Supporting Information

Macrocyclic Peptoid–Peptide Hybrids as Inhibitors of Class I Histone Deacetylases

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Contents:

Materials and Methods

Enzymes and Chemicals. Fmoc α-amino acids, chlorotrityl resin, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophospate (HBTU), 2-(1*H*-azabenzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophospate (HATU), and PyBroP were purchased from Novabiochem (San Diego, CA). Fmoc-β^3-leucine was purchased from Peptech Corporation (Burlington, MA). Unless otherwise noted, all solvents and chemicals were reagent grade and were used as received. ^{1}H NMR spectra were obtained on a Bruker AMX-500 (500 MHz). Solvent peaks (DMSO-d_6) were used as an internal reference for ^{1}H at 2.50 ppm. Analytical and preparative RP-HPLC were carried out with C_{18} columns using gradients of solvent A (99:1:0.1 [water/acetonitrile/TFA]) and solvent B (10:90:0.07 [water/acetonitrile/TFA]). HDAC1, HDAC2, and HeLa nuclear extract were from Biomol International (Plymouth Meeting, PA). HDAC3–NCoR2, HDAC6, and HDAC8 were purchased from BPS Biosciences (San Diego, CA). Trypsin (TPCK treated, from bovine pancreas; 12,500 units/mg) was from Sigma-Aldrich (Milwaukee, WI). HDAC assay buffer was prepared as described in the Biomol International protocol AK-500 [Tris/Cl (50 mM), NaCl (137 mM), KCl (2.7 mM), $MgCl_2$ (1 mM), pH 8.0]. Anti acetylated α-tubulin antibody was from Sigma (St. Louis, MO). Histone H3 antibody was from Abcam (Cambridge, MA), acetylated K9+K14 histone antibody was from Upstate (Temecula, CA), and acetylated α-tubulin antibody was from Sigma (St. Louis, MO).

Fmoc-β*N*leu-OH [or Fmoc-(*N*-*i*Bu)-βAla-OH]. *tert*-Butyl acrylate (4.4 mL, 30 mmol) and isobutylamine (1.5 mL, 15 mmol) were dissolved in DMSO (5 mL) and stirred in a sealed vessel under microwave irradiation at 150 ºC for 30 min (using a Biotage Initiater M.W. reactor). The mixture was cooled to room temperature, diluted with EtOAc (200 mL), and washed with water (4 × 100 mL) and brine (100 mL). The organic layer was then dried (Na_2SO_4), filtered, concentrated, and dried under vacuum. The crude Michael adduct was used without further purification, dissolved in DMF (10 mL), and treated with Fmoc-OSu (1.1 equiv) and *i*Pr_2EtN (1.1 equiv) for 2 hours at room temperature. The DMF was evaporated in vacuo, EtOAc (200 mL) was added, and the solution was washed with water (2 × 50 mL), 1 N HCl (2 × 50 mL), sat. $NaHCO_3$ (2 × 50 mL), and brine (50 mL). The organic layer was then dried (Na_2SO_4), filtered, concentrated, and the residue was purified by vacuum liquid chromatography (4 × 4 cm column) to give the *t*Bu ester as a colorless syrup. TLC: R_f 0.38 (hexanes–EtOAc 5:1). Finally, the product was obtained after treatment with 40% TFA–CH_2Cl_2 for 2 h, followed by extensive co-evaporation and drying under high vacuum. Yield = 3.1 g (43% for three steps). ^{13}C NMR (125 MHz, $CDCl_3$): δ 177.2, 156.4, 144.2 (2 C), 141.6 (2 C), 127.8 (2 C), 127.3 (2 C), 124.9 (2 C), 120.1 (2 C), 67.0, 55.4, 47.6, 44.3* + 43.3* (1 C rotamers), 33.0, 27.5, 20.0 (2 C); $HPLC_{230nm}$ >98% purity; ESI-MS: $[M + Na]^+$ obsd. = 390.13 (calc. = 390.17).

