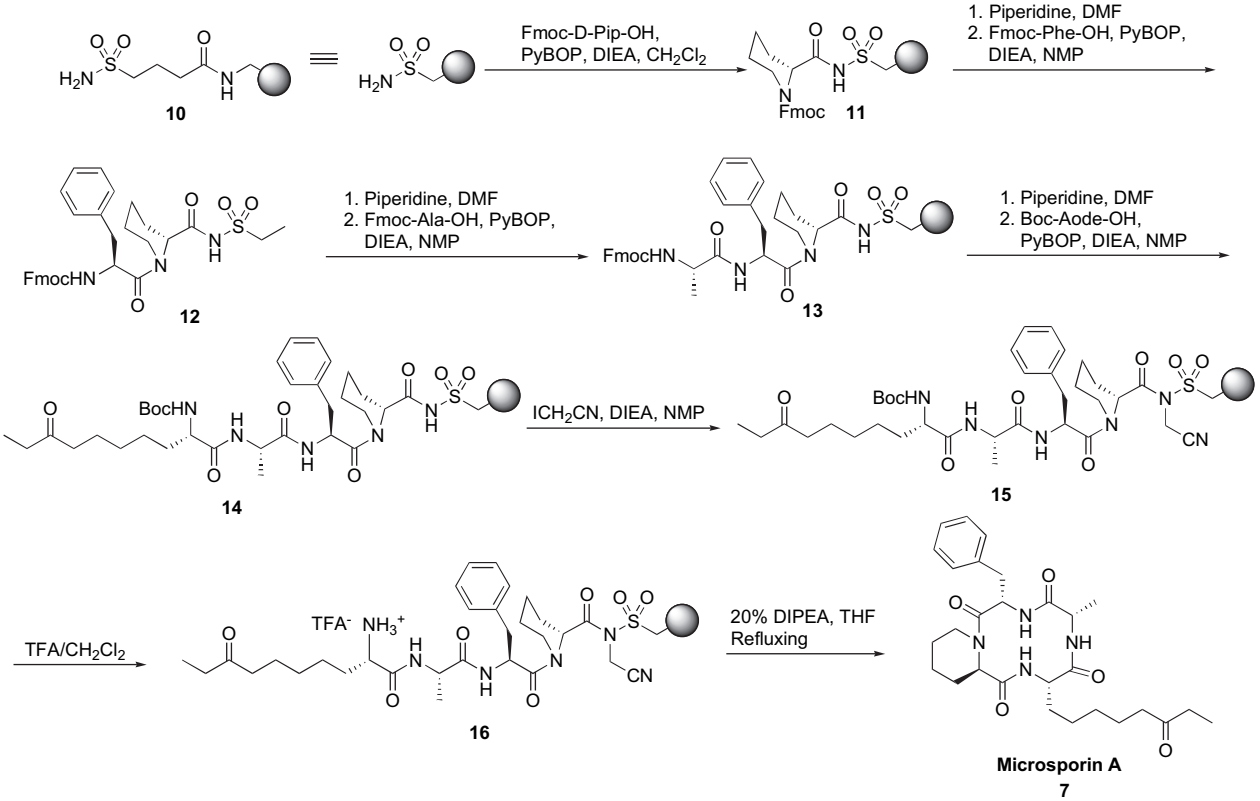
Microsporin A (**7**) showed in vitro cytotoxicity against human colon adenocarcinoma HCT-116 (IC_{50} 0.6 μg/mL) and a mean IC_{50} value of 2.7 μM in the National Cancer Institute's diverse 60-cell line panel. Microsporin B showed reduced in vitro cytotoxicity against HCT-116 (IC_{50} 8.5 μg/mL), which may indicate the importance of the Aoda ketone carbonyl group for biological activity. Microsporin A (**7**) was tested as an inhibitor of histone deacetylase, and it exhibited greater in vitro inhibition against both a mixture of HDACs and HDAC8 than the known antitumor agent HDAC inhibitor SAHA (**4**) (Table 2).

Table 2. HDAC inhibition by microsporin A (**7**) and SAHA (**4**)

Compound	HDACs IC_{50} (μM)	HDAC8 IC_{50} (μM)
Microsporin A (**7**)	0.14±0.01	0.55±0.01
SAHA (**4**)	0.30±0.02	0.78±0.03

3. Conclusions

The microsporins A and B are the first cyclic tetrapeptide HDAC inhibitors with significant cancer cell cytotoxicities



Scheme 1. Total synthesis of microsporin A.

isolated from a marine microorganism. Their potent inhibition of HDAC, coupled with significant in vitro cytotoxicity against numerous cancer cell lines, provides ample justification to suggest these peptides to be evaluated in in vivo antitumor assays. The first synthesis of microsporin A reported here utilizes a sulfonamide linker resin for the first time in a solid-phase synthesis of a cyclic tetrapeptide.

