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Synthesis and Biochemical Evaluation of Biotinylated Conjugates of Largazole Analogues: Selective Class I Histone Deacetylase Inhibitors

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This paper is dedicated to Prof. Stuart L. Schreiber on the occasion of his 60th birthday. We are also very pleased to dedicate this manuscript to Profs. Stuart L. Schreiber and K. C. Nicolaou on being awarded the Wolf Prize.

Abstract: The synthesis of biotinylated conjugates of synthetic analogues of the potent and selective histone deacetylase (HDAC) inhibitor largazole is reported. The thiazole moiety of the parent compound's cap group was derivatized to allow the chemical conjugation to biotin. The derivatized largazole analogues were assayed across a panel of HDACs 1–9 and retained potent and selective inhibitory activity towards the class I HDAC isoforms. The biotinylated conjugate was further shown to pull down HDACs 1, 2, and 3.

Keywords: biotin · cancer · inhibitors · largazole · peptidomimetics

1. Introduction

The marine natural product largazole was isolated from marine cyanobacteria *Symploca* sp. off of the Florida coastline by Luesch and co-workers.[1] Largazole's sixteen-membered cyclodepsipeptide structure (Figure 1), containing both a thioester tail and (*S*)-3-hydroxy-7-mercaptohept-4-enoic acid moieties, constitutes the structural basis of a promising antitumor prodrug.[2] Research efforts have explored the therapeutic use of largazole towards not only cancer therapy but also corneal angiogenesis, rheumatoid arthritis, neurodegenerative diseases, osteoporosis, and numerous other disease areas.[3] The cyclodepsipeptide natural product and histone deacetylase inhibitor (HDACi) romidepsin (FK228) is currently an FDA-approved therapeutic for the treatment of cutaneous T-cell lymphoma (CTCL).[4] Vorinostat (SAHA), a simple hydroxamate, was the first HDACi approved by the FDA for human clinical use and is a non-selective "pan" inhibitor of all the HDAC isoforms.[5] Panobinostat, the pan-HDACi LBH-589 developed by Novartis, has been approved for multiple myeloma combination therapy as well.[6] Largazole, FK228, and the spiruchostatins,[7] which all contain the same (*S*)-3-hydroxy-7-mercaptohept-4-enoic acid moiety, are all potent biochemical inhibitors of the class I HDACs and exert their HDAC inhibitory activity through binding of the sulfhydryl residue to the active site zinc ion of the class I HDACs. Our laboratory and others have incorporated the (*S*)-3-hydroxy-7-mercaptohept-4-enoic acid moiety into numerous synthetic analogues, which in most cases provides selective and potent class I HDAC inhibitors.[8]

Selective HDAC isoform inhibition is essential for the development of a safe and efficient therapeutic.[9] This has also been the most challenging aspect in the development of HDAC inhibitors to date. Eighteen HDACs have been isolated, the Schreiber laboratory spearheading this effort.[10] The HDACs of general therapeutic interest are within classes I and II; specifically, class I includes four HDACs (1, 2, 3, and 8) that are much smaller and primarily located in the nucleus, compared to class II HDACs (4, 5, 6, 7, and 9), which each contain ~1000 amino acid residues and are expressed in both the cytoplasm and nucleus.[11] Adverse side effects have been observed in currently marketed pan-HDAC inhibitors, which

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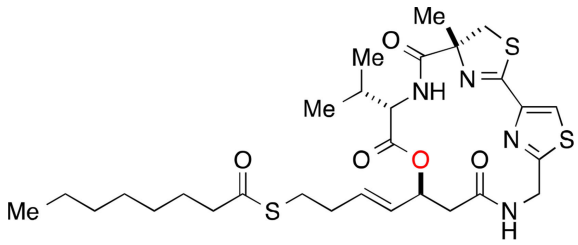
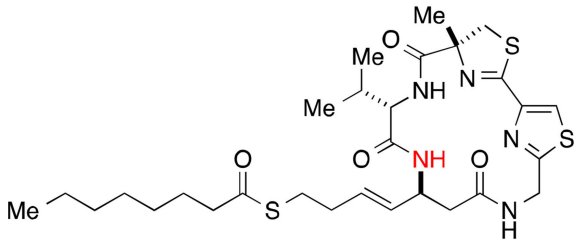


*Largazole (**1**)*



*Largazole Peptide Isostere (**2**)*

Figure 1. Largazole (**1**), the parent natural product, and largazole peptide isostere (**2**).

has been partially attributed to the lack of selectivity towards HDAC isoforms.[12]

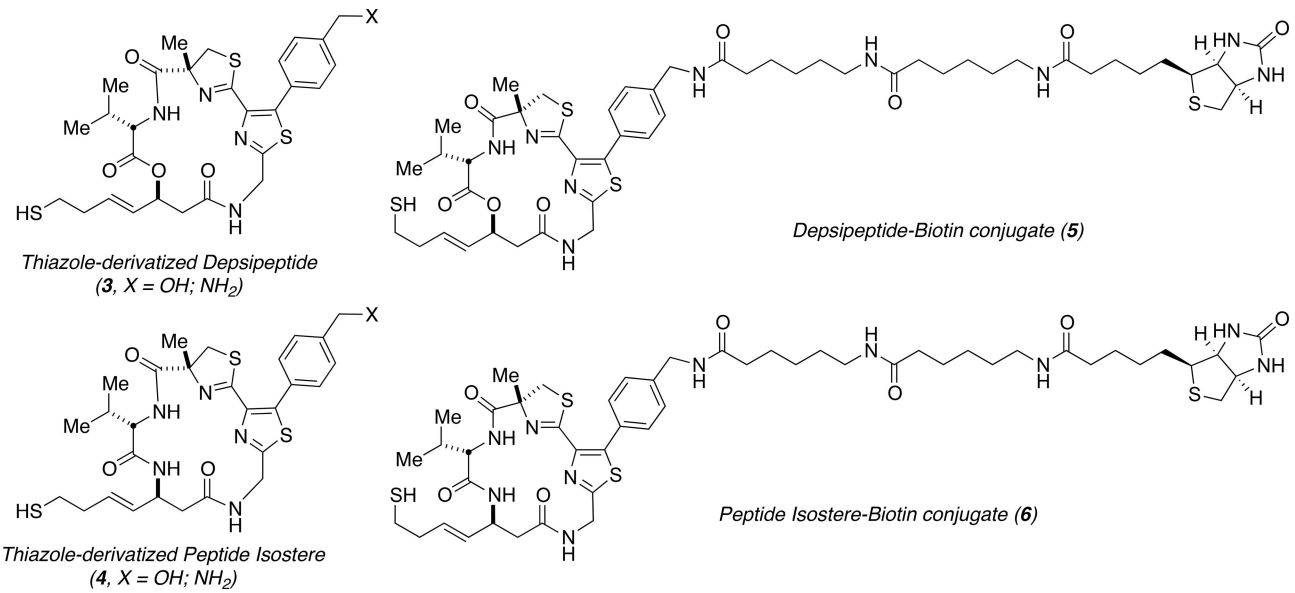
To date we have developed syntheses of more than sixty analogues of largazole by modifying three main motifs of the parent natural product.[8,13] We have interrogated structural changes to (1) the zinc-binding domain, (2) the surface recognition unit or so-called "cap group" (thiazole–thiazoline fragment), and (3) the depsipeptide versus the corresponding peptide isosteres (Figure 1).[13a]

Herein we describe synthetic routes to access both depsipeptide and peptide isostere analogues of the parent natural product largazole with a functional handle projecting from the thiazole moiety of the cap group that permits the chemical conjugation of other molecular entities that might prove valuable for cell-specific targeting and as probes for biochemical and cellular function (Figure 2). Our synthetic route utilizes our original strategic four-component convergent disconnection deployed in the efficient total synthesis of largazole reported by this laboratory in 2008.[8a]

2. Results and Discussion

2.1 Design

Prior molecular modeling studies of largazole, performed in collaboration with the Wiest laboratory, revealed that the cap group of largazole has the unique ability to interact with the protein surface of the HDAC enzyme and projects away from the active site in unencumbered space, providing an opportunity for chemical functionalization.[13a,14] We reasoned that thiazole derivatization would be chemically tractable and would allow for the installation of functionality that would not hinder the Zn^{2+} binding arm of this scaffold from interaction with the Zn^{2+} atom ensconced at the active site of the enzyme. With the dual objective of not abrogating the Zn^{2+} binding role of the sulfhydryl-containing side chain[15] and incorporating a chemically flexible handle for conjugating cancer-cell-specific targeting motifs and other reporter molecules, we