6-Oxo-octylamine submonomer. *N*-Boc-6-amino-hexanoic acid was converted to the corresponding Weinreb amide under standard peptide coupling conditions (*N*,*O*-dimethyl-hydroxylamine, HBTU, *i*Pr_2EtN, DMF). The crude product was reacted with ethylmagnesium bromide Grignard without further purification after the work-up. The Weinreb amide (22 mmol theoretical) was dissolved in dry THF–diethylether 1:1 (40 mL) and stirred on an ice-bath. BrMgEt (3.0 M in Et_2O, 23 mL) was added portionwise over 30 min, and after further stirring for 30 min the bath was removed and the reaction was allowed to proceed at RT for 3 h. Then the mixture was quenched by addition of 4 N HCl, EtOAc (250 mL) was added, and the organic phase was washed with 1 N HCl (2 × 50 mL), saturated $NaHCO_3$ (2 × 50 mL), and brine (50 mL). The organic layer was then dried ($MgSO_4$), filtered, and concentrated. The resulting residue was purified by vacuum liquid chromatography (5 × 5 cm column) to give 1.6 g (29% for three steps) of the Boc-protected submonomer building block, which was deprotected with 40% TFA–CH_2Cl_2 immediately prior to use. ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.51 (1 H, br. s), 3.09 (2 H, m) 2.41 (4 H, m), 1.59 (2 H, m), 1.48 (2 H, m), 1.43 (9 H, s), 1.30 (2 H, m), 1.04 (3 H, t, J = 7.3 Hz) ; ESI-MS: $[M + Na]^+$ obsd. = 265.86 (calc. = 266.17).

Compound S4. The 2-nitrobenzenesulfonyl (nosyl)-protected building block was prepared from β-alanine methylester, under standard Fukuyama conditions.[S1] Briefly, β-alanine methylester (3.85 g, 27.6 mmol) was N-nosylated by treatment with 2-nitrobenzenesulfonyl chloride (6.58 g, 29.7 mmol, 1.08 equiv) and *i*Pr_2EtN (12.8 mL, 77.3 mmol, 2.8 equiv) in dry CH_2Cl_2 (50 mL) at RT for 16 h. Aqueous work-up and drying under high vacuum yielded **S2** (7.73 g, 97%) as a yellow syrup that was used without further purification. Compound **S2** (1.38 g, 4.78 mmol) was then dissolved in DMF (10 mL), and K_2CO_3 (1.32 g, 9.56 mmol, 2 equiv) was added followed by 3,3-dimethylallylbromide (613 µL, 5.26 mmol, 1.1 equiv) and the mixture was stirred at RT for 16 h. The solvent was evaporated under reduced pressure, and the residue was subjected to aqueous work-up to give crude **S3**, which was deprotected with LiOH (402 mg, 16.87 mmol, 3.5 equiv) in water–MeOH (1:1) for 2 h at RT to give the title compound **S4** (1.15 g, 70% for two steps). This was used without purification after the final work-up. ^{1}H NMR (500 MHz, CD_3OD): δ 8.03 (1 H, dd, J = 7.5 Hz and J = 1.8 Hz), 7.79 (3 H, m), 5.08 (1 H, m), 3.97 (2 H, d, J = 7.2 Hz), 3.55 (2 H, t, J = 7.4 Hz), 2.57 (2 H, t, J = 7.4 Hz), 1.68 (3 H, m), 1.67 (3 H, m); ESI-MS: $[M + Na]^+$ obsd. = 364.93 (calc. = 365.08).

Peptoid/Peptide Synthesis. Compounds were prepared on solid phase using standard Fmoc protocols using HBTU for coupling to primary and PyBroP for coupling to secondary amines, respectively, or using peptoid submonomer synthesis as follows: resin-bound amines were treated with a 1 M solution of bromoacetic acid in DMF and DIC (25 equiv) for 2 h at RT; then, after washing with DMF (3×) and NMP (3×), the bromide was displaced with the proper primary amine "submonomer" (30 equiv) in NMP under shaking for 2.5 h at RT. The linear peptide–peptoid hybrids were cleaved from the solid support using TFA–CH_2Cl_2 (1:1), and were precipitated using diethylether before being subjected to head-to-tail ring closure without further purification. Cyclizations were performed in DMF solution at 0.5–1 mM concentration using HATU as the coupling reagent, and were purified by preparative HPLC when LC-MS analysis showed full conversion, as previously described.[S2] All compounds were purified to homogeneity by preparative reversed-phase

HPLC (Phenomenex C-18 Luna column) and the obtained fractions were lyophilized to give the compounds as fluffy solids that were characterized by LC-MS (Table S1). DMSO stock solutions were prepared using UV [ε(indole) = 5690 $M^{-1} \times cm^{-1}$ (280 nm)] to adjust the concentrations where possible. Stock solutions of compounds lacking chromophores were prepared by weight.

