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Modular Synthesis and Biological Activity of Pyridyl-based Analogs of the Potent Class I Histone Deacetylase Inhibitor Largazole

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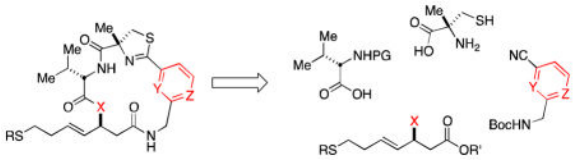
Abstract

The formation of a series of analogs containing a pyridine moiety in place of the natural thiazole heterocycle, based on the potent, naturally occurring HDAC inhibitor Largazole has been accomplished. The synthetic strategy was designed modularly to access multiple inhibitors with different aryl functionalities containing both the natural depsipeptide and peptide isostere variant of the macrocycle. The cytotoxicity and biochemical activity of the library of HDAC inhibitors is described herein.

Graphical abstract



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Supporting Information. ^{1}H-NMR and ^{13}C-NMR of all compounds reported in the experimental section, LC_{50} plots, sequence alignment and analysis of surface residues surrounding the active site, RMSD plots and pymol sessions files of all complexes of HDAC with Largazole analogs discussed. This material is available free of charge via the Internet at http://pubs.acs.org. Pymol sessions with additional snapshots from the MD simulations can be obtained from the authors upon request.

Keywords

Largazole; Class I Histone Deacetylase Inhibitor; pyridine analogs; cytotoxic and biochemical assays

1. INTRODUCTION

Largazole (**1**) has received a great deal of attention from the synthetic and medical communities ever since its isolation from the marine cyanobacterium of the genus *Symploca* by the Leusch laboratory.[1,2] This depsipeptide natural product has been synthesized and modified by several research groups due to its potent biochemical activity as a Class I Histone Deacetylase Inhibitor (HDACi) and consequent anticancer properties.[3–5] Largazole (**1**) is a pro-drug with the active form being the free thiol species **2** (Figure 1) that is formed *in vitro* and *in vivo* by esterase or lipase-based cleavage of the octanoyl residue.[6] It has been suggested by us that the octanoyl tail present on Largazole allows for better cell-permeability relative to the active, free thiol species.[6] The nascent thiol group tightly coordinates to the Zn^{2+}-domain within the HDAC enzyme active site resulting in tight, non-covalent binding of the drug and consequent enzymatic inhibition.

There are currently eighteen known HDAC enzymes present in human cells that are separated into four different classes based on their structural homology with yeast proteins.[7] Of these, Class I (HDACs 1, 2, 3, and 8), Class II (HDAC 4, 5, 6, 7, 9, and 10), and Class IV (HDAC 11) are Zn^{2+}-dependent while Class III (SirT1-7, known as the Sirtuins) are NAD^{+}-dependent. A substantial body of data has elucidated that the inhibition of the ubiquitous Class I HDACs is a viable anticancer therapeutic target.[8,9] Current research efforts in our laboratories have been to access inhibitors that are selective for Class I HDACs over the tissue-dependent Class II HDACs. The goal of our efforts has been to generate compounds based on the molecular scaffold of Largazole that exhibit superior potency, selectivity and drug-like properties relative to that of the natural product.

We have been active in pursuing new analogs of Largazole (**1**) since the completion of our concise total synthesis in 2008.[6] The first key structural alteration we examined, was to replace the depsipeptide oxygen atom present in the macrocycle with a lactam nitrogen atom providing the corresponding more chemically robust peptide isostere **3** (Figure 1).[10] We also examined other functional group changes in the macrocycle, as well as the zinc-binding domain.[11,12] The present work described herein continues this exploration by investigating the exchange of the thiazole heterocycle in the macrocyclic cap group for a pyridyl residue in both the natural depsipeptide and peptide isostere variants of the macrocyclic core (Figure 2).

2. RESULTS AND DISCUSSION

2.1. Design

The macrocyclic cap group of Largazole not only contributes significantly to the overall binding, but also offers unique opportunities for the design of even more potent and selective analogs because it allows the precise positioning of functional groups on the

surface of the protein by appropriate functionalization of the macrocycle. The analysis of the position of Largazole in our model of the HDAC1-Largazole thiol complex[13] as well as the recently published crystal structure of the HDAC8-Largazole thiol complex (pdb 3EQD)[14] provide the structural basis for rational design that exploits specific surface interactions. Based on the sequence alignment of the residues surrounding the active site (Figure S1 in the Supporting Information), we envisioned the replacement of the thiazole ring by a protonated pyridine to form a charge interaction with a nearby negatively charged residue (Asp), as shown in Figure 3. In addition, the inclusion of a positive charge could modify the conformational preferences of the macrocycle, which was shown previously to be important for the relative potency of the depsipetide and its amide isostere,[10] and control its overall shape, which is known to control isoform selectivity.[15] With these considerations in mind, two different isomers of the pyridyl-based analogs of Largazole, compounds **6** and **8** their respective amide isosteres **10** and **12** were chosen for synthetic studies and biochemical profiling.

2.2. Chemistry

Our published total synthesis of Largazole (**1**) provided the technological framework from which we have been able to access numerous new analogs.[6] The retrosynthetic analysis displayed in Scheme 1 outlines the modular nature of our approach. The key macrolactamization reaction we have found is best performed on the least-hindered amide located at the bottom of macrocycle **13**. The target macrocycle has been bisected into two halves through the top amide linkage. Bottom fragment **14** is constituted from L-Valine derivative **17** and both the requisite β-hydroxy ester (**16**, X = OH, for the depsipeptides) and the corresponding β-amino ester (**16**, X = NH_2, for the peptide isosteres). The thiazoline/pyridyl-containing fragment **15** will be formed through a cyclocondensation reaction between α-methyl-L-cysteine (**18**) and aryl nitrile **19**.

The synthesis of fragment **23** for the depsipeptide version of the macrocycle is displayed in Scheme 2. The formation of aldehyde **21** was achieved through a hetero-Michael addition of trityl mercaptan into acrolein followed by a Wittig olefination. The stereocenter present in alcohol **22** was set using a Crimmins's type chiral auxiliary.[16] This asymmetric aldol transformation occurred to produce alcohol **22** in very high diastereomeric purity. This material was submitted to cleavage of the chiral auxillary using 2-(trimethylsilyl)ethanol. Installation of the L-valine derived amino acid was mediated by EDCI to access depsipeptide bottom fragment **23**.