*Thiazole-derivatized Depsipeptide (**3**, X = OH; NH_2)*

*Depsipeptide-Biotin conjugate (**5**)*

*Thiazole-derivatized Peptide Isostere (**4**, X = OH; NH_2)*

*Peptide Isostere-Biotin conjugate (**6**)*

Figure 2. Thiazole-derivatized depsipeptides (**3**), thiazole-derivatized peptide isosteres (**4**), depsipeptide–biotin conjugate (**5**), and peptide isostere–biotin conjugate (**6**).

modified our convergent synthetic route, utilizing previously established disconnections.[8b] This design was probed using our previously established[16] molecular dynamics (MD) protocol for the study of HDAC inhibitors. Figure 3 shows the model of **6** bound to the crystal structure of HDAC8 (PDB code: 4RN0).[14] As envisioned in the original design, the biotin and the amide linker protrude away from the binding site, leaving all contacts of the protein with the cap group and the zinc binding domain intact. It is therefore expected that the binding of **5** and **6** will mimic largazole and its amide isostere, respectively. Although no crystal structure of largazole derivatives to HDAC1–3 is currently available, these findings should also apply to these HDACs due the previously described similarities in binding modes.[13a,14]

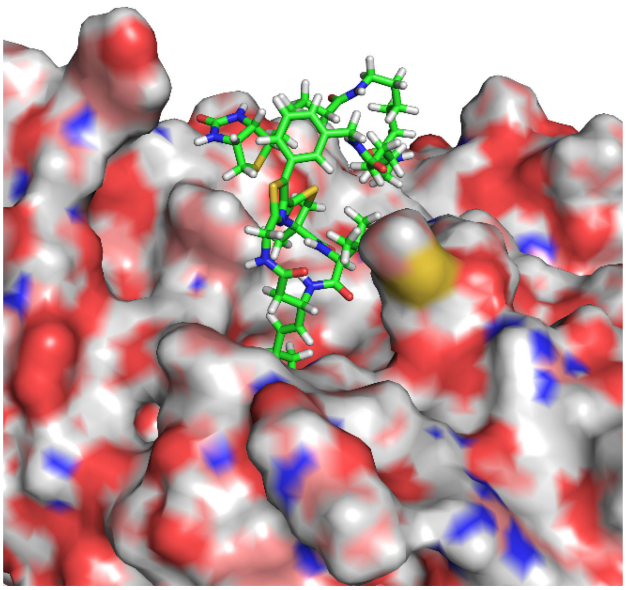


Figure 3. MD refined model of the complex of **6** bound to HDAC8.

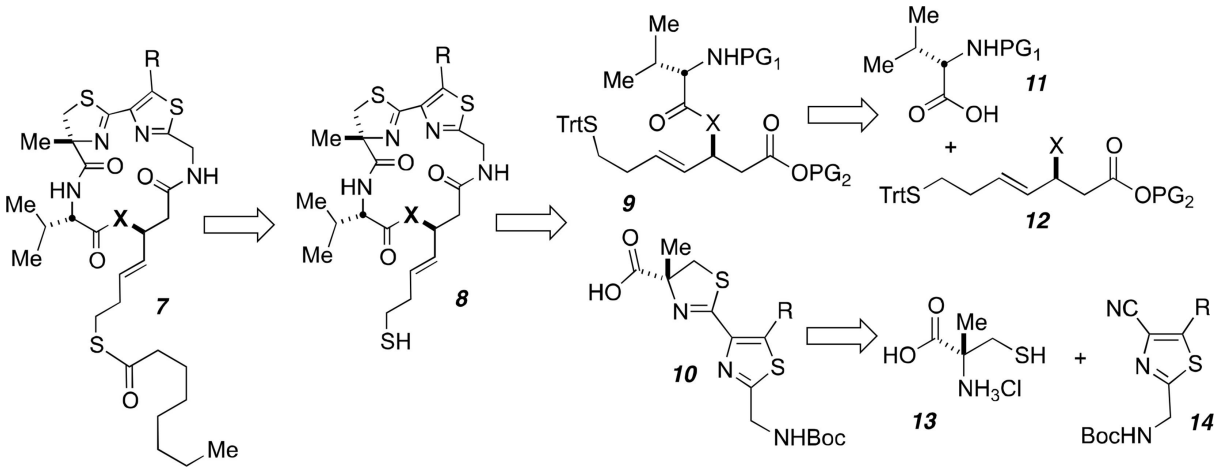
We chose to evaluate the installation of both hydroxyl group handles and amino group handles for chemical conjugation studies (Figure 2). The amine handle allows for extensive conjugation across a series of cancer-relevant biological molecules via robust amide linkages, which might prove resistant to proteolytic cleavage in vitro and in vivo, whereas the hydroxyl residue allows for chemical conjugation through more labile ester linkages.[15] Biotin conjugation has been widely used to study and identify small-molecule drug targeting to cellular proteins and has been used in the context of numerous pull-down experiments.[16]

2.2 Synthesis

Scheme 1 shows the synthetic strategy we have successfully used to access largazole derivatives and we have applied this to the preparation of compounds **3** and **4**.[8c] The two halves, depsipeptide or peptide isostere fragments (**9**) and thiazole–thiazoline moiety (**10**), are utilized to access the pre-macrocyclization species. Macrocyclization to *S*-trityl-protected precursors is accessed through standard peptide coupling techniques. The two halves can then be further dissected into the β-amino acid or β-hydroxy acid fragments (**12**, X = NH_2, OH, respectively), protected L-valine (**11**), α-methyl cysteine (**13**), and the substituted thiazole cyano precursor (**14**).

As shown in Scheme 2, commercially available acrolein (**15**) was reacted with trityl mercaptan followed by a Wittig olefination to yield aldehyde **16**. Alcohol stereochemistry was established through the use of a Crimmins-type aldol condensation, the product of which was then cleaved using 2-(trimethylsilyl)ethanol. Amino acid EDCI coupling between the free alcohol of **18** and L-valine was successful in providing compound **19**.

As illustrated in Scheme 3, the corresponding peptide isostere precursor was accessed via Boc-protected amino acid



Scheme 1. Retrosynthetic pathway for cap group derivatization of the thiazole fragment of parent compounds of largazole (X = O or NH).

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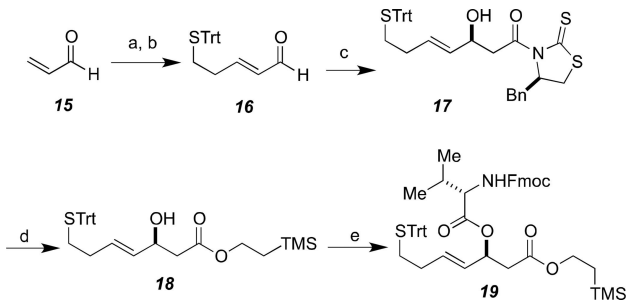
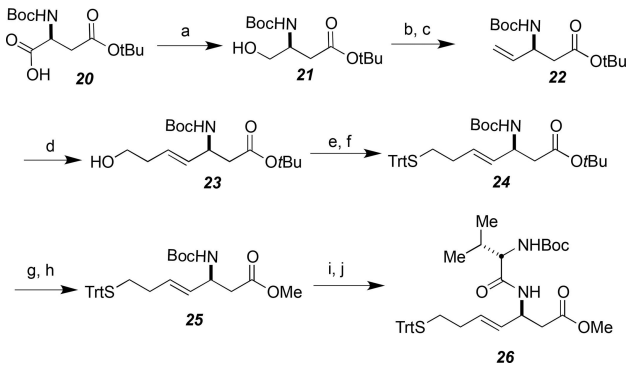


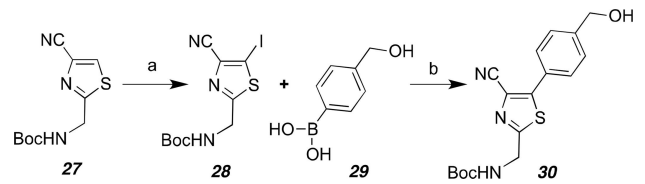
Scheme 2. Synthesis of compound **19** for depsipeptide series. Reagents and conditions: (a) TrtSH, Et_3N, CH_2Cl_2; (b) (formylmethylene)triphenylphosphorane, PhH, 80 °C, 77% over two steps; (c) (*R*)-1-(4-benzyl-2-thioxothiazolidin-3-yl)ethanone, $TiCl_4$, DIPEA, CH_2Cl_2, −78 °C, 76%; (d) 2-(trimethylsilyl)ethanol, imidazole, CH_2Cl_2, 83%; (e) *N*-Fmoc-Val-OH, EDCI, DIPEA, DMAP, CH_2Cl_2, 77%.