Table S1. Compound Characterization Data.

compound	isolated yields (based on resin loading)	$HPLC_{230\ nm}$	SSI-MS $[M+H]^+$ calcd./found
2[2]	3.3 mg (9%)	>98%	585.36/585.87
3	2.9 mg (8%)	>98%	585.36/585.93
4	2.1 mg (10%)	>98%	585.36/585.93
5[a]	4.0 mg (10%)	>98%	595.37/596.00
6	3.6 mg (9%)	>98%	595.37/595.80
7a	1.0 mg (5%)	>98%	592.36/592.80
7b	4.6 mg (13%)	>98%	557.37/557.13
7c	4.6 mg (13%)	>98%	557.37/557.13
7d	4.0 mg (11%)	>98%	543.35/543.06
7e	4.2 mg (12%)	>98%	549.39/549.26
8	1.6 mg (4%)	>98%	579.35/579.06
9[b]	4.8 mg (12%)	>98%	667.42/667.13
10[c]	4.2 mg (18%)	>98%	681.43/681.4
11[d]	1.7 mg (7%)[e]	>95%	679.43/679.13

[a]ESI-TOF high-resolution mass spec.: $[M+Na]^+$ obsd. = 618.3624 (calc. = 618.3626); [b]$[M+H]^+$ obsd. = 667.4290 (calc. = 667.4290); [c]$[M+H]^+$ obsd. = 681.4315 (calc. = 681.4334). [d]$[M+H]^+$ obsd. = 679.4284 (calc. = 679.4290). [e]Based on the protected intermediate (**S5**), as only half of the material was deprotected.

Conformational search. A conformational search was carried out using the OPLS-2005 force-field[S3] and a solvation model for water as incorporated in Macromodel v. 9.8 release 107.[S4] The OPLS-2005 force field was chosen because it is a general organic force field suitable for both small molecules and larger biomolecules; further, the OPLS force field was recently shown to be among the most suitable force fields for modeling the conformations of peptoids and peptide-peptoid hybrids.[S5] The search was run for 100,000 steps using the mixed torsional/low-mode[S6] search. All conformations within 10 kcal/mol of the global minimum were saved to give a total of 21,700 structures. Since the main interest of the current study was the conformation of the backbone, an additional filtering was carried out to remove duplicate backbone conformations, which resulted in 269 structures with significantly different positions of the backbone atoms. For each backbone conformation, only the most favorable orientation of the side chains was saved. Table S2 lists relative energies of the 25 lowest energy conformations; the pdb files for these structures are available for download as online supporting material. Figure S4 shows the ten lowest energy conformations, which are within 3 kcal/mol of the lowest energy conformation. At energy gaps larger than that, some conformations contained all *trans* amide configurations. Whereas the cis-trans-trans-trans conformations overlay well with the pharmacophoric region of apicidin (Figure S4), the all-trans conformations do not.

Table S2. Summary of 25 lowest energy backbone conformations identified by conformational search.

Entry	Potential energy (kcal/mol)	Relative potential energy (kcal/mol)	Amide configuration
backbone 1	-67.11	0.00	cis-trans-trans-trans
backbone 2	-66.87	0.24	cis-trans-trans-trans
backbone 3	-66.07	1.04	cis-trans-trans-trans
backbone 4	-65.75	1.36	cis-trans-trans-trans
backbone 5	-65.04	2.07	cis-trans-trans-trans
backbone 6	-64.72	2.39	cis-trans-trans-trans
backbone 7	-64.72	2.39	cis-trans-trans-trans
backbone 8	-64.61	2.50	cis-trans-trans-trans
backbone 9	-64.27	2.84	cis-trans-trans-trans
backbone 10	-64.17	2.94	cis-trans-trans-trans
backbone 11	-64.04	3.07	all trans
backbone 12	-64.03	3.08	cis-trans-trans-trans
backbone 13	-64.02	3.09	cis-trans-trans-trans
backbone 14	-63.80	3.31	all trans
backbone 15	-63.78	3.33	all trans
backbone 16	-63.71	3.40	cis-trans-trans-trans
backbone 17	-63.69	3.42	cis-trans-trans-trans
backbone 18	-63.49	3.62	cis-trans-trans-trans
backbone 19	-63.45	3.66	cis-trans-trans-trans
backbone 20	-63.23	3.88	cis-trans-trans-trans
backbone 21	-63.15	3.97	cis-trans-trans-trans
backbone 22	-63.03	4.08	all trans
backbone 23	-63.02	4.09	cis-trans-trans-trans
backbone 24	-63.00	4.11	cis-trans-trans-trans
backbone 25	-62.89	4.22	cis-trans-trans-trans