Peptide precursor **28** was furnished using the previously established protocol (Scheme 3).[10] Commercially available amino acid derivative **24** was converted to the primary alcohol through reduction of a mixed anhydride. Swern oxidation followed by Wittig olefination achieved formation of terminal alkene **25**. Scrambling of the stereogenic center was not apparent through an aqueous $KHSO_4$ workup of the oxidation step and submission of the aldehyde directly to the olefination reaction. The cross metathesis to construct alkene **26** proved to be troublesome in producing high yielding and reproducible results.[17] The primary alcohol was converted to the protected thiol through activation with TsCl and displacement with trityl thiol anion. After methyl ester formation, the amine present on

substrate **27** was unmasked using TFA and coupled with an L-valine derived amino acid to yield peptide precursor **28**.

Aryl fragment **32** was constructed starting with dicarboxylic acid **29** (Scheme 4). Fischer esterification and reduction of one of the ethyl esters provided the requisite primary alcohol functionality. After using NH_4OH to generate an amide from the corresponding carboxylic acid, $POCl_3$ successfully dehydrated the amide to generate the requisite nitrile and chloride present in compound **30**. Gabriel's protocol was utilized to convert the chloride into an amine, which was readily protected to provide cyclization partner **31**. Cyclocondensation with α-methyl-L-cysteine[18] proceeded in high yields to generate the pyridyl fragment **32**.

The route for the pyridyl "OUT" aryl fragment **36** is displayed in Scheme 5. The synthetic strategy follows the same method mentioned previously for the pyridine "IN" variant except dicarboxylic acid **33** was used instead.

The final steps to access the desired inhibitors were performed upon the successful assembly of the four key fragments. The depsipeptide class construction is shown in Scheme 6. The protected amine present in substrate **23** was unmasked using diethylamine. The successful union between the nascent amine and acid **32** or **36** was accomplished cleanly using PyBOP. The macrolactamization was performed using HATU and HOBt after exposing the requisite functionality through acid-mediated deprotection. Accessing pure material from the ring closure transformation required rigorous column chromatography. Trityl cleavage granted the formation of inhibitors **6** and **8**. Unfortunately, adding the octanoyl residue was only viable using standard acid chloride procedure on pyridine "OUT" depsipeptide thiol **8**. Pyridine "IN" thiol **6** gave rise to dioctanoylation when exposed to the same conditions. The *N*-octanoyl-imidazole reagent was prepared and then was exposed to thiol **6**. The change in the reactivity of the carbonyl gave rise to only the thioester being formed.

The creation of the peptide class of pyridine-thiazoline inhibitors **10** and **12** followed the same method discussed for their depsipeptide counterparts. The only difference in their route arose during the deprotection steps. The same strategy was employed as previously discussed to add the octanoyl fragment to arrive at inhibitors **9** and **11**.

2.3. Biochemical & Biological Evaluation

Compounds **1**–**12** were tested for inhibitory activity against HDACs 1–9, using an optimized homogenous assay performed in a 384-well plate, as described previously.[6] The results of these studies, summarized in Table 1 and Figure S5, identify that the thiol derivatives of Largazole including compounds **2, 4, 6, 8, 10, and 12,** are among the most potent with IC_{50} values below 25nM for HDACs 1–3. Of these thiols, the depsipeptide pyridine thiols **6, 8** show similar potency to Largazole thiol (**2**) and Largazole peptide isostere thiol (**4**) with IC_{50} values in the single digit nM range for HDACs 1–3. Conversely, the peptide pyridine thiols **10, 12** show a decrease in potency with IC_{50}s in the double-digit nM range for HDACS 1–3. Largazole (**1**) and derivatives **3, 5, 7, 9, 11** display IC_{50} values in the 200nM–1μM range. Of these compounds, Largazole (**1**) and peptide pyridine "OUT" derivative **11** were similar in potency for HDACs 1–3 with IC_{50}s in the high nM–μM range while the depsipeptide pyridines and peptide pyridine "IN" derivative **5, 7, 11** exhibited an increased

potency with IC_{50}s in the 200nM–350nM range for HDAC 1. These studies demonstrate that the free thiol is important for increased inhibition, as previously reported, with the depsipeptide pyridine derivatives displaying increased inhibition over the peptide isostere derivatives.

Next, we tested the activity of the novel analogs in the 797 and 10326 NUT midline carcinoma cell line. As can be seen in Table 2 and Figure S6, depsipeptide pyridine "OUT" **7** showed the greatest potency against both cell lines with an IC_{50} of 10nM. This was comparable to both Largazole and depsipeptide pyridine "IN" **1, 5** with IC_{50}s of 20nM against both 797 and 30nM and 40nM against 10326 respectively. Compound **10** was not evaluated due to it's low biochemical activity.

Conversely, the peptide isosteres **9, 11** exhibited ~10-fold decreased inhibition in comparison to the depsipeptide pyridine derivatives, demonstrating that the depsipeptide mimetic may be important for increased inhibition. Compound **11** is more active in cell culture but it appears to have less activity in the biochemical assay compared to the other prodrugs derivatives. While this is an unexpected result in cell culture as the other prodrugs and their thiols derivatives show stronger activity biochemically, it may be possible that the compound 11 is cleaved and activated within cells to a higher extent than it's prodrug counterparts yielding higher activity in cell culture.

2.4. Computational Studies of Selectivity

The biochemical profiling provides a number of interesting results that, when combined with the results from molecular dynamics simulations, probe the structural basis for the observed selectivity. We studied Largazole thiol **2** and its analogues **6**, **8**, **10** and **12**, which are postulated to be the active form of the corresponding Largazole derivatives,[6] in our homology model of HDAC1,[13] HDAC6,[15] and the crystal structure of HDAC8 (pdb 3EQD).[14] Here, we will discuss the cases of the largest experimentally observed selectivity in detail, while the full results for all five compounds in the three HDAC isoforms studied can be found in the Supporting Information.