4. Experimental

4.1. Isolation of the microsporins

Microsporum cf. *gypseum*, strain CNL-629, was cultured without shaking for 32 days in 20×2.8 L Fernbach flasks each containing 1 L of the seawater-based nutrient medium YPG (5 g yeast extract, 5 g peptone, 10 g glucose, 1 L seawater). The mycelium and the broth were combined and extracted together with ethyl acetate. The extract was then concentrated and subjected to reversed-phase flash chromatography followed by size exclusion chromatography (Sephadex LH-20) and reversed-phase HPLC to afford the two new cyclic tetrapeptides, microsporin A (**7**, 4 mg) and microsporin B (**8**, 3.5 mg). For microsporin A (**7**): colorless oil, $[\alpha]_D$ +11.6 (*c* 0.17, CH_2Cl_2); UV λ_{max} ($CHCl_3$): 270 (ε=3510), 242 (ε=1670); IR ν_{max} (film, NaCl) 3283, 2931, 1684, 1525, 1449, 1266 cm^{-1}; for microsporin B (**8**): colorless oil, $[\alpha]_D$ −39.8 (*c* 0.44, CH_2Cl_2); UV λ_{max} ($CHCl_3$): 242 (ε=13260); IR ν_{max} (film, NaCl) 3307, 2920, 1684, 1660, 1519, 1449, 1261 cm^{-1}.

4.2. General synthetic methods

Common organic solvents were purchased from Fisher. All of the reagents were purchased from Aldrich Chemical Co. and were used without further purification unless stated otherwise. Methylene chloride was distilled under N_2 from calcium hydride. Flash chromatography was performed with Merck silica gel (230–400 mesh). TLC plates (silica gel 60-F254) were purchased from VWR Scientific. All ^1H NMR spectra were recorded on Varian Gemini 300 MHz, Mercury 400, or Inova 500 spectrometers (75, 100, or 125 MHz for ^{13}C NMR spectra). Chemical shifts (δ) are reported downfield from tetramethylsilane (Me_4Si) in parts per million (ppm). Compounds were visualized with a ninhydrin spray reagent or a UV–vis lamp. Mass spectra were recorded either on a VG Instrument VG70-250SE high-resolution mass spectrometer (ESI) or on a Micromass Quattro II spectrometer (APCI).

4.3. (*S*)-2-*tert*-Butoxylcarbonylamino-8-oxo-decanoic acid (Boc–Aoda–OH, Boc-9)

To a stirred solution of 2 M NaOH (3 mL) was added methyl (*S*)-2-amino-8-oxo-decanoate[26] (100 mg, 0.47 mmol) and then Boc_2O (150 mg, 0.70 mmol) at 0 °C. The reaction mixture was allowed to rise to room temperature; TLC analysis showed the reaction was completed overnight. The mixture was acidified with 0.5 N HCl (20 mL) and extracted with EtOAc (3×20 mL). The combined organic phases were washed with brine, dried over sodium sulfate, and concentrated in vacuo to give compound **3** (117 mg, 86%) as a colorless oil; ^1H NMR (500 MHz, $CDCl_3$) δ 1.02 (t, *J*=7.5 Hz, 3H), 1.30 (m, 2H), 1.39 (m, 2H), 1.42 (s, 9H), 1.56 (m, 2H), 1.65 (br s, 1H), 1.82 (br s, 1H), 2.40 (m, 4H), 4.27 (br s, 1H), 5.14 (d, *J*=6.5 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 7.98, 23.70, 25.21, 28.46, 32.41, 36.06, 42.29, 53.44, 80.24, 155.82, 177.18, 212.48. MS (ESI): 302.2 (M^++1), 324 (M^++Na^+). HRMS (CI): *m*/*z* calcd for $C_{15}H_{28}NO_5$ [M+H^+], 302.1967; found, 302.1975. Anal. calcd for $C_{15}H_{27}NO_5$ C, 59.78; H, 9.03; N, 4.65. Found C, 59.90; H, 9.10; N, 4.45.