Scheme 3. Synthesis of fragment **26** for peptide isostere macrocycle. Reagents and conditions: (a) 4-methylmorpholine, isobutyl chloroformate, THF, −40 °C; $NaBH_4$, MeOH, −20 °C, 66%; (b) oxalyl chloride, DMSO, DIPEA, CH_2Cl_2, −65 °C; (c) methyltriphenylphosphonium bromide, KHMDS, THF, −78 °C, 80% over two steps; (d) 3-buten-1-ol, Grubbs catalyst, 2nd generation, CH_2Cl_2, 50 °C, 25%; (e) TsCl, Et_3N, DMAP, CH_2Cl_2, 82%; (f) TrtSH, KO^{t}Bu, THF, 87%; (g) LiOH, THF, MeOH, 50 °C, 96%; (h) MeOH, EDCI, DIPEA, DMAP, CH_2Cl_2, 77%; (i) TFA, CH_2Cl_2; (j) *N*-Boc-Val-OH, PyBOP, DIPEA, CH_2Cl_2, 88% over two steps.

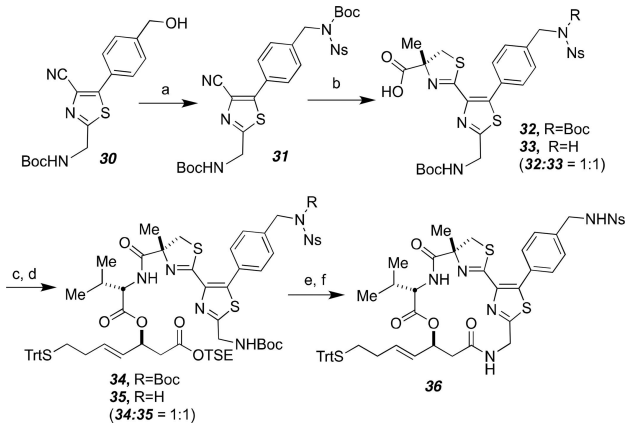
derivative **20**.[8b] Reduction of the carboxylic acid (**20** to **21**), Swern oxidation, and subsequent Wittig olefination provided terminal alkene **22**. Grubb's cross-metathesis with 3-buten-1-ol was employed to provide compound **23**. The yield at this stage was very modest and alternative routes were explored but were either unsuccessful or resulted in epimerization of the amino group.[7,17] Tosylation and reaction with trityl thiol provided protected thiol **24**, which then underwent methyl ester formation to provide compound **25**. *N*-*t*-Boc deprotection using TFA exposed the free amine group, which was then coupled to *N*-*t*-Boc protected L-valine through the use of PyBOP, furnishing fragment **26**.

Functionalization of the thiazole ring is depicted in Scheme 4.[18] Compound **30** with the hydroxymethyl handle was accessed via cyano-thiazole compound **27**. LDA deprotonation followed by iodination provided cross-coupling precursor **28**. Suzuki coupling with boronic acid **29** provided compound **30.** It is important to note that additional aromatic boronic acids have been used to access derivatives successfully and will be reported elsewhere.



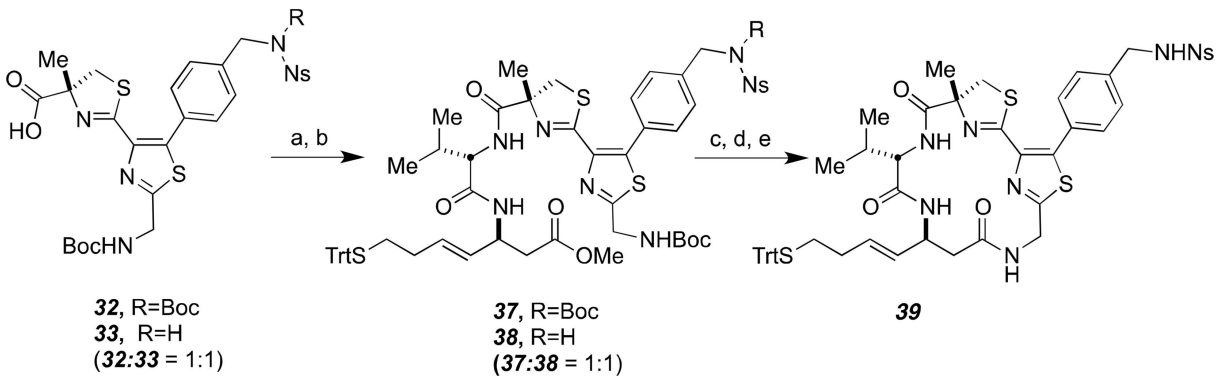
Scheme 4. Synthesis of fragment **30** for cap group assembly. Reagents and conditions: (a) LDA, I_2, THF, −78 °C, 74%; (b) tetrakis (triphenylphosphine)palladium(0), sodium bicarbonate, DME–H_2O (3:1), reflux, 68%.

Scheme 5 illustrates the depsipeptide macrocycle assembly. The free alcohol of **30** could either be protected with TBDPS for direct conjugation via ester linkages or subjected to Mitsunobu reaction using *tert*-butyl ((4-nitrophenyl) sulfonyl)carbamate, which provided compound **31.** Cyclocondensation of **31** with α-methyl cysteine gave carboxylic acids **32:33** as a 1:1 mixture that was carried forward as a mixture.[19] Peptide coupling of fragment **19** provided **34** + **35** as a 1:1 mixture that was deprotected and cyclized to furnish the desired species **36.**



Scheme 5. Synthesis of depsipeptide macrocycle compound **36.** Reagents and conditions: (a) *tert*-butyl ((4-nitrophenyl)sulfonyl) carbamate, diethyl azodicarboxylate, triphenylphosphine, THF, 90%; (b) α-methyl cysteine, sodium carbonate, triethylamine, MeOH, reflux, 59%; (c) **19**, MeCN–diethylamine (10:1); (d) PyBOP, DIPEA, DCM, 58% over two steps; (e) DCM–TFA (5:1); (f) HOBt, HATU, DIPEA, MeCN, 20% over two steps.

For the assembly of the corresponding peptide isostere, **37** was reacted with LiOH–H_2O to provide a free carboxylic acid that underwent intramolecular HOBt and HATU coupling with the deprotected Boc amino group after subjection to TFA to provide compound **39** (Scheme 6).



Scheme 6. Synthesis of peptide isostere macrocycle compound **39**. Reagents and conditions: (a) **26**, DCM–TFA (10:1); (b) PyBOP, DIPEA, DCM; (c) lithium hydroxide–H_2O, THF–MeOH–H_2O (2:2:1); (d) DCM–TFA (10:1); (e) HOBt, HATU, DIPEA, MeCN, 12% over five steps.

Table 1. IC_{50} values (nM) of compounds **1**, **2**, **5**, and **43** across HDAC isoforms 1–3, 6, and 8.

Compound	HDAC isoform IC_{50} (nM)				
	1	2	3	6	8
Largazole (**1**)	10.09	18.65	9.09	165.6	1068
2	1.95	3.38	2.59	102	255.3
43	0.166	2.61	0.734	14.9	94
5	0.2388	2.84	1.064	36.51	129.3

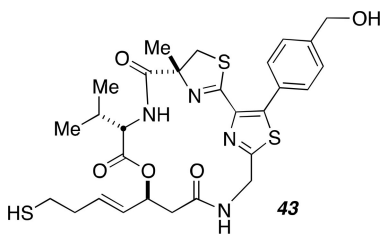


Figure 4. Structure of depsipeptide analogue with a hydroxymethyl handle off of the thiazole cap group.

Compounds **36** and **39** were each subjected to treatment with thiophenol and 2 M KOH in acetonitrile to remove the nosyl protecting group and expose the free amine for coupling to biotin.[20] $NaHCO_3$ was used to promote the amide coupling of biotin for both the depsipeptide and peptide largazole derivatives. Biotin linker **40** was used due to the stability of the amide bond, reliable synthesis for highly functionalized molecules, and the increased length allowed for solubility. Trityl deprotection using DCM–TFA and iPr$_3$SiH provided largazole–biotin derivatives **5** and **6** in 41% and 36% yield, respectively, as shown in Scheme 7. This convergent route can be utilized to access additional amide-linked biological conjugates.