We will start the discussion of the potency of Largazole thiol and its derivatives in HDAC1. Experimentally, the most notable finding is the 16-fold decrease in activity in going from **6**, which is similar to Largazole thiol **2**, to its amide isostere **10**. Figure 4 shows snapshots from the MD simulations of **6** (blue, with protein in red) and **10** (green) in HDAC1. For comparison purposes, the previously analyzed[10] position of **2** is shown in grey. As can be seen, the overall positions of **2** and **6** at the mouth of the active site pocket are similar. In addition, **6** does indeed establish an interaction between the protonated pyridyl ring and the key aspartate D99, although the NH-O distance fluctuates to up to >3.5 Å over the course of the simulation. However, the macrocyclic ring of **6** has to rotate relative to the position of **2** to enable this interaction, decreasing the covered surface area. As a result, the overall IC_{50} of the two compounds is similar. In contrast, **10** does not establish over the course of the simulation, presumably because it would require a ring conformation that is unfavorable for the more rigid amide isostere. Instead, the pyridyl and thiazole ring establish hydrophobic interactions at the opposite side of the binding pocket, predominately with F205 and leaving

the protonated pyridyl ring solvent exposed. The resulting weaker interactions are consistent with the 16-fold decrease in binding affinity for **10**.

Compounds **2** and **6** are experimentally found to be 11 and 18 times less potent in HDAC6 than in HDAC1 while **12** is >200 times less potent. The results for **2** and **6** can be understood in terms of the previously discussed[15] differences in the shape of the protein surface between HDAC6 and the class 1 HDACs such as HDAC1 at the mouth of the active site. Specifically, HDAC6 selective inhibitors such as tubacin or WT161 have a Y-shaped cap group that is quite dissimilar to the macrocycle in the compounds studied here. As a result of this shape mismatch, significant loop reorganization in this region is necessary to establish binding contacts, as can be seen from the RMSD plots in Figure S3 in the Supporting Information.

Compounds **2** and **12** occupy very similar positions in HDAC6, as shown in the snapshots from the MD simulations of HDAC6 in Figure 5. However, the long timescale simulations of **12** bound to HDAC6 indicate a reorientation of a helix (shown on the top left in Figure 5) that is only observed in this system. It can therefore be hypothesized that it is energetically unfavorable despite the short NH-O hydrogen bond of 1.96 Å and correlates with the low activity of **12**.

All compounds studied are found to have a ~100:1 selectivity favoring the class I HDAC1-3 over HDAC8, another member of the same isoform class. These results can be rationalized by the computational finding that, in order to accommodate the large macrocyclic cap group, HDAC8 has to adopt the “open” conformation where the two binding sites observed in the crystal structures of HDAC8 (e.g. pdb code 1T64[19]) are connected through the movement of F152 and Y306, leading to a large groove resembling the one observed experimentally (pdb code 1VKG).[19] This groove is stable over the course of the simulation (see Figure S4 in the Supporting Information) and specific for HDAC8.[20] Compound **2** and analogs can only establish contacts to one side of the binding pocket, leading to the experimentally observed selectivity. In the representation shown in Figure 6, 2 only binds to the right side of the pocket, while the interaction with D88 leads to an interaction of **12** with the left side of the binding pocket.

3. CONCLUSION

Potent HDAC is have been synthesized by manipulating the macrocycle present on the natural product Largazole. Inhibitors with excellent selectivity for only HDAC1, HDAC2, and HDAC3 were formed through altering the depsipeptide framework to the peptide isostere variant and changing the thiazole to a pyridine.[29] The depsipeptide pyridyl variants proved to be equipotent (pyridyl “IN”) and twice as potent (pyridyl “OUT”) when tested against two cell lines. These biological observations were further rationalized through molecular modeling.

4. EXPERIMENTAL SECTION

General experimental method

Unless otherwise noted, all reactions were run under an argon atmosphere in flame or oven dried glassware. Reactions were monitored using thin layer silica gel chromatography (TLC) using 0.25 mm silica gel 60F plates with fluorescent indicator from Merck. Plates were visualized by treatment with anisaldehyde stain with gentle heating. Products were purified via column chromatography using the solvent system(s) indicated. Silica gel 60, 230–400 mesh, was purchased from Sorbent Technologies. Tetrahydrofuran (THF), dichloromethane (CH_2Cl_2), acetonitrile (CH_3CN), triethylamine (Et_3N), toluene, diethyl ether (Et_2O), and *N*,*N*-dimethylformamide (DMF) were passed through an alumina drying column (Solv-Tek Inc.) using argon pressure. All other reagents were purchased from commercial sources and used as received without additional purification. ^1H NMR and ^{13}C NMR spectra were recorded on Varian 300, 400, or 500 MHz NMR spectrometers. Chemical shifts are reported in ppm relative to $CHCl_3$ at δ = 7.27 (^1H NMR) and δ = 77.23 (^{13}C NMR) or tetramethylsilane (TMS) δ = 0.00, where noted. Mass spectra were obtained on Fisions VG Autospec. Optical rotations were collected at 589 nm on a Rudolph Research Automatic Polarimeter Autopol III. All compounds tested in the biological assays were shown to have a purity of >95% via NMR.

(*S*,*E*)-2-(Trimethylsilyl)ethyl 3-(((*S*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanoyl)oxy)-7-(tritylthio)hept-4-enoate (23)—To a solution of 2-(trimethylsilyl)ethyl (*S*,*E*)-3-hydroxy-7-(tritylthio)hept-4-enoate[6] (300 mg, 0.578 mmol) in CH_2Cl_2 (15.0 mL) at ambient temperature was added *N*-Fmoc-L-valine (981 mg, 2.89 mmol), EDCI•HCl (665 mg, 3.47 mmol), DMAP (7.1 mg, 0.058 mmol), and DIPEA (0.60 mL, 3.47 mmol). After stirring for 18 h, the reaction mixture was concentrated. The crude residue was purified using flash chromatography (1% to 20% ethyl acetate in hexanes) to provide ester (375 mg, 77%): ^1H-NMR ($CDCl_3$, 400 MHz) δ 7.71–7.75 (m, 2H), 7.56–7.58 (m, 2H), 7.18–7.38 (m, 19H), 5.58–5.69 (m, 2H), 5.34 (dd, *J* = 15.6, 7.6 Hz, 1H), 5.25 (d, *J* = 9.2 Hz, 1H), 4.30–4.40 (m, 2H), 4.25 (dd, *J* = 8.8, 4.4 Hz, 1H), 4.20 (t, *J* = 6.8 Hz, 1H), 4.12 (t, *J* = 8.8 Hz, 2H), 2.65 (dd, *J* = 16.0, 8.0 Hz, 1H), 2.52 (dd, *J* = 15.6, 5.2 Hz, 1H), 2.10–2.17 (m, 3H), 2.02 (q, *J* =6.8 Hz, 2H), 0.91–0.99 (m, 2H), 0.89 (d, *J* = 6.8 Hz, 3H), 0.77 (d, *J* = 6.8 Hz, 3H), −0.01 (s, 9H); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 170.9, 169.6, 156.1, 144.8, 143.9, 143.8, 141.3, 134.0, 129.6, 127.9, 127.7, 127.0, 126.6, 125.1, 120.0, 71.8, 67.0, 66.6, 63.1, 58.7, 47.2, 39.7, 31.4, 31.3, 31.1, 19.0, 17.3, −1.5; IR (neat) 3059, 2979, 1731, 1509, 1447, 1249, 1182, 1033, 858, 741; HRMS (ESI) *m/z* calcd for $C_{51}H_{57}NNaO_6SSi$ $[M+Na]^+$ 862.3574, found 862.3583; $[\alpha]_D$ = −15.0 (*c* 1.18, $CHCl_3$).