IC_{50} Determination. The IC_{50} values against HeLa extracts were determined using the standard Biomol HDAC fluorimetric assay protocol (AK-500).[S7] For inhibition of recombinant human HDACs the dose–response experiments with internal controls were performed in black or white low binding Nunc 96-well microtiter plates. The dilution series were prepared in Milli-Q water from 1 mM DMSO stock solutions. The appropriate dilution of the inhibitor (10 µL of a 5× concentration solution) was added to each well followed by HDAC assay buffer (38 µL) containing Ac-Lys(Ac)-7-amino-4-methylcoumarin substrate (67 µM) and bovine serum albumin (0.75 µg/µL). For HDAC8 the Arg-His-Lys(Ac)-Lys(Ac)-AMC substrate (27 µM) was used. Finally, a solution of the appropriate HDAC (2 µL) was added and the plate was incubated at 37 °C for 30 min in the dark [HDAC1, 0.15 µg/µL; HDAC2, 0.08 µg/µL; HDAC3, 0.03 µg/µL; HDAC6, 0.011 µg/µL; HDAC8, 0.4 µg/µL]. Then trypsin (50 µL, 0.4 µg/µL) was added and the assay development was allowed to proceed for 15 min at RT, before the plate was read using a Tecan GENios plate reader with excitation at 360 nm and detecting emision at 460 nm.

Cancer Cell Cytotoxicity Assays. Cells were cultivated in flat 96-well tissue culture plates in 90 µL of medium supplemented with 10% FBS and antibiotics. HeLa cells were seeded at a density of 5,000 cells/well, Jurkat, Hct-116, and MCF-7 at a density of 10,000 cells/well, and K-562 and KYO-1 at a density of

15,000 cells/well. Twenty-four hours later, for adherent cells, 10 μL of medium containing various concentrations of the desired compounds were added in triplicate, and the cells were incubated at 37 °C for 72 h in a 5% CO atmosphere. Cell survival was determined using the XTT (Sigma-aldrich, St. Louis, MO) colorimetric assay. Immediately prior to use, a mixture of XTT (1 mg/mL) and PMS (*N*-methyl dibenzopyrazine methyl sulfate, 0.383 mg/mL) in phenol red free RPMI was prepared (0.1 mL of PMS per 5 mL of XTT solution). After adding 50 μL of this mixture to each well, plates were incubated at 37 °C for 2 h in the case of HeLa, Hct-116, and MCF-7 cells, and 4 h for Jurkat, K-562, and KYO-1 cells. Plates were shaken to evenly distribute the dye in the wells and the absorbance was measured using a Tecan GENios plate reader spectrometer at a wavelength of 485 nm.

Cell Culture and Western Blot. Jurkat cells were cultivated in RPMI 1640 medium supplemented with 10 % Fetal Bovine Serum and antibiotics. At confluency, cells were seeded in 6-well plates and incubated with various concentrations of compound **9** at 37 °C for 24 h. The cells were then washed, harvested, and lysed, and the lysates were run on 4–12% polyacrylamide SDS PAGE gels (Invitrogen), transferred to nitrocellulose membranes and visualized as described previously.[S8] TSA and the vehicle (DMSO) were used as positive and negative controls, respectively. The figure S5 represents a single experiment.

Supporting References:

(S1) Fukuyama, T.; Jow, C. K.; Cheung, M. *Tetrahedron Lett*. **1995**, *36*, 6373-6374.
(S2) Montero, A.; Beierle, J. M.; Olsen, C. A.; Ghadiri, M. R. *J. Am. Chem. Soc*. **2009**, *131*, 3033-41.
(S3) Kaminski, G. A.; Friesner, R. A.; Tirado-Rives, J.; Jorgensen, W. J. *J. Phys. Chem. B* **2001**, *105*, 6474.
(S4) Macromodel v. 9.8 release 107, Schrödinger, LLC, New York, NY, **2010**.
(S5) Brandt, W.; Herberg, T.; Wessjohann, L. *Pept. Sci*. **2011**, *96*, 651-668.
(S6) Kolossváry, I.; Guida, W. C. *J. Comput. Chem*. **1999**, *20*, 1671.
(S7) Wegener, D.; Hildmann, C.; Riester, D.; Schwienhorst, A. *Anal. Biochem*. **2003**, *321*, 202-8.
(S8) Chou, C. J.; Herman, D.; Gottesfeld, J. M. *J. Biol. Chem*. **2008**, *283*, 35402-9.

Figure S1. Solid-phase peptoid–peptide synthesis.