2.3 Biochemical Evaluation

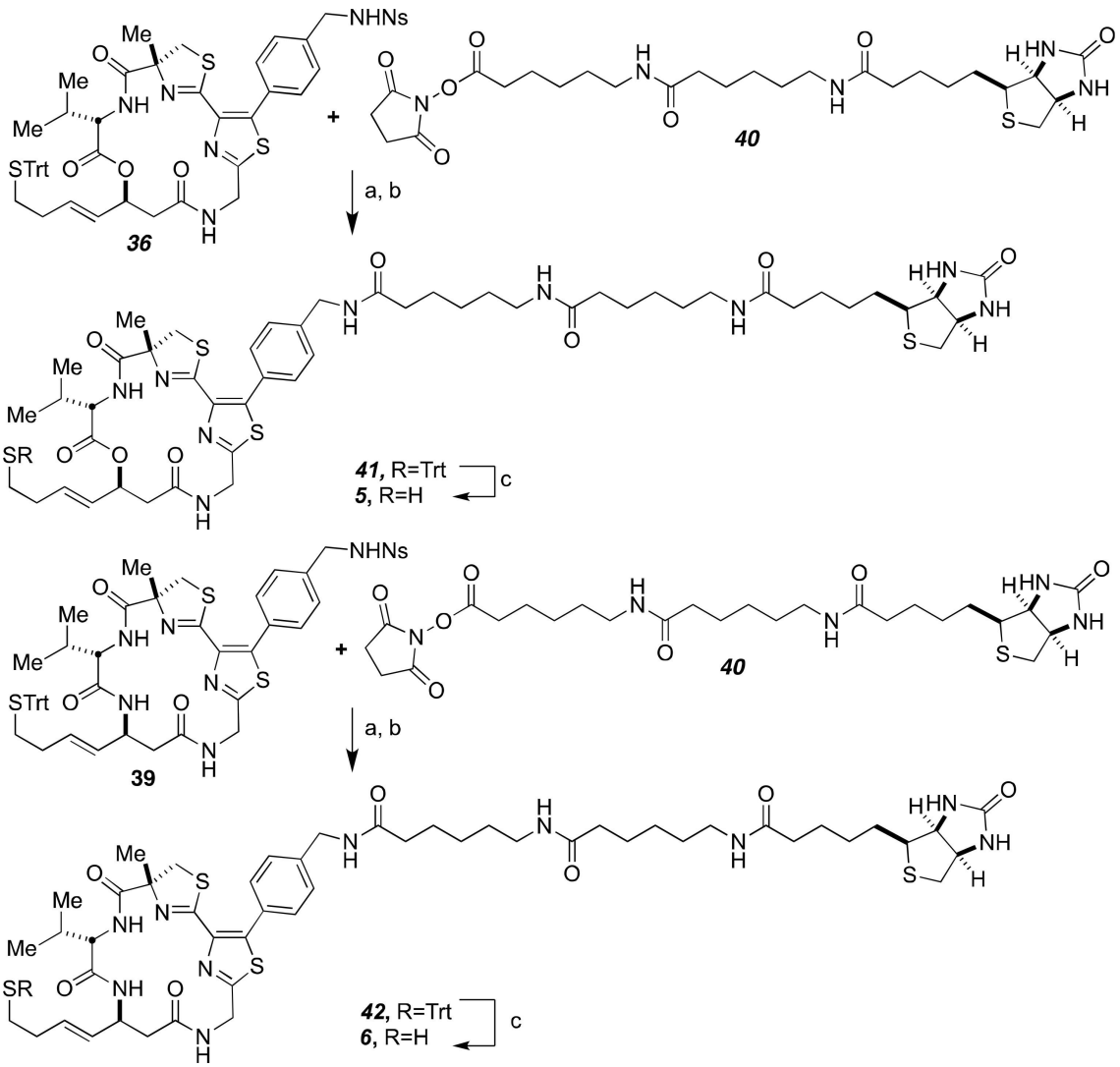
Compounds **1**, **2**, **5**, and **43** were tested across an HDAC isoform panel of HDACs 1–9 to determine the biochemical inhibitory potency of these species relative to the parent natural product. The IC_{50} values, reported in nM in Table 1, represent a summary of HDAC inhibitory activity. The peptide isostere thiol of parent largazole, **2**, displayed inhibitory activity below 4 nM for HDACs 1–3, proving more potent than the parent natural product **1**. The newly derivatized compound at the thiazole cap group of parent largazole, **43** (Figure 4), displayed sub-nanomolar inhibitory activity for HDACs 1 and 3 and below 3 nM for HDAC 2. A decrease in potency was seen across all derivatives for HDACs 6 and 8, as expected.

Biotin–largazole conjugate **5** exhibited very little change in IC_{50} activity when compared to compound **43**. IC_{50} values below 3 nM were maintained across HDACs 1–3 for **5** and **43**. A full representation of the full HDAC panel for **43** (Figure 5A) and **5** (Figure 5B) is depicted in Figure 5.

Affinity chromatography assays were performed with the largazole–biotin derivative **5** to detect HDACs 1–3 binding in cell lysate. Streptavidin beads were added after a 16 h incubation with compound **5** or vehicle in 200 µg of HeLa nuclear extract. Figure 6 shows immunoblot analysis of the resulting eluates, confirming the enrichment of HDACs 1–3 from streptavidin beads conjugated to compound **5** over mock pull down (vehicle only) or input samples.

3. Conclusion

We have developed a versatile, successful synthesis of new cap group derivatives of the potent natural product largazole with functional handles for conjugating probe molecules, reporters, and potential cancer-cell-specific targeting moieties. The potent biochemical activity observed for the thiazole-derivatized analogues reported herein demonstrates that the unencumbered cap group chemical space is a fertile area for



Scheme 7. Synthesis of depsipeptide–biotin conjugate (**5**) and peptide isostere–biotin conjugate (**6**). Reagents and conditions: (a) thiophenol, 2 M potassium hydroxide, MeCN; (b) sodium bicarbonate, MeCN; (c) DCM–TFA (20:1), triisopropylsilane, 41% (depsipeptide derivative) over three steps, 36% (peptide isostere) over three steps.

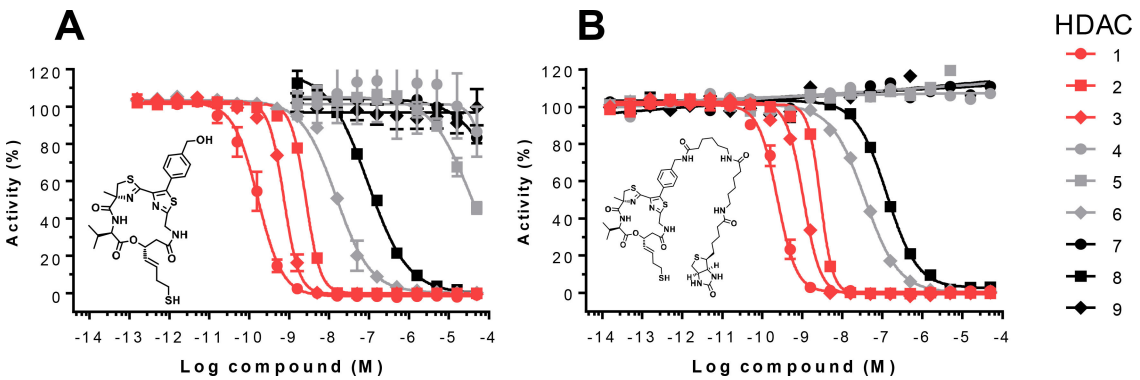


Figure 5. Inhibition of HDAC activity by largazole thiol and biotinylated largazole thiol. (A,B) Inhibition of HDAC 1–9 activity by (A) **43** and (B) **5** is presented in dose–response format. Data represent mean values of quadruplicate data ± s.d. IC_{50} curves were fit by logistic regression.

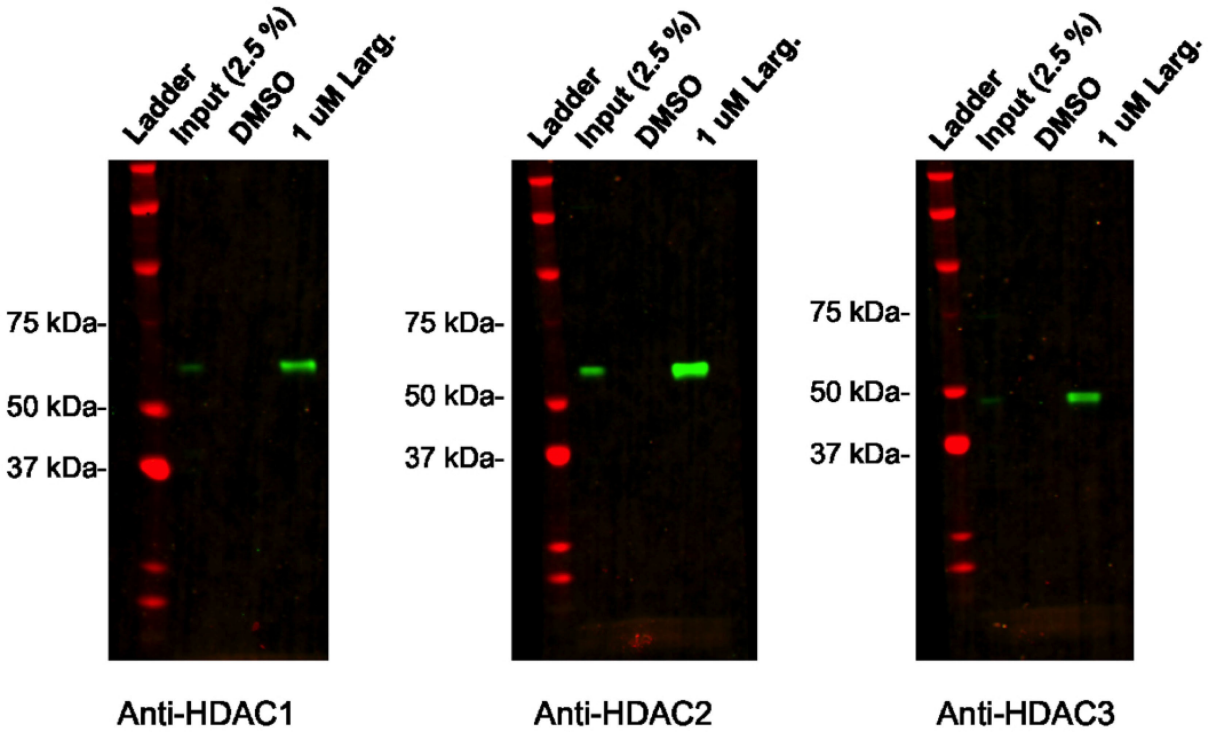


Figure 6. 1 μM largazole–biotin (**5**) in 200 μL of HeLa nuclear extract (~200 μg); 1 mg of streptavidin beads added after 16 h incubation with **5**.