(*S*,*E*)-Methyl 3-((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-methylbutanamido)-7-(tritylthio)hept-4-enoate (28)—To a solution of Boc protected amine **27**[10] (0.650 g, 1.22 mmol) in CH_2Cl_2 (37 mL) at 0 °C was treated with TFA (0.37 mL). After 2 h, the reaction mixture was concentrated under vacuum, taken back up in toluene (15 mL), and concentrated again. In another flask, Boc-L-valine (0.531 g, 2.44 mmol), PyBOP (1.27 g, 2.44 mmol), and DIPEA (0.65 mL, 3.66 mmol) were combined in CH_2Cl_2 (60 mL). The

freshly deprotected amine was added via CH_2Cl_2 (10 mL) to the flask containing the activated acid at ambient temperature. After 3 h, the resulting mixture was concentrated under reduced pressure. The crude residue was purified using flash chromatography (5% to 50% ethyl acetate in hexanes) to obtain the desired amide (0.680 g, 88%): ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.15–7.37 (m, 15H), 6.49 (d, *J* = 8.0 Hz, 1H), 5.45 (dtd, *J* = 15.2, 6.4, 0.8 Hz, 1H), 5.34 (dd, *J* = 15.6, 6.0 Hz, 1H), 4.97–5.02 (m, 1H), 4.67–4.74 (m, 1H), 3.82–3.86 (m, 1H), 3.59 (s, 3H), 2.53 (d, *J* = 5.2 Hz, 2H), 2.05–2.16 (m, 3H), 2.01 (q, *J* = 6.8 Hz, 2H), 1.40 (s, 9H), 0.89 (d, *J* = 6.8 Hz, 3H), 0.83 (d, *J* = 6.8 Hz, 3H); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 171.6, 170.5, 155.8, 144.8, 130.5, 129.5, 127.8, 126.5, 79.7, 66.5, 59.9, 51.7, 47.2, 38.7, 31.3, 31.0, 28.3, 19.3, 17.6; IR (neat) 3304, 2965, 1738, 1685, 1650, 1522, 1492, 1365, 1172, 751; HRMS (ESI) *m/z* calcd for $C_{37}H_{46}N_2NaO_5S$ $[M+Na]^+$ 653.3025, found 653.3032; $[\alpha]_D = -14.0$ (*c* 1.21, $CHCl_3$).

6-((((*tert*-Butoxy)carbonyl)amino)methyl)picolinonitrile (31)—To a solution of commercially available chloride **30** (9.66 g, 63.3 mmol) in DMF (400 mL) at ambient temperature was treated with potassium phthalimide (11.7 g, 63.3 mmol). After stirring for 5 h, the mixture was concentrated under vacuum. The remaining mixture was taken up in H_2O (200 mL) and was filtered to collect the solid. The solid was washed with H_2O (100 mL) and THF (100 mL) to obtain the desired phthalimide derivitive (11.5 g, 69%) and was moved forward without further purification. To a solution of the crude phthalimide derivative (5.84 g, 22.2 mmol) in THF/MeOH (200 mL, 1:1, v/v) at ambient temperature was treated with hydrazine monohydrate (1.18 mL, 24.4 mmol). After 2 h, 1.0 M HCl (24.5 mL) was added to the mixture and was stirred for another 3 h before concentrating the reaction mixture under vacuum. The remaining residue was taken up in H_2O (200 mL) and the unwanted solid was removed through filtration. The filtrate was concentrated and placed under vacuum to remove the remaining H_2O. The crude solid was taken up in CH_2Cl_2 (175 mL) and triethylamine (9.28 mL, 66.6 mmol) and Boc_2O (4.86 g, 24.4 mmol) was added. After stirring for 12 h at room temperature, the reaction was quenched with a saturated solution of $NaHCO_3$ (200 mL), extracted with CH_2Cl_2 (3 × 150 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified using flash chromatography (10% to 45% ethyl acetate in hexanes) to provided the aryl pyridine "IN" fragement (2.24 g, 43%): ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.78 (t, *J* = 7.6 Hz, 1H), 7.56 (d, *J* = 7.6 Hz, 1H), 7.49 (d, *J* = 8.0 Hz, 1H), 5.51 (s, 1H), 4.44 (d, *J* = 5.6 Hz, 2H), 1.43 (s, 9H); ^{13}C-NMR ($CDCl_3$, 100 MHz) 160.1, 155.9, 137.6, 133.0, 127.0, 125.1, 117.1, 79.9, 45.5, 28.3; IR (neat) 3347, 2979, 2934, 2239, 1699, 1518, 1453, 1250, 1170, 862; HRMS (ESI) *m/z* calcd for $C_{12}H_{15}N_3NaO_3$ $[M+Na]^+$ 256.1062, found 256.1062.