4.4. Resin-bound linear tetrapeptide 14

A suspension of resin **10**[28] (100 mg, 0.4 mmol/g) in anhydrous CH_2Cl_2 (10 mL) was treated with Fmoc–Pip–OH (5 equiv), PyBOP (5 equiv), and DIPEA (15 equiv) at −20 °C for 12 h. After being washed with DMF (5×5 mL), THF (5×5 mL), CH_2Cl_2 (5×5 mL), and anhydrous DMF (5×5 mL), the resin was again treated with Fmoc–Pip–OH (5 equiv), PyBOP (5 equiv), and DIPEA (15 equiv) at −20 °C for 12 h. After being washed with DMF (5×5 mL), THF (5×5 mL), CH_2Cl_2 (5×5 mL), resin **11** was treated with 20% piperidine in DMF (10 mL) for 2 h to remove the Fmoc group. After being washed with DMF, THF, CH_2Cl_2, and anhydrous DMF, resin **12** was immersed in anhydrous NMP (10 mL), and treated with Fmoc–Phe–OH (5 equiv), PyBOP (5 equiv), and DIPEA (15 equiv) at room temperature for 12 h. After being washed with DMF (5×5 mL), THF (5×5 mL), CH_2Cl_2 (5×5 mL), and anhydrous DMF (5×5 mL), the resin was cycled through the same set of conditions for coupling, washing, deprotection, and washing as above, using Fmoc–Ala–OH (5 equiv) then Boc-**9** (5 equiv) successively in the peptide elongation for the generation of **14**. The resin was washed sequentially with NMP (5×5 mL), DMF (5×5 mL), and CH_2Cl_2 (5×5 mL), and dried in a stream of nitrogen.

4.5. Activation of the carboxyl terminus of 14 to give 15

Resin **14** was swollen and washed with NMP (5×5 mL) and then immersed in a mixture of NMP, DIPEA, and iodoacetonitrile, which was filtered through a basic alumina plug. After being stirred for 24 h in a 10 mL reaction vessel wrapped in aluminum foil, the resin was washed sequentially with NMP (5×5 mL), DMF (5×5 mL), and CH_2Cl_2 (5×5 mL), and dried in a stream of nitrogen, giving **15**.

4.6. Removal of the Boc protecting group of 15

Protected linear tetrapeptide **15** was treated with a 1:1 mixture of TFA/CH_2Cl_2 (5 mL) for 0.5 h at room temperature and was washed sequentially with CH_2Cl_2 (5×5 mL), methanol (5×5 mL), and THF (5×5 mL), and dried in a stream of nitrogen to give **16**.

4.7. Synthetic microsporin A (7)

Dried resin **16** was immersed in 50% DIPEA (5 mL) in DMF for 24 h at 50 °C, filtered, and washed with THF. The filtrates were combined, and the solution was dried in vacuo to obtain the crude product. Flash chromatography of the crude product gave microsporin A (**7**, 2.0 mg) as a colorless oil, which had the virtually identical spectral and chromatographic properties as the natural product. $[\alpha]_D^{23}$ +12.2 (*c* 0.06,

$CHCl_3$). MS (ESI): 513.3 (M^++1). HRMS (CI): *m/z* calcd for $C_{28}H_{41}N_4O_5$ [M+H^+], 513.3077; found, 513.3077.

4.8. HDACs and HDAC8 enzyme assays

The HDAC inhibition assay was performed using the HDAC fluorescent activity assay kit (Biomol Research Laboratories, Plymouth Meeting, PA). HeLa cell nuclear extract, which contains several HDAC isozymes and other nuclear factors, was used as the source of HDAC activity. The final substrate concentration in the assay mixture was 50 μM. The reaction was allowed to proceed for 30 min at 37 °C before the reaction was terminated. Test compounds were prepared as 1 mM stock solutions in DMSO (Molecular Biology grade, Sigma–Aldrich Co., St. Louis, MO) and stored at 0 °C. The final DMSO concentration in the wells was not more than 2%. Assays were performed in white polystyrene 96-well half-area assay plates (Corning, NY), and the fluorescence was measured on a Gemini EM Fluorescence/Chemiluminescence Plate reader with an excitation wavelength of 355 nm and an emission wavelength of 460 nm, using SOFTmax PRO software. The HDAC8 inhibition assay was performed as above using the HDAC8 fluorescent activity assay kit (Biomol Research Laboratories, Plymouth Meeting, PA). The final substrate concentration in the assay mixture was 25 μM. The reaction was allowed to proceed for 45 min at 37 °C before being terminated.

4.9. HCT-116 human colon adenocarcinoma assay

Human colon adenocarcinoma cells (HCT-116) were plated in 96-well plates and incubated overnight at 37 °C in 5% CO_2/air. Peptides **7** and **8** were added to the plate and serially diluted. The plate was then incubated for a further 72 h. Cell viability was assessed at the end of this period through the use of a CellTiter 96 AQ_{ueous} non-radioactive cell proliferation assay (Promega). Inhibition concentration (IC_{50}) values are interpreted from the bioreduction of MTS/PMS by living cells into a formazan product. The first step of the assay is the addition of MTS/PMS to the sample wells followed by a 3 h incubation. The quantity of the formazan product (proportional to the number of living cells) in each well was then determined using a Molecular Devices Emax microplate reader that measured the amount of 490 nm absorbance in each well, and the IC_{50} value was calculated by a SOFTMax analysis program. Etoposide (Sigma) and DMSO (solvent) were used as positive and negative controls, respectively.

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