future investigations into increasing the selectivity and therapeutic potential of largazole analogues. We are intensively investigating the chemical conjugation of other molecules to the largazole scaffold via the approach described herein and will report on this in due course.

4. Experimental Section

4.1 General Experimental Methods

All reactions were run under argon in oven- or flame-dried glassware. Thin-layer silica gel chromatography (TLC) was used to monitor reactions on 0.25 mm silica gel 60F plated with fluorescent indicator from Merck. Various stains were used to visualize TLC plates, including but not limited to anisaldehyde and Seebach's with gentle heating. Column chromatography and preparatory TLC were used to purify all compounds. The solvent systems described below were run on silica gel 60 (230–400 mesh) purchased from Sorbent Technologies. Reagents were purchased from commercial sources and used either directly or after drying. ^{1}HNMR and ^{13}CNMR were recorded using Varian 300, 400, or 500 MHz NMR spectrometers. Chemical shifts are reported in ppm relative to $CHCl_3$ at $\delta = 7.27$ (^{1}HNMR) and $\delta = 77.23$ (^{13}CNMR) or tetramethylsilane (TMS) at $\delta = 0.00$. Mass spectra were obtained on a Fisions VG Autospec. Optical rotations were collected at 589 nm on a Rudolph Research Automatic Polarimeter Autopol III. FT-IR spectra were collected on a Thermo Scientific Nicolet iS50.

4.2 *tert*-Butyl ((4-Cyano-5-iodothiazol-2-yl)methyl)carbamate (28)

To a solution of thiazole **27** (85 mg, 0.36 mmol) in 7.2 mL of THF at −78 °C, 1.1 mL of LDA (0.88 mmol, 0.8 M in THF) was added dropwise (it should be noted that the addition of more than three equivalents of LDA would have resulted in decomposition of SM). The mixture was stirred at −78 °C for 20 min, then a THF solution (1.44 mL) of I_2 (365 mg, 1.44 mmol) was added. After stirring at the same temperature for 5 min, the solution was treated with saturated aq. NH_4Cl, diluted with ethyl acetate, separated, washed with brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (0 to 9% ethyl acetate in DCM) to afford 97.4 mg (74% yield) of thiazole iodide **28** as a brown solid.

^{1}HNMR (400 MHz, $CDCl_3$) δ 5.29 (s, 1H), 4.57 (d, J = 6.4 Hz, 2H), 1.46 (s, 9H); **^{13}CNMR** (100 MHz, $CDCl_3$) δ 176.5, 155.6, 134.6, 113.8, 85.0, 81.0, 42.6, 28.3; **IR** (neat) 3364, 2927, 1678, 1516; **HRMS** (ESI): *m/z* calcd. for $C_{10}H_{12}IN_3NaO_2S^+$ $(M+Na)^+$ 387.9587, found 387.9579.

4.3 *tert*-Butyl ((4-Cyano-5-(4-(hydroxymethyl)phenyl) thiazol-2-yl)methyl)carbamate (30)

Thiazole iodide **28** (550 mg, 1.5 mmol), 4-(hydroxymethyl) phenylboronic acid **29**, (343 mg, 2.3 mmol), $NaHCO_3$ (380 mg, 4.5 mmol), and $Pd(PPh_3)_4$ were combined, and 16 mL of solvent ($DME/H_2O = 3/1$, v/v) was added. The mixture was heated to 120 °C and refluxed at that temperature for 14 h, then cooled to room temperature and diluted with water. DME was evaporated, and the aqueous layer was extracted with ethyl acetate and separated. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (0 to 11 % ethyl acetate in DCM) to afford 67 mg (19 % yield) of protonated compound **27** and 354 mg (68 % yield) of desired coupling product **30** as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61 (d, $J = 8.2$ Hz, 2H), 7.41 (d, $J = 8.2$ Hz, 2H), 5.64 (br s, 1H), 4.70 (s, 2H), 4.52 (d, $J = 4.6$ Hz, 2H), 2.81 (br s, 1H), 1.45 (s, 9H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 169.1, 143.7, 128.7, 128.1, 127.5, 127.0, 120.4, 115.3, 114.6, 80.8, 64.2, 42.3, 28.3 (×3); **IR** (neat) 3332, 2978, 2227, 1689, 1513, 1279, 1248, 910, 728; **HRMS** (ESI): *m/z* calcd. for $C_{17}H_{19}N_3O_2S^+$ $(M+Na)^+$ 368.1039, found 368.1030.

4.4 *tert*-Butyl ((4-Bromophenyl)sulfonyl)(4-(2-(((*tert*-butoxycarbonyl)amino)methyl)-4-cyanothiazol-5-yl)benzyl)-carbamate (31)

Benzyl alcohol **30** (35 mg, 0.1 mmol), NsNHBoc (45 mg, 0.15 mmol), and PPh_3 (39 mg, 0.15 mmol) were combined and 1 mL of THF was added. To the mixture was added DEAD (40 % in toluene, w/w, 75 µL, 0.15 mmol) at 0 °C. After stirring at 0 °C for 30 min, the solution was washed with 1 N HCl, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (0 % to 4 % ethyl acetate in DCM) to afford 57 mg (90 % yield) of **31** as a yellow solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.32 (dd, $J = 8.3$, 1.7 Hz, 1H), 7.79–7.69 (m, 5H), 7.54 (d, $J = 8.2$ Hz, 1H), 5.54 (br s, 1H), 4.99 (s, 2H), 4.57 (d, $J = 5.9$ Hz, 2H), 1.46 (s, 9H), 1.28 (s, 9H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 176.2, 169.2, 150.2, 147.6, 140.0, 134.5, 133.8, 133.1, 132.4, 132.0, 128.5, 128.4, 127.5, 124.6, 120.7, 114.7, 85.7, 80.8, 50.6, 42.5, 28.3 (×3), 27.8 (×3); **IR** (neat) 3353, 2980, 1730, 1543, 1367, 1280, 1249, 1151, 1123, 1062, 918, 852, 760; **HRMS** (ESI): *m/z* calcd. for $C_{28}H_{31}N_5NaO_8S_2^+$ $(M+Na)^+$ 652.1506, found 652.1498.

4.5 (*R*)-2′-(((*tert*-Butoxycarbonyl)amino)methyl)-4-methyl-5′-(4-(((4-nitrophenyl)sulfonamido)methyl)phenyl)-4,5-dihydro-[2,4′-bithiazole]-4-carboxylic Acid (33)

Thiazole nitrile **31** (377 mg, 0.6 mmol), α-methyl-cysteine-HCl (124 mg, 0.72 mmol), and $NaHCO_3$ (76 mg, 0.9 mmol) were combined and 10 mL of methanol was added. To the mixture was added Et_3N (0.16 mL, 1.2 mmol). After refluxing at 70 °C for 36 h, the solvent was removed under reduced pressure. The residue was purified by flash column chromatography on silica gel (9 % to 30 % ethyl acetate in DCM) to afford 230 mg (**32 : 33** = 1 : 1, 59 %) of **32** and **33** as a yellow foam. **32** and **33** were used as a mixture for the next reaction without further purification. An analytically pure sample could be obtained by esterification, separation, then hydrolysis.