(*R*)-2-(6-(((*tert*-Butoxycarbonyl)amino)methyl)pyridin-2-yl)-4-methyl-4,5-dihydrothiazole-4-carboxylic acid (32)—To a solution of nitrile **31** (1.50 g, 6.43 mmol) in MeOH/pH 6.0 buffer (105 mL, 3:2, v/v) was added α-methyl-L-cysteine (1.32 g, 7.72 mmol) and $NaHCO_3$ (1.08 g, 12.9 mmol). The reaction mixture was heated to 70 °C for 48 h. The mixture was cooled to ambient temperature and the MeOH was removed under reduced pressure. The aqueous layer was extracted with diethyl ether (3 × 30 mL) before being acidified to a pH of ~2 using 1.0 M HCl. The aqueous layer was then extracted with ethyl acetate (3 × 30 mL) and the ethyl acetate layer was dried over $MgSO_4$. The solution

was concentrated to give the desired acid (1.89 g, 84%): ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.97 (d, *J* = 7.6 Hz, 1H), 7.74 (t, *J* = 8.0 Hz, 1H), 7.37 (d, *J* = 7.6 Hz, 1H), 5.45 (bs, 1H), 4.46 (d, *J* = 5.2 Hz, 2H), 3.77 (d, *J* = 11.6 Hz, 1H), 3.29 (d, *J* = 11.6 Hz, 1H), 1.65 (s, 3H), 1.45 (s, 9H); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 176.3, 171.5, 157.5, 156.1, 149.6, 137.3, 123.9, 120.5, 84.9, 79.7, 45.3, 40.3, 28.4, 24.1; IR (neat) 3415, 2977, 1688, 1515, 1455, 1366, 1278, 1160, 754; HRMS (ESI) *m/z* calcd for $C_{16}H_{22}N_3O_4S$ $[M+H]^+$ 352.1331, found 352.1329; $[\alpha]_D$ = −36.6 (*c* 0.98, $CHCl_3$).

2-((((*tert*-Butoxy)carbonyl)amino)methyl)isonicotinonitrile (35)—To a solution of commercially available chloride **34** (5.84 g, 38.3 mmol) in DMF (250 mL) at ambient temperature was treated with potassium phthalimide (7.09 g, 38.3 mmol). After stirring for 5 h, the mixture was concentrated under vacuum. The remaining mixture was taken up in H_2O (100 mL) and was filtered to collect the solid. The solid was washed with H_2O (50 mL) and THF (50 mL) to obtain the desired phthalimide derivative (5.04 g, 50%) and was moved forward without further purification. To a solution of the crude phthalimide derivative (5.04 g, 19.1 mmol) in THF/MeOH (170 mL, 1:1, v/v) at ambient temperature was treated with hydrazine monohydrate (1.02 mL, 21.1 mmol). After 2 h, 1.0 M HCl (21.4 mL) was added to the mixture and was stirred for another 3 h before concentrating the reaction mixture under vacuum. The remaining residue was taken up in H_2O (200 mL) and the unwanted solid was removed through filtration. The filtrate was concentrated and placed under vacuum to remove the remaining H_2O. The crude solid was taken up in CH_2Cl_2 (150 mL) and triethylamine (8.00 mL, 57.4 mmol) and Boc_2O (4.59 g, 21.1 mmol) was added. After stirring for 12 h at room temperature, the reaction was quenched with a saturated solution of $NaHCO_3$ (200 mL), extracted with CH_2Cl_2 (3 × 150 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified using flash chromatography (15% to 50% ethyl acetate in hexanes) to provided the aryl pyridine “IN” fragement (3.74 g, 84%): ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 8.69 (d, *J* = 5.2 Hz, 1H), 7.51 (s, 1H), 7.39 (dd, *J* = 5.2, 0.8 Hz, 1H), 5.45 (s, 1H), 4.47 (d, *J* = 5.6 Hz, 2H), 1.44 (s, 9H); ^{13}C-NMR ($CDCl_3$, 100 MHz) 159.9, 155.9, 150.0, 123.6, 123.1, 121.1, 116.4, 80.1, 45.6, 28.3; IR (neat) 3337, 2978, 2934, 2245, 1709, 1514, 1246, 1168, 949; HRMS (ESI) *m/z* calcd for $C_{12}H_{16}N_3O_3$ $[M+H]^+$ 234.1243, found 234.1237.

(*R*)-2-(2-(((*tert*-Butoxycarbonyl)amino)methyl)pyridin-4-yl)-4-methyl-4,5-dihydrothiazole-4-carboxylic acid (36)—To a solution of nitrile **35** (1.50 g, 6.43 mmol) in MeOH/pH 6.0 buffer (105 mL, 3:2, v/v) was added α-methyl-L-cysteine (1.32 g, 7.72 mmol) and $NaHCO_3$ (1.08 g, 12.9 mmol). The reaction mixture was heated to 70 °C for 48 h. The mixture was cooled to ambient temperature and the MeOH was removed under reduced pressure. The aqueous layer was extracted with diethyl ether (3 × 30 mL) before being acidified to a pH of ~2 using 1.0 M HCl. The aqueous layer was then extracted with ethyl acetate (3 × 30 mL) and the ethyl acetate layer was dried over $MgSO_4$. The solution was concentrated to give the desired acid (1.89 g, 85%): ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 8.61 (d, *J* = 4.8 Hz, 1H), 7.68 (s, 1H), 7.58 (dd, *J* = 5.2, 1.6 Hz, 1H), 5.64 (bs, 1H), 4.48 (d, *J* = 4.8 Hz, 2H) 3.94 (d, *J* = 11.6 Hz, 1H), 3.38 (d, *J* = 11.6 Hz, 1H), 1.66 (s, 3H), 1.43 (s, 9H); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 175.5, 166.5, 158.5, 156.1, 148.7, 141.4, 121.2, 120.9, 85.0, 79.8, 45.1, 41.8, 28.3, 23.8; IR (neat) 3355, 2979, 2359, 1707, 1517, 1284, 1167, 754;

HRMS (ESI) *m/z* calcd for $C_{16}H_{22}N_3O_4S$ $[M+H]^+$ 352.1331, found 352.1334; $[\alpha]_D$ = −39.3 (*c* 1.08, $CHCl_3$).