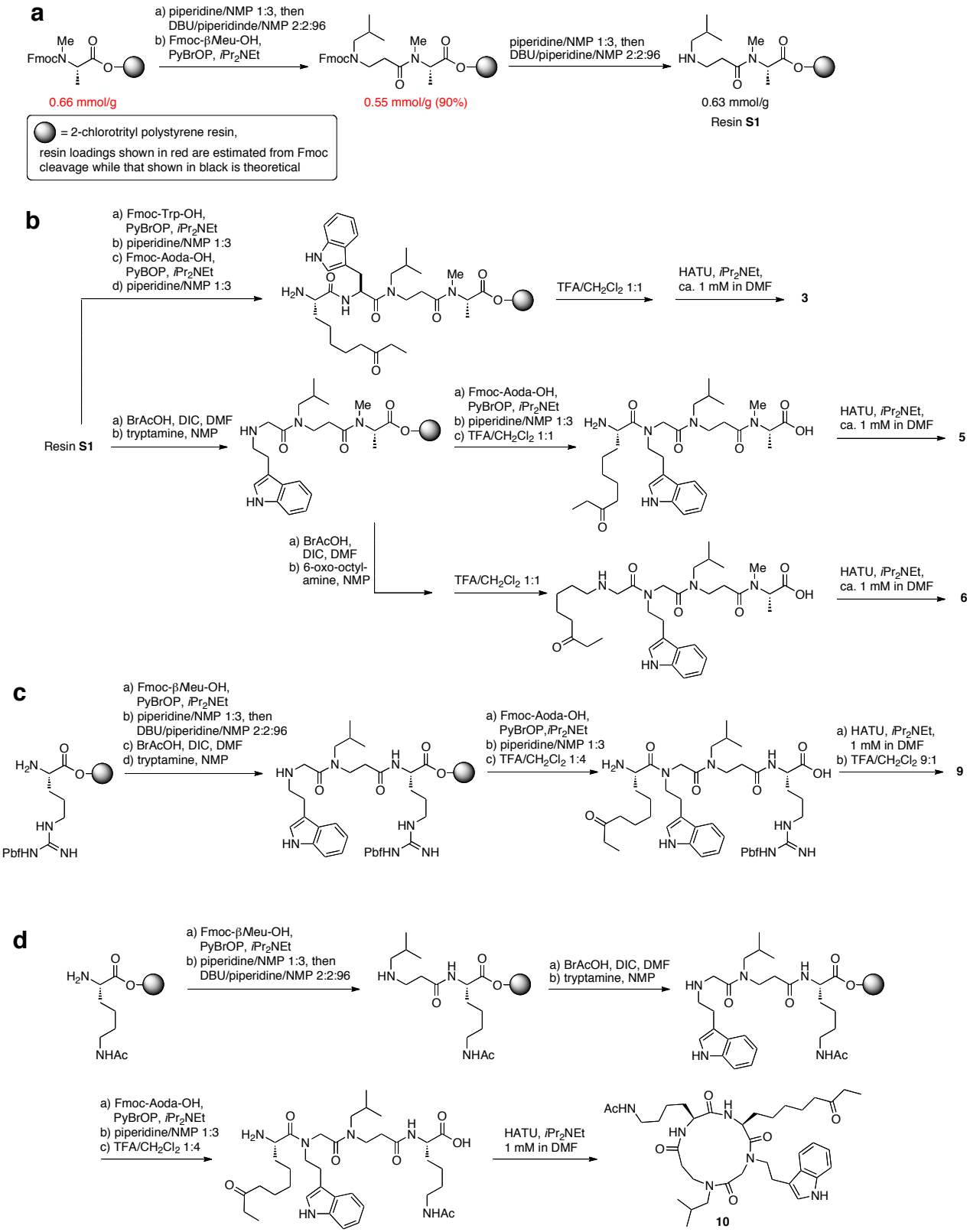


Figure S2. Synthesis of building block **S4**.