(R=H, **33**), $[\alpha]_D^{28} = -8°$ ($c = 1.126$ in CH_3OH); **^{1}H NMR** (400 MHz, CD_3OD) δ 7.81 (dd, $J = 7.8$, 1.2 Hz, 1H), 7.74 (dd, $J = 7.6$, 1.4 Hz, 1H), 7.66 (ddd, $J = 7.8$, 7.6, 1.4 Hz, 1H), 7.59 (ddd, $J = 7.8$, 7.6, 1.2 Hz, 1H), 7.33 (d, $J = 8.1$ Hz, 2H), 7.25 (d, $J = 8.1$ Hz, 2H), 4.46 (s, 2H), 4.29 (s, 2H), 3.76 (d, $J = 11.3$ Hz, 1H), 3.22 (d, $J = 11.3$ Hz, 1H), 1.45 (s, 3H), 1.45 (s, 9H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 176.0, 169.5, 162.6, 156.8, 147.7, 141.6, 140.0, 138.2, 133.8, 133.2, 132.1, 130.2, 129.7, 128.9, 127.5, 124.3, 84.8, 79.6, 46.3, 41.7, 41.5, 27.3, 22.6; **IR** (neat) 3120, 2974, 2938, 1693, 1665, 1392, 1365, 1347, 1172, 1154, 1079, 863; **HRMS** (ESI): *m/z* calcd. for $C_{27}H_{30}N_5O_8S_3^+$ $(M+H)^+$ 648.1251, found 648.1231.

4.6 2-(Trimethylsilyl)ethyl (*S,E*)-3-((((*R*)-2′-(((*tert*-Butoxycarbonyl)amino)methyl)-4-methyl-5′-(4-(((4-nitrophenyl)sulfonamido)methyl)phenyl)-4,5-dihydro-[2,4′-bithiazole]-4-carbonyl)-L-valyl)oxy)-7-(tritylthio)hept-4-enoate (35)

19 (336 mg, 0.4 mmol) was dissolved in 40 mL of CH_3CN. 4 mL of diethylamine was added at 0 °C. The bath was removed and the resulting solution was stirred at room temperature for 2 h, then evaporated, azeotroped with toluene (2 × 2 mL), and dried under vacuum. In another round flask, acid **32/33** (230 mg, 0.35 mmol, mixture of **32** and **33**, 1 : 1) was dissolved in 55 mL of DCM. PyBOP (416 mg, 0.8 mmol) and DIPEA (210 µL, 1.2 mmol) were added and the mixture was allowed to stir at room temperature for 20 minutes. To the resulting solution was added a DCM solution (total 25 mL) of crude amine. After 3 h, the reaction was concentrated and submitted immediately to column chromatography (0 % to 25 % ethyl acetate in DCM) to afford the macrocyclization precursor (236 mg, 58 %, **34 : 35** = 1 : 1) as a yellow foam. **34** and **35** were used as a mixture for the next reaction without further purification.

4.7 *N*-(4-((1²*Z*,2²*Z*,2⁴*R*,5*S*,8*S*)-5-Isopropyl-2⁴-methyl-3,6,10-trioxo-8-((*E*)-4-(tritylthio)but-1-en-1-yl)-2⁴,2⁵-dihydro-7-oxa-4,11-diaza-1(4,2),2(2,4)-dithiazolacyclododecaphane-1⁵-yl)benzyl)-4-nitrobenzenesulfonamide (36)

Acyclic precursor (236 mg, 0.2 mmol, mixture of **34** and **35**, 1:1) was dissolved in 30 mL of DCM, and 6 mL of TFA was added to the solution at 0 °C. The reaction was allowed to warm to room temperature and stirred for 16 h. Solvents were evaporated and the crude amino acid was azeotroped with toluene (10×3 mL) to remove residual TFA. The crude amino acid was then dissolved in 300 mL of CH_3CN (to ~0.001 M) and DIPEA (0.32 mL, 1.8 mmol) was added. The resulting moderately opaque solution was allowed to stir for 10 min, before an MeCN (10 mL) solution of HATU (230 mg, 0.6 mmol) and HOBt (81 mg, 0.6 mmol) was added dropwise. The reaction was allowed to stir for 26 h, then concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (0% to 11% AcOEt in DCM for the first column, 25% to 75% ethyl acetate in hexane for the second column, and 0% to 9% AcOEt in DCM for the third column) to afford 40 mg (20% yield for two steps) of **36** as a yellow foam.

$[\alpha]_D^{26} = +19^\circ$ ($c = 0.147$ in $CHCl_3$); **^{1}H NMR** (500 MHz, CD_3OD) δ 7.91 (dd, $J = 7.8$, 1.3 Hz, 1H), 7.80 (dd, $J = 7.8$, 1.3 Hz, 1H), 7.71 (ddd, $J = 7.9$, 7.5, 1.4 Hz, 1H), 7.64 (dddd, $J = 7.7$, 7.6, 5.1, 1.2 Hz, 1H), 7.57–7.18 (m, 15H), 5.71 (ddd, $J = 16.1$, 8.4, 6.7 Hz, 1H), 5.63 (m, 1H), 5.48 (dd, $J = 15.4$, 6.3 Hz, 1H), 5.11 (d, $J = 17.5$ Hz, 1H), 4.55 (d, $J = 3.8$ Hz, 1H), 4.37 (s, 1H), 4.34 (d, $J = 17.5$ Hz, 1H), 3.77 (d, $J = 11.6$ Hz, 1H), 3.24 (d, $J = 11.6$ Hz, 1H), 3.20 (m, 1H), 2.89 (dd, $J = 15.9$, 10.6 Hz, 1H), 2.67 (dd, $J = 15.9$, 3.1 Hz, 1H), 2.20 (dd, $J = 17.3$, 17.0 Hz, 1H), 2.12–2.02 (m, 2H), 1.92–1.88 (m, 2H), 1.77 (s, 3H), 1.59 (m, 1H), 0.73 (d, $J = 6.9$ Hz, 3H), 0.57 (d, $J = 6.9$ Hz, 3H); **^{13}C NMR** (125 MHz, CD_3OD) δ 175.8, 171.8, 170.3, 168.3, 167.2, 149.3, 146.3, 144.6, 142.9, 140.8, 135.4, 134.8, 133.8, 133.5, 133.0, 131.6, 131.3, 130.8, 129.3, 129.2, 128.9, 127.7, 125.8, 83.9, 73.6, 58.9, 47.6, 47.4, 44.4, 41.3, 41.0, 35.2, 32.5, 32.4, 30.8, 30.5, 27.4, 24.5, 23.8, 19.8, 17.3, 14.4; **IR** (neat) 3362, 2960, 2926, 2853, 1734, 1668, 1540, 1443, 1344, 1259, 1165, 1071, 972, 853; **HRMS** (ESI): *m/z* calcd. for $C_{53}H_{53}N_6O_8S_4^+$ $(M+H)^+$ 1029.2802, found 1029.2778.

4.8 Largazole–Biotin Conjugate (5)

Thiophenol (10.4 µL, 0.1 mmol), KOH (3.6 mg, 64 µmol), water (30 µL), and acetonitrile (1.2 mL) were combined to give a colorless solution. To 8.2 mg (9.1 µmol) of Ns amine **36** was added 0.4 mL of the above solution at room temperature. The reaction was allowed to stir for 2 h then diluted with 4 mL of hexane and submitted directly to column chromatography (100% AcOEt then 25% MeOH in DCM) to afford 6.0 mg (78% yield) of free amine as a yellow oil.

Free amine (6.0 mg, 9 µmol), biotin derivative **40** (8.1 mg, 18 µmol), and $NaHCO_3$ (1.5 mg, 18 µmol) were combined. DMF (0.3 mL) was added and the reaction was allowed to stir for 14 h then concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (100% AcOEt then 5% to 11% MeOH in DCM) then by preparative TLC (9% MeOH in DCM three times) to afford the pure coupling product **41** as a yellow oil.

Coupling product **41** was dissolved in 0.6 mL of DCM and cooled to 0 °C. TFA (30 µL) and iPr$_3$SiH (1.0 µL, 6 µmol) were added to the solution at 0 °C. The bath was removed and the reaction was allowed to stir at room temperature for 1 h. The solvent was removed by argon flow and the residue was purified by flash column chromatography on silica gel (0 to 9% MeOH in $CHCl_3$) to afford 4.0 mg of free thiol **5** (53% for two steps). This was further purified by preparative TLC (9% MeOH in DCM two times) to afford the pure sample for biological evaluation.