(*S*,*E*)-2-(Trimethylsilyl)ethyl 3-(((*S*)-2-((*R*)-2-(6-(((*tert*-butoxycarbonyl)amino)methyl)pyridin-2-yl)-4-methyl-4,5-dihydrothiazole-4-carboxamido)-3-methylbutanoyl)oxy)-7-(tritylthio)hept-4-enoate (37)—To a solution of Fmoc protected amine **23** (2.39 g, 2.85 mmol) in acetonitrile (195 mL) at ambient temperature was added diethylamine (15.0 mL). After 2 h, the reaction mixture was concentrated under reduced vacuum, taken back up in ethyl acetate (50 mL), and concentrated again. In a separate flask, acid **32** (1.00 g, 2.85 mmol), PyBOP (2.97 g, 5.70 mmol), and DIPEA (1.49 mL, 8.55 mmol) was combined in CH_2Cl_2 (195 mL). The freshly deprotected amine was added via CH_2Cl_2 (10 mL) to the flask containing the activated acid at ambient temperature. After 3 h, the resulting mixture was concentrated under reduced pressure. The crude residue was purified using flash chromatography (5% to 40% ethyl acetate in hexanes) to obtain the desired amide (2.13 g, 79%): ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 8.04 (d, *J* = 8.0 Hz, 1H), 7.69 (t, *J* = 7.6 Hz, 1H), 7.15–7.38 (m, 16H), 5.67 (dt, *J* = 15.2, 6.8 Hz, 1H), 5.59–5.65 (m, 1H), 5,45–5.47 (m, 1H), 5.36 (dd, *J* = 15.6, 7.6 Hz, 1H), 4.45–4.48 (m, 3H), 4.14 (dd, *J* = 9.6, 7.6 Hz, 2H), 3.74 (d, *J* = 11.6 Hz, 1H), 3.28 (d, *J* = 12.0 Hz, 1H), 2.68 (dd, *J* = 15.6, 7.6 Hz, 1H), 2.54 (dd, *J* = 15.6, 5.6 Hz, 1H), 2.00–2.18 (m, 5H), 1.60 (s, 3H), 1.46 (s, 9H), 0.93–0.97 (m, 2H), 0.81 (d, *J* = 6.8 Hz, 3H), 0.74 (d, *J* = 6.8 Hz, 3H), 0.01 (s, 9H); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 174.4, 170.9, 170.4,169.6, 157.6, 156.0, 149.9, 144.8, 137.3, 133.9, 129.5, 127.8, 127.7, 126.6, 123.8, 120.0, 85.6, 79.6, 71.8, 66.6, 63.1, 56.6, 45.4, 40.5, 39.7, 31.3, 31.2, 31.0, 28.4, 24.8, 19.0, 17.4, 17.3, −1.5; IR (neat) 3393, 2960, 1736, 1684, 1508, 1446, 1390, 1249, 1171, 859; HRMS (ESI) *m/z* calcd for $C_{52}H_{66}N_4NaO_7S_2Si$ $[M+Na]^+$ 973.4040, found 973.4042; $[\alpha]_D$ = −19.0 (*c* 1.11, $CHCl_3$).

(*S*,*E*)-2-(Trimethylsilyl)ethyl 3-(((*S*)-2-((*R*)-2-(2-(((*tert*-butoxycarbonyl)amino)methyl)pyridin-4-yl)-4-methyl-4,5-dihydrothiazole-4-carboxamido)-3-methylbutanoyl)oxy)-7-(tritylthio)hept-4-enoate (38)—To a solution of Fmoc protected amine **23** (1.55 g, 1.84 mmol) in acetonitrile (125 mL) at ambient temperature was added diethylamine (10.0 mL). After 2 h, the reaction mixture was concentrated under reduced vacuum, taken back up in ethyl acetate (50 mL), and concentrated again. In a separate flask, acid **36** (0.650 g, 1.84 mmol), PyBOP (1.92 g, 3.68 mmol), and DIPEA (0.96 mL, 5.52 mmol) was combined in CH_2Cl_2 (125 mL). The freshly deprotected amine was added via CH_2Cl_2 (10 mL) to the flask containing the activated acid at ambient temperature. After 3 h, the resulting mixture was concentrated under reduced pressure. The crude residue was purified using flash chromatography (5% to 40% ethyl acetate in hexanes) to obtain the desired amide (1.42 g, 81%): ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 8.51–8.52 (m, 1H), 7.72–7.73 (m, 1H), 7.15–7.38 (m, 15H), 7.11 (d, *J* = 8.8 Hz, 1H), 5.69 (dt, *J* = 15.2, 6.8 Hz, 1H), 5.60–5.65 (m, 1H), 5.36 (dd, *J* = 15.2, 7.6 Hz, 1H), 4.54–4.56 (m, 2H), 4.48 (dd, *J* = 8.8, 4.4 Hz, 1H), 4.14 (dd, *J* = 10.0, 8.0 Hz, 2H), 3.84 (d, *J* = 11.6 Hz, 1H), 3.41 (d, *J* = 11.6 Hz, 1H), 2.67 (dd, *J* = 15,6, 8.0 Hz, 1H), 2.54 (dd, *J* = 16.0, 5.6 Hz, 1H), 2.03–2.18 (m, 5H), 1.56 (s, 3H), 1.44 (s, 9H), 0.92–0.97 (m, 2H), 0.81 (d, *J* = 6.8 Hz, 3H), 0.74 (d, *J* = 6.8 Hz, 3H), 0.01 (s, 9H); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 173.8, 170.3, 169.6, 167.0, 158.7, 155.9, 149.7, 144.8, 140.3, 133.9, 129.5, 127.8, 127.7, 126.6, 120.3,

119.9, 85.4, 79.6, 71.8, 66.6, 63.1, 56.8, 45.7, 41.7, 39.7, 31.3, 31.1, 31.0, 28.4, 24.5, 19.0, 17.4, 17.2, −1.5; IR (neat) 3390, 2960, 1737, 1681, 1593, 1504, 1444, 1248, 1170, 836, 744; HRMS (ESI) *m/z* calcd for $C_{52}H_{66}N_4NaO_7S_2Si$ $[M+Na]^+$ 973.4040, found 973.4058; $[\alpha]_D$ = −32.5 (*c* 1.03, $CHCl_3$).