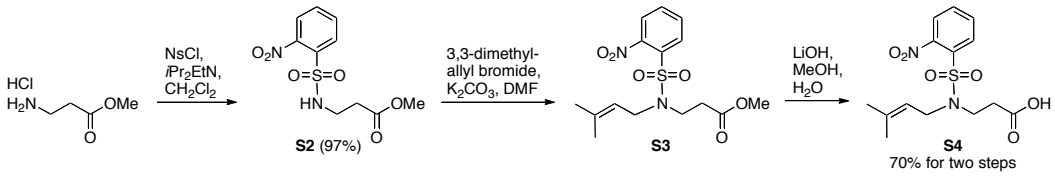
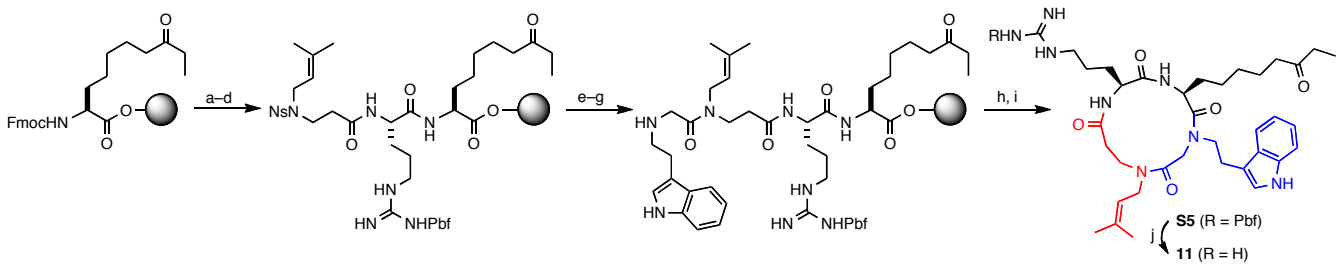


Figure S3. Solid-phase peptoid-peptide synthesis of compound **11**.



Reagents and conditions: a) piperidine–NMP 1:3, 2 × 15 min; b) Fmoc-Arg(Pbf)-OH (5 equiv), HBTU (5 equiv), *i*Pr$_2$EtN (10 equiv), NMP, 2 h; c) piperidine–NMP 1:3, 2 × 15 min; d) building block **S4**, (5 equiv), HBTU (5 equiv), *i*Pr$_2$EtN (10 equiv), NMP, 2 h; e) DBU (5 equiv), 2-mercaptoethanol (10 equiv), DMF, 2 × 1 h; f) BrAcOH (27 equiv), DIC (25 equiv), DMF, 1 h, RT; g) tryptamine (30 equiv), NMP, 2 h, RT; h) CH_2Cl_2–TFA (98:2), 1 h; i) HATU (2 equiv), *i*Pr$_2$EtN (5 equiv), DMF (1 mM), 16 h; j) CH_2Cl_2–TFA (1:1), 1 h.

Figure S4. Calculated molecular conformations of peptoids **9**/**10**. a) The 10 lowest energy conformations obtained by a conformational search are shown, with side chain atoms removed for clarity. b) Overlay of the NMR structure of apicidin (white) onto one of the calculated structures of compounds **9**/**10** (green). Side chain atoms have been removed for clarity. The three side chains known to comprise the pharmacophoric region of the inhibitor are labeled.

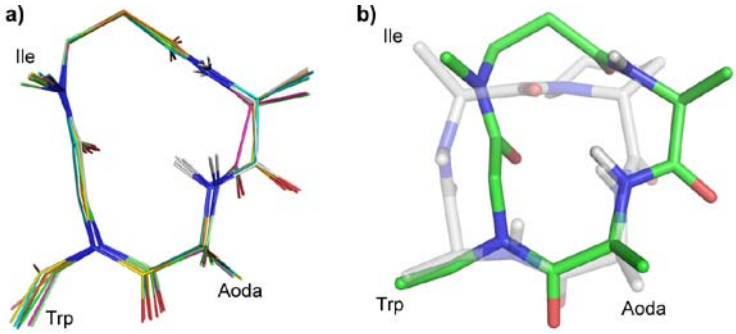


Figure S5. Western blot analysis of cell lysates from Jurkat cells treated with DMSO, TSA, or compound **9** (0.2 μM, 2 μM, or 20 μM, respectively). Histone H3 serves as a control of protein loading. A GI_{50} = 47 μM was measured for **9** against Jurkat cells using a standard XTT assay.

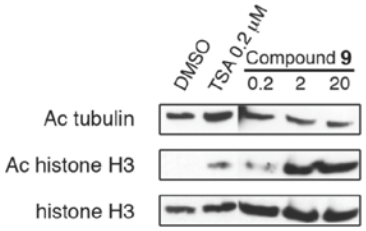


Figure S6. ^{1}H NMR of compound **2** (multiple conformations).