$[\alpha]_D^{25} = +52.0^\circ$ ($c = 0.050$ in CH_3OH); **^{1}H NMR** (600 MHz, CD_3OD,) 7.48 (d, $J = 8.0$ Hz, 2H), 7.40 (d, $J = 8.0$ Hz, 2H), 5.92–5.82 (m, 2H), 5.72–5.63 (m, 2H), 5.13 (d, $J = 8.0$ Hz, 1H), 4.59–4.55 (m, 2H), 4.49 (dd, $J = 7.7$, 4.7 Hz, 1H), 4.44–4.40 (m, 3H), 4.30 (dd, $J = 7.7$, 4.7 Hz, 1H), 3.77 (d, $J = 11.6$ Hz, 1H), 3.60 (s, 1H), 3.27 (d, $J = 11.6$ Hz, 1H), 3.21–3.15 (m, 5H), 2.98–2.91 (m, 2H), 2.76–2.69 (m, 3H), 2.56 (dd, $J = 6.9$, 6.9 Hz, 1H), 2.45 (dd, $J = 13.5$, 6.5 Hz, 1H), 2.36 (m, 1H), 2.28 (dd, $J = 7.4$, 7.4 Hz, 2H), 2.23–2.09 (m, 6H), 1.81 (s, 3H), 1.76–1.29 (m, 23H), 0.74 (d, $J = 6.9$ Hz, 3H), 0.59 (d, $J = 6.9$ Hz, 3H); **^{13}C NMR** (125 MHz, CD_3OD) 176.1, 176.0, 175.9, 175.8, 171.8, 170.4, 168,3, 167.2, 166.0, 144.7, 143.0, 142.5, 133.7, 131.4, 129.7, 129.1, 128.8, 84.0, 73.8, 64.3, 63.3, 61.6, 58.9, 57.0, 44.4, 43.6, 41.2, 41.0, 40.2, 38.7, 37.0, 36.9, 36.8, 35.2, 32.7, 30.1, 29.8, 29.5, 27.5, 26.9, 26.7, 26.6, 24.5, 19.8, 17.3; **IR** (neat) 3308, 2930, 2859, 1656, 1551, 1462, 1262, 667; **HRMS** (ESI): *m/z* calcd. for $C_{50}H_{72}N_9O_8S_4^+$ $(M+H)^+$ 1054.4381, found 1054.4346.

4.9 Methyl (*S*,*E*)-3-((*S*)-2-((*R*)-2′-(((*tert*-Butoxycarbonyl)amino)methyl)-4-methyl-5′-(4-(((4-nitrophenyl)sulfonamido)-methyl)phenyl)-4,5-dihydro-[2,4′-bithiazole]-4-carboxamido)-3-methylbutanamido)-7-(tritylthio)hept-4-enoate (38)

Boc-amine **26** (420 mg, 0.66 mmol) was dissolved in 10 mL of DCM and 1 mL of TFA was added to the solution at 0 °C. The reaction was allowed to warm to room temperature and stirred for 2 h. Solvents were evaporated and the crude amino acid was azeotroped with toluene (2×2 mL) to remove residual TFA. Acid **32/33** (470 mg, ca. 0.72 mmol, **32**:**33** = 1:1) was dissolved in 50 mL of DCM. PyBOP (730 mg, 1.4 mmol) and DIPEA (630 µL, 3.6 mmol) were added and the mixture was allowed to stir at room temperature for 20 minutes. To the resulting solution was added a DCM solution (total 10 mL) of crude amine. After 4 h, the reaction was concentrated and submitted immediately to column chroma-

tography (16% to 33% ethyl acetate in DCM) to afford macrocyclization precursor **37 : 38** (1 : 1) as a yellow foam.

For R=H **38**, $[\alpha]_D^{26} = -55.8^\circ$ ($c = 0.163$ in $CHCl_3$); **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.04 (m, 1H), 7.67 (m, 1H), 7.61–7.58 (m, 2H), 7.36–7.34 (m, 10H), 7.27–7.14 (m, 13H), 6.63 (d, $J = 8.9$ Hz, 1H), 6.46 (d, $J = 9.4$ Hz, 1H), 5.87 (br s, 1H), 5.46 (ddd, $J = 15.4$, 6.8, 6.3 Hz, 1H), 5.37–5.31 (m, 2H), 4.76 (m, 1H), 4.57 (d, $J = 6.0$ Hz, 2H), 4.31 (m, 2H), 3.93 (dd, $J = 8.9$, 8.6 Hz, 2H), 3.67 (m, 2H), 3.56 (m, 1H), 3.53 (s, 3H), 3.45 (d, $J = 11.6$ Hz, 1H), 3.19 (d, $J = 11.6$ Hz, 1H), 2.52 (m, 2H), 2.13–1.98 (m, 2H), 1.73 (m, 1H), 1.44 (s, 12H), 1.26–1.20 (m, 4H), 0.77 (d, $J = 6.7$ Hz, 3H), 0.62 (d, $J = 6.7$ Hz, 3H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ 174.4, 171.7,169.8, 167.4, 162.3, 148.0, 144.8, 141.6, 137.8, 134.2, 132.3, 130.6, 130.5, 130.3, 129.6, 129.5, 129.4, 127.8, 127.6, 126.5, 124.5, 85.5, 80.4, 66.5, 58.6, 51.7, 47.2, 47.1, 43.7, 42.3, 40.6, 38.6, 31.4, 31.3, 30.5, 29.6, 24.1, 19.2, 18.4, 18.1, 17.0, 14.1, 12.6; **IR** (neat) 3367, 2964, 2927, 1717, 1652, 1541, 1511, 1366, 1164, 972, 752; **HRMS** (ESI): *m/z* calcd. for $C_{59}H_{65}N_7NaO_{10}S_4^+$ $(M+Na)^+$ 1182.3568, found 1182.3583.

4.10 *N*-(4-((1²*Z*,2²*Z*,2⁴*R*,5*S*,8*S*)-5-Isopropyl-2⁴-methyl-3,6,10-trioxo-8-((*E*)-4-(tritylthio)but-1-en-1-yl)-2⁴,2⁵-dihydro-4,7,11-triaza-1(4,2),2(2,4)-dithiazolacyclododecaphane-1⁵-yl)benzyl)-4-nitrobenzenesulfonamide (39)

Acyclic precursor **37/38** obtained above was azeotroped with toluene (2 × 2 mL) to remove residual AcOEt, then combined with $LiOH \cdot H_2O$ (139 mg, 3.3 mmol). Solvent (THF–MeOH–H_2O, 2 + 2 + 1 mL) was added and the reaction mixture was allowed to stir for 2.5 h. The reaction mixture was diluted with 10 mL of water, then the pH was adjusted to 2 by using 1 N HCl. Organic solvents were evaporated and the remaining aqueous layer was extracted with DCM (3 × 10 mL) then dried over Na_2SO_4, filtered, and evaporated. The crude acid was dissolved in 15 mL of DCM and 3 mL of TFA was added to the solution at 0 °C. The reaction was allowed to warm to room temperature and stirred for 3 h. Solvents were evaporated and the crude amino acid was azeotroped with toluene (2 × 2 mL) to remove residual TFA. The crude amino acid was then taken up in 700 mL MeCN and DIPEA (1 mL, 5.7 mmol) was added. The resulting moderately opaque solution was allowed to stir for 10 min, before HATU (500 mg, 1.32 mmol) and HOBt (178 mg, 1.32 mmol) were added. The reaction was allowed to stir for 14 h, then concentrated and redissolved in AcOEt. The solution was washed with saturated aqueous NH_4Cl, $NaHCO_3$, and brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (0% to 33% AcOEt in DCM for the first column, 16% to 33% AcOEt in DCM for the second column, then 33% to 66% AcOEt in DCM for PTLC) to afford 80 mg (12% yield for five steps) of **39** as white foam. (The product does dissolve in pure DCM and mixtures of DCM/AcOEt, DCM/MeOH and $CHCl_3$/MeOH but does not dissolve in pure $CHCl_3$, AcOEt, MeCN, or MeOH. Use 66% AcOEt in DCM for developing TLC.)

$[\alpha]_D^{30} = +17.5^\circ$ ($c = 0.257$ in CH_2Cl_2); **^{1}H NMR** (400 MHz, CD_2Cl_2) δ 8.03 (dd, $J = 7.5$, 2.2 Hz, 1H), 7.82 (dd, $J = 7.8$, 1.5 Hz, 1H), 7.72–7.68 (m, 2H), 7.31–7.18 (m, 23H), 6.92 (d, $J = 8.1$ Hz, 1H), 6.72 (d, $J = 9.5$ Hz, 1H), 6.63 (d, $J = 10.5$ Hz, 1H), 6.21 (dd, $J = 6.4$, 6.4 Hz, 1H), 5.52–5.40 (m, 2H), 5.07 (dd, $J = 17.5$, 8.4 Hz, 1H), 4.86 (m, 2H), 4.47 (dd, $J = 10.4$, 3.3 Hz, 1H), 4.35 (dd, $J = 6.4$, 1.8 Hz, 1H), 4.24 (dd, $J = 17.5$, 3.1 Hz, 1H), 3.71 (d, $J = 11.7$ Hz, 1H), 3.24 (d, $J = 11.7$ Hz, 1H), 2.67 (dd, $J = 14.6$, 4.2 Hz, 1H), 2.52 (dd, $J = 14.8$, 9.1 Hz, 1H), 2.37 (m, 1H), 2.20 (dd, $J = 7.7$, 7.1 Hz, 2H), 2.02–1.98 (m, 2H), 1.77 (s, 3H), 0.87 (dd, $J = 6.9$, 6.6 Hz, 1H), 0.71 (d, $J = 6.8$ Hz, 3H), 0.40 (d, $J = 6.8$ Hz, 3H); **^{13}C NMR** (100 MHz, CD_2Cl_2) δ 173.7, 170.1, 170.0, 166.0, 147.7, 144.8, 142.1, 141.9, 138.4, 133.7, 133.5, 132.9, 130.8, 130.5, 130.2, 129.6, 129.5, 128.3, 127.9, 127.8, 126.6, 126.5, 125.2, 83.8, 66.9, 58.5, 48.2, 47.4, 44.6, 41.3, 40.5, 38.7, 32.3, 31.9, 31.7, 31.6, 30.0, 24.0, 19.7, 16.0, 14.3; **IR** (neat) 3351, 2960, 2927, 1661, 1539, 1499, 1364, 1265, 1163, 1070, 844, 733, 700, 583; **HRMS** (ESI): *m/z* calcd. for $C_{53}H_{53}N_7NaO_7S_4$ $(M+Na)^+$ 1050.2781, found 1050.2801.