Trityl protected depsipeptide pyridyl "IN" macrocycle (39)—To a solution of depsipeptide **37** (2.10 g, 2.21 mmol) in CH_2Cl_2 (100 mL) at 0 °C was treated with TFA (20.0 mL) dropwise. The reaction mixture was allowed to stir for 18 h. The mixture was concentrated under reduced pressure, taken back up in toluene (50 mL), and concentrated again. The remaining residue was taken up in acetonitrile (2300 mL) and was treated with HATU (1.68 g, 4.42 mmol), HOBt (0.597 g, 4.42 mmol), and DIPEA (2.32 mL, 13.3 mmol) at room temperature. After 18 h, the reaction mixture was concentrated under reduced pressure. The crude residue was passed through a silica plug using an eluent of 10% MeOH in CH_2Cl_2. The filtrate was concentrated and taken up in ethyl acetate (150 mL). The solution was washed with a saturated solution of $NaHCO_3$ (100 mL). The aqueous layer was extracted ethyl acetate (3 × 50 mL) and then the combined organic layers were washed with a saturated solution of NaCl (100 mL). After drying over Na_2SO_4 and concentrating under reduced vacuum, the crude mixture was purified using flash chromatography (1% to 6% MeOH in DCM) to obtain the macrocycle (0.334 g, 20%): ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.78 (t, *J* = 7.6 Hz, 1H), 7.62 (d, *J* = 7.6 Hz, 1H), 7.15–7.37 (m, 16H), 7.06 (d, *J* = 4.8 Hz, 1H), 5.74 (dtd, *J* = 15.6, 6.8, 1.2 Hz, 1H), 5.62 (q, *J* = 6.0 Hz, 1H), 5.38 (dd, *J* = 15.6, 6.4 Hz, 1H), 4.97 (dd, *J* = 18.0, 6.8 Hz, 1H), 4.66 (dd, *J* = 10.0, 4.0 Hz, 1H), 4.29 (dd, *J* = 18.0, 2.0 Hz, 1H), 4.07 (d, *J* = 11.2 Hz, 1H), 3.36 (d, *J* = 11.6 Hz, 1H), 2.68–2.70 (m, 2H), 2.08–2.17 (m, 3H), 2.02 (q, *J* = 7.2 Hz, 2H), 1.82 (s, 3H), 0.73 (d, *J* = 7.2 Hz, 3H), 0.52 (d, *J* = 6.8 Hz, 3H); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 173.4, 170.8, 168.9, 168.7, 156.5, 149.4, 144.8, 137.7, 133.0, 129.4, 127.8, 127.3, 126.5, 124.1, 123.1, 84.9, 72.9, 66.5, 57.4, 43.8, 43.5, 41.4, 33.5, 31.5, 31.1, 24.7, 18.9, 16.7; IR (neat) 3368, 2960, 1735, 1675, 1575, 1512, 1444, 1242, 1037, 974, 744; HRMS (ESI) *m/z* calcd for $C_{42}H_{44}N_4NaO_4S_2$ $[M+Na]^+$ 755.2702, found 755.2696; $[\alpha]_D$ = +27.4 (*c* 0.57, $CHCl_3$).

Trityl protected depsipeptide pyridyl "OUT" macrocycle (40)—To a solution of depsipeptide **38** (2.00 g, 2.10 mmol) in CH_2Cl_2 (100 mL) at 0 °C was treated with TFA (20.0 mL) dropwise. The reaction mixture was allowed to stir for 18 h. The mixture was concentrated under reduced pressure, taken back up in toluene (50 mL), and concentrated again. The remaining residue was taken up in acetonitrile (2100 mL) and was treated with HATU (1.60 g, 4.20 mmol), HOBt (0.570 g, 4.20 mmol), and DIPEA (2.19 mL, 12.6 mmol) at room temperature. After 18 h, the reaction mixture was concentrated under reduced pressure. The crude residue was passed through a silica plug using an eluent of 10% MeOH in CH_2Cl_2. The filtrate was concentrated and taken up in ethyl acetate (150 mL). The solution was washed with a saturated solution of $NaHCO_3$ (100 mL). The aqueous layer was extracted ethyl acetate (3 × 50 mL) and then the combined organic layers were washed with a saturated solution of NaCl (100 mL). After drying over Na_2SO_4 and concentrating under reduced vacuum, the crude mixture was purified using flash chromatography (1% to 6% MeOH in DCM) to obtain the macrocycle (0.350 g, 23%): ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 8.65 (d, *J* = 5.2 Hz, 1H), 7.77 (s, 1H), 7.15–7.40 (m, 16H), 6.97 (d, *J* = 9.2 Hz, 1H), 6.33

(dd, *J* = 8.0, 5.2 Hz, 1H), 5.78 (dtd, *J* = 15.6, 6.4, 0.8 Hz, 1H), 5.71 (q, *J* = 6.0 Hz, 1H), 5.50 (dd, *J* = 15.6, 6.0 Hz, 1H), 4.97 (dd, *J* = 17.6, 8.0 Hz, 1H), 4.52 (dd, *J* = 8.8, 4.0 Hz, 1H), 4.02 (dd, *J* = 18.0, 5.2 Hz, 1H), 3.99 (d, *J* = 11.6 Hz, 1H), 3.38 (d, *J* = 11.6 Hz, 1H), 2.74–2.78 (m, 2H), 2.01–2.29 (m, 5H), 1.74 (s, 3H), 0.76 (d, *J* = 6.8 Hz, 3H), 0.66 (d, *J* = 6.8 Hz, 3H); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 173.1, 169.9, 168.4, 168.2, 159.3, 150.5, 144.6, 140.0, 132.6, 129.4, 128.1, 127.9, 126.7, 120.8, 116.9, 84.5, 71.5, 66.7, 58.0, 44.5, 43.5, 40.8, 33.4, 31.2, 31.0, 24.9, 18.9, 17.2; IR (neat) 3405, 2962, 1739, 1683, 1552, 1508, 1404, 1255, 1183, 1029, 748; HRMS (ESI) *m/z* calcd for $C_{42}H_{44}N_4NaO_4S_2$ $[M+Na]^+$ 755.2702, found 755.2694; $[\alpha]_D$ = +27.6 (*c* 0.58, $CHCl_3$).

Depsipeptide pyridyl "IN" thiol (6)—To a solution of trityl protected thiol **39** (300 mg, 0.409 mmol) in CH_2Cl_2 (55 mL) at 0 °C was treated with TFA (2.25 mL) and triisopropylsilane (0.17 mL, 0.818 mmol). The reaction mixture was warmed to ambient temperature and stirred for 2 h. The mixture was concentrated and purified using flash chromatography (1% to 6% MeOH in CH_2Cl_2) to obtain the desired thiol (164 mg, 82%): ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.86 (t, *J* = 7.6 Hz, 1H), 7.68 (d, *J* = 7.6 Hz, 1H), 7.42 (d, *J* = 8.0 Hz, 1H), 7.35 (bs, 1H), 7.25 (d, *J* = 9.6 Hz, 1H), 5.85 (dtd, *J* = 15.2, 7.2, 1.2 Hz, 1H), 5.64–5.69 (m, 1H), 5.54 (ddt, *J* = 15.6, 6.8, 1.2 Hz, 1H), 5.11 (dd, *J* = 18.0, 7.6 Hz, 1H), 4.69 (dd, *J* = 10.0, 4.0 Hz, 1H), 4.34 (dd, *J* = 18.0, 1.6 Hz, 1H), 4.14 (d, *J* = 11.2 Hz, 1H), 3.46 (d, *J* = 11.6 Hz, 1H), 2.87 (dd, *J* = 15.2, 10.0 Hz, 1H), 2.71 (dd, *J* = 15.2, 2.4 Hz, 1H), 2.53 (qd, *J* = 7.6, 0.8 Hz, 1H), 2.31–2.37 (m, 2H), 2.09–2.17 (m, 1H), 1.91 (s, 3H), 1.40 (t, *J* = 8.0 Hz, 1H), 0.71 (d, *J* = 6.8 Hz, 3H), 0.49 (d, *J* = 6.8 Hz, 3H); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 174.8, 172.6, 169.7, 168.5, 156.9, 148.0, 138.3, 132.8, 128.4, 125.1, 123.7, 83.5, 72.6, 57.5, 43.7, 43.5, 41.2, 36.4, 33.3, 23.8, 23.8, 18.6, 16.5; IR (neat) 3369, 2962, 1735, 1670, 1572, 1515, 1426, 1295, 1174, 989, 799; HRMS (ESI) *m/z* calcd for $C_{23}H_{31}N_4O_4S_2$ $[M+H]^+$ 491.1787, found 491.1791; $[\alpha]_D$ = +58.5 (*c* 0.53, $CHCl_3$).