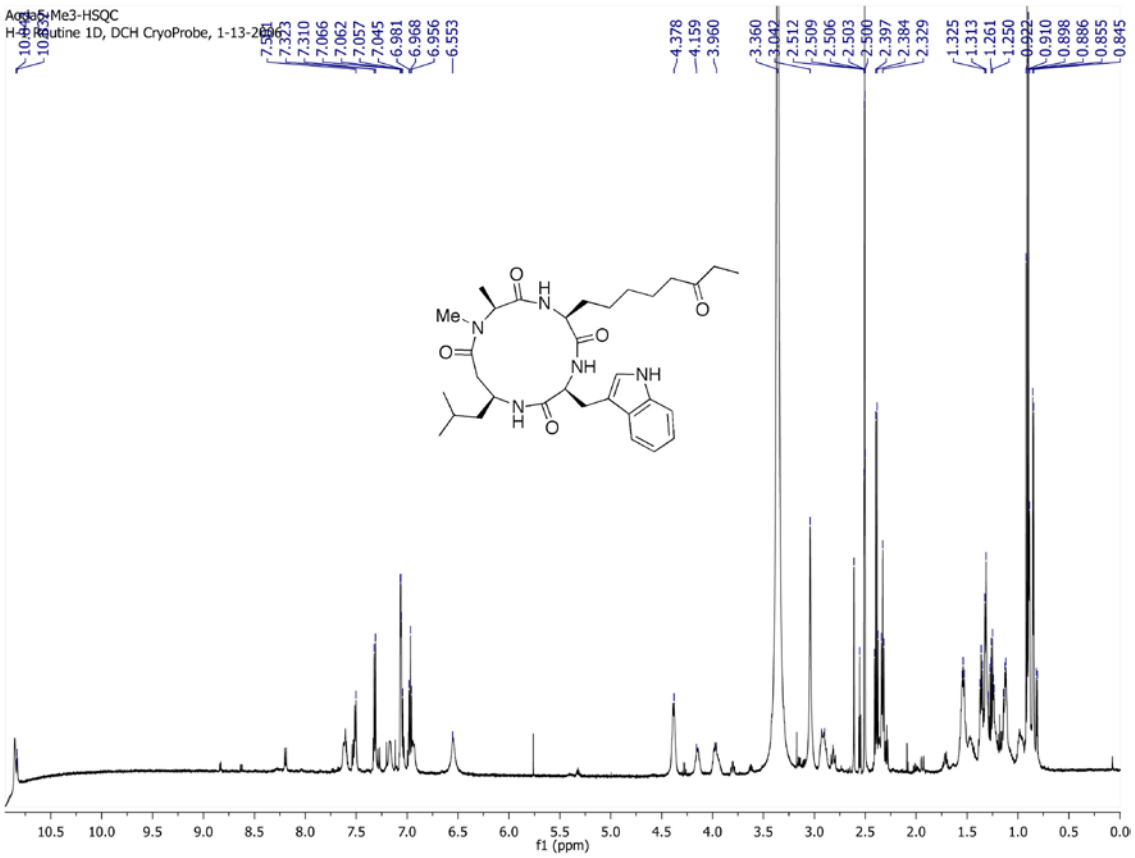


Figure S7. ^{1}H NMR of compound **5** (multiple conformations).