4.11 Largazole Peptide Isostere–Biotin Conjugate (6)

Thiophenol (10.4 µL, 0.1 mmol), KOH (3.6 mg, 64 µmol), water (30 µL), and DMF (1.2 mL; acetonitrile was used as solvent to deprotect the Ns group of the depsipeptide macrocyclic substrate **36** mentioned above, however this peptide macrocycle **39** is not soluble in acetonitrile therefore DMF was used here) were combined to give a colorless solution. To 8.5 mg (8.3 µmol) of Ns amine **39** was added 1.0 mL of above solution at room temperature. The reaction was allowed to stir for 3 h then evaporated and purified by column chromatography (50% AcOEt in DCM then 50% MeOH in DCM) to afford 6.1 mg (87% yield) of free amine as a yellow solid.

Free amine (6.1 mg, 7.2 µmol), biotin derivative **40** (8.2 mg, 14 µmol), and $NaHCO_3$ (1.2 mg, 14 µmol) were combined. DMF (0.5 mL) was added and the reaction was allowed to stir for 15 h then concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (100% AcOEt then 5% to 50% MeOH in DCM) then by preparative TLC (11% MeOH in DCM two times) to afford the pure coupling product **42** as a yellow solid.

Coupling product **42** was combined with 0.6 mL of DCM to give a suspension. TFA (30 µL) was added at 0 °C and the suspension soon became a yellow solution. iPr$_3$SiH (1.0 µL, 6 µmol) was added to the solution at 0 °C. The bath was removed and the reaction was allowed to stir at room temperature for 1 h. Solvent was removed by argon flow and the residue was purified by flash column chromatography on silica gel (4 to 50% MeOH in DCM) to afford 3.1 mg of free thiol **6** (41% for two steps). This was further purified by

preparative TLC (11% MeOH in DCM two times) to afford the pure sample for biological evaluation.

$[\alpha]_D^{29}$ = +88.0° (*c* = 0.10 in CH_3OH); **^{1}H NMR** (600 MHz, CD_3OD,) 7.49 (d, *J* = 8.2 Hz, 2H), 7.41 (d, *J* = 8.2 Hz, 2H), 5.70–5.56 (m, 3H), 5.17 (d, *J* = 17.5 Hz, 1H), 4.93 (m, 1H), 4.91 (ddd, *J* = 12.2, 5.9, 4.1 Hz, 1H), 4.52 (d, *J* = 3.0 Hz, 1H), 4.48 (m, 1H), 4.44 (s, 2H), 4.37 (d, *J* = 17.5, 1H), 4.29 (dd, *J* = 7.9, 4.5 Hz, 1H), 3.71 (d, *J* = 11.9 Hz, 1H), 3.65 (s, 1H), 3.60 (s, 1H), 3.40 (d, *J* = 11.9 Hz, 1H), 3.21–3.14 (m, 4H), 2.92 (dd, *J* = 12.8, 5.0 Hz, 1H), 2.77–2.69 (m, 2H), 2.64 (dd, *J* = 14.9, 4.0 Hz, 1H), 2.55 (1H, dd, *J* = 6.9, 6.9 Hz), 2.47–2.40 (m, 2H), 2.35 (dd, *J* = 13.5, 6.8 Hz, 1H), 2.28 (dd, *J* = 7.4, 7.4 Hz, 1H), 2.18 (dd, *J* = 13.5, 7.2 Hz, 2H), 1.88 (3H, s), 1.75–1.36 (m, 23H), 0.73 (d, *J* = 6.9 Hz, 3H), 0.41 (d, *J* = 6.9 Hz, 3H); **^{13}C NMR** (125 MHz, CD_3OD) 176.2, 176.0, 175.9, 175.8, 172.2, 172.1, 169.6, 167.7, 160.0, 144.8, 142.8, 142.7, 132.5, 131.3, 129.8, 129.0, 128.7, 84.2, 64.3, 63.4, 61.6, 59.2, 57.0, 44.7, 43.6, 41.1, 41.0, 40.1, 39.1, 37.5, 37.0, 36.9, 36.8, 33.3, 30.7, 30.1, 29.7, 29.5, 27.5, 26.9, 26.7, 26.6, 24.6, 24.3, 20.0, 15.8; **IR** (neat) 3300, 2927, 2854, 1677, 1548, 1435, 1205, 1132, 801, 724; **HRMS** (ESI): *m/z* calcd. for $C_{50}H_{72}N_{10}NaO_7S_4^+$ $(M+Na)^+$ 1075.4360, found 1075.4379.

4.12 HDAC Biochemical Assay

Compounds **1**, **2**, **5**, and **43** were tested against HDACs 1–9 and the activity was determined with an optimized homogenous assay performed in a 384-well plate. Reactions were performed in assay buffer (50 mM HEPES, 100 mM KCl, 0.001% Tween-20, 0.05% BSA, pH 7.4; additional 200 μM TCEP was added for HDAC6) and followed by fluorogenic release of 7-amino-4-methylcoumarin from the substrate upon deacetylase and trypsin enzymatic activity. Fluorescence measurements were obtained every five minutes using a multilabel plate reader and plate-stacker (Envision; Perkin-Elmer). Each plate was analyzed by plate repeat, and the first derivative within the linear range was imported into analytical software (Spotfire DecisionSite). Replicate experimental data from incubations with inhibitor were normalized to DMSO controls ([DMSO] < 0.5%). The IC_{50} was determined by logistic regression with unconstrained maximum and minimum values. The recombinant, full-length HDAC protein (BPS Biosciences) was incubated with fluorophore conjugates substrate, MAZ1600 and MAZ1675 at K_m = [substrate].

4.13 Biochemical Pull-Down Studies

HeLa cells (ATCC) were cultured in DMEM supplemented with 10% FBS and Pen Strep (100 U/mL). Nuclear extracts were collected from ~250 μL of HeLa cell pellet using NE-PER extraction reagents (ThermoFisher Scientific) according to the manufacturer's protocol. For pull-down studies, compound **5** or DMSO was added to 200 μL of HeLa nuclear extract (1 mg/mL total protein) at a final concentration of 1 μM and 0.1%, respectively. After 16 h incubation at 4 °C, 200 μg streptavidin Dynabeads (MyOne Streptavidin T1; ThermoFisher Scientific), equilibrated in NER buffer at 1 mg/mL, was added to each protein–compound mixture and incubated for 1 h at 4 °C with gentle rotation. Beads were then collected, washed four times with wash buffer (10 mM HEPES pH 8.0, 300 mM NaCl, 10 mM KCl, 1.5 mM $MgCl_2$, and 0.2% Triton X-100), and finally resuspended and boiled in 90 μL of 1X NuPAGE LDS sample buffer (ThermoFisher Scientific) to elute bound protein. Eluate and input samples were run on 4–12% Bis-Tris NuPAGE gels (ThermoFisher Scientific) and transferred to PVDF membranes. Membranes were blocked for 1 h at RT in Odyssey blocking buffer (TBS; LI–COR Biosciences) and then incubated overnight at 4 °C in blocking buffer supplemented with 0.2% Tween-20 and a primary antibody diluted 1 : 1000 (anti-HDAC1, Cell Signaling #2062; anti-HDAC2, Cell Signaling #2540; anti-HDAC3, Cell Signaling #2632). Next, membranes were washed three times with TBST at RT (5 min each) and incubated for 1 h at RT in blocking buffer supplemented with 0.2% Tween-20, 0.01% SDS, and IRDye 800CW goat anti-rabbit IgG (LI–COR Biosciences) diluted 1 : 7000. Following three 5 min washes in TBST, membranes were rinsed in TBS and imaged on the Odyssey CLx imaging system (auto intensity, 84 μm resolution, high quality; LI–COR Biosciences).

Notes

The authors declare no competing financial interest. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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