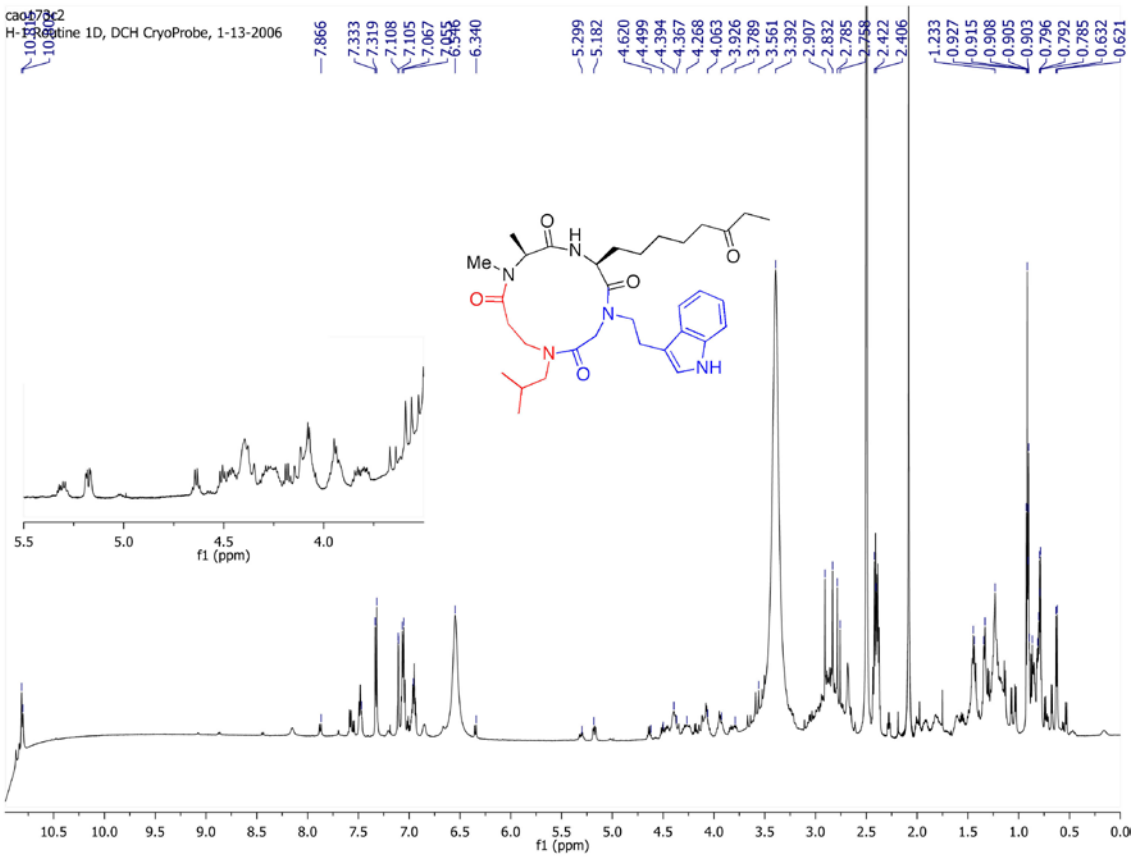


Figure S8. ^{1}H NMR of compound **9**.

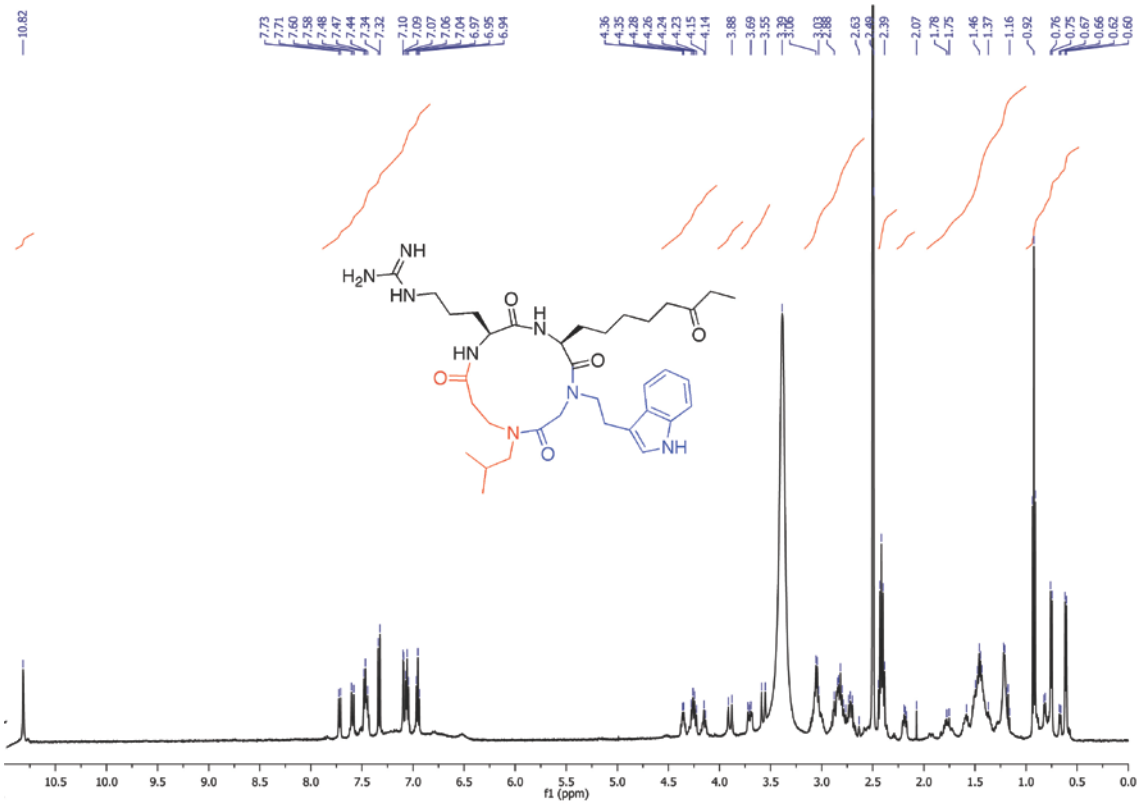


Figure S9. ^{1}H NMR of compound **10**.