Depsipeptide pyridyl "OUT" thiol (8)—To a solution of trityl protected thiol **40** (320 mg, 0.437 mmol) in CH_2Cl_2 (60 mL) at 0 °C was treated with TFA (2.30 mL) and triisopropylsilane (0.18 mL, 0.873 mmol). The reaction mixture was warmed to ambient temperature and stirred for 2 h. The mixture was concentrated and purified using flash chromatography (1% to 6% MeOH in CH_2Cl_2) to obtain the desired thiol (188 mg, 89%): ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 8.70 (d, *J* = 5.2 Hz, 1H), 7.89 (s, 1H), 7.45 (dd, *J* = 5.2, 1.6 Hz, 1H), 6.92 (d, *J* = 9.2 Hz, 1H), 6.36–6.39 (m, 1H), 5.87 (dt, *J* = 15.6, 6.4 Hz, 1H), 5.73–5.78 (m, 1H), 5.67 (ddt, *J* = 15.6, 6.4, 1.2 Hz, 1H), 5.09 (dd, *J* = 17.6, 8.0 Hz, 1H), 4.57 (dd, *J* = 9.2, 4.0 Hz, 1H), 4.37 (dd, *J* = 17.6, 4.8 Hz, 1H), 4.05 (d, *J* = 11.6 Hz, 1H), 3.40 (d, *J* = 11.6 Hz, 1H), 2.76–2.89 (m, 2H), 2.50–2.64 (m, 2H), 2.30–2.44 (m, 2H), 2.11–2.20 (m, 1H), 1.78 (s, 3H), 1.38 (t, *J* = 7.6 Hz, 1H), 0.75 (d, *J* = 6.8 Hz, 3H), 0.66 (d, *J* = 6.8 Hz, 3H); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 173.1, 169.3, 168.7, 168.4, 158.7, 149.4, 141.1, 132.4, 128.8, 121.3, 117.8, 84.5, 72.2, 57.8, 43.6, 43.6, 40.3, 36.1, 33.6, 24.9, 23.7, 18.9, 17.0; IR (neat) 3319, 2962, 1736, 1670, 1551, 1508, 1242, 1184, 1027, 971, 730; HRMS (ESI) *m/z* calcd for $C_{23}H_{31}N_4O_4S_2$ $[M+H]^+$ 491.1787, found 491.1795; $[\alpha]_D$ = +68.3 (*c* 0.74, $CHCl_3$).

Depsipeptide pyridyl "IN" (5)—To a solution of depsipeptide pyridine "IN" thiol **6** (20.0 mg, 0.041 mmol) in THF (0.8 mL) at ambient temperature was treated with *N*-octanoyl–imidazole (15.8 mg, 0.082 mmol), imidazole (5.6 mg, 0.082 mmol), and DMAP (0.5 mg, 0.004 mmol). The reaction mixture was warmed to 50 °C and stirred for 6 h. The mixture was cooled and concentrated and purified using flash chromatography (0.5% to 4% MeOH in CH_2Cl_2) to obtain the desired octanoyl masked thiol (17.4 mg, 69%): ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.82 (t, *J* = 7.6 Hz, 1H), 7.65 (d, *J* = 7.6 Hz, 1H), 7.39 (d, *J* = 8.0 Hz, 1H), 7.30 (d, *J* = 9.6 Hz, 1H), 5.85 (dt, *J* = 15.6, 6.8 Hz, 1H), 5.63 (td, *J* = 16.0, 2.0 Hz, 1H), 5.52 (dd, *J* = 15.2, 6.8 Hz, 1H), 5.06–5.12 (m, 1H), 4.67 (dd, *J* = 9.6, 3.6 Hz, 1H), 4.32 (d, *J* = 18.0 Hz, 1H), 4.15 (d, *J* = 11.6 Hz, 1H), 3.42 (d, *J* = 11.6 Hz, 1H), 2.81–2.90 (m, 2H), 2.63 (dd, *J* = 14.0, 1.2 Hz, 1H), 2.47 (t, *J* = 7.6 Hz, 2H), 2.27 (q, *J* = 7.6 Hz, 2H), 2.06–2.13 (m, 1H), 1.92 (s, 3H), 1.56–1.61 (m, 2H), 1.23–1.27 (m, 10H), 0.84 (t, *J* = 6.8 Hz, 3H), 0.69 (d, *J* = 6.8 Hz, 3H), 0.49 (d, *J* = 6.8 Hz, 3H); ^{13}C-NMR (CD_3OD, 100 MHz) δ 199.4, 174.2, 173.7, 170.7, 168.7, 157.5, 147.7, 138.7, 132.3, 128.7, 125.0, 123.2, 83.4, 72.9, 54.1, 43.4, 42.8, 42.7, 40.2, 33.1, 31.9, 31.4, 28.6, 28.5, 27.4, 25.3, 23.0, 22.2, 18.1, 15.3, 13.0; IR (neat) 2959, 2928, 2856, 1737, 1682, 1571, 1419, 1239, 990; HRMS (ESI) *m/z* calcd for $C_{31}H_{45}N_4O_5S_2$ $[M+H]^+$ 617.2826, found 617.2832; $[\alpha]_D$ = +125 (*c* 0.80, $CHCl_3$).

Depsipeptide pyridyl "OUT" (7)—To a solution of depsipeptide pyridine "OUT" thiol **8** (85.0 mg, 0.179 mmol) in CH_2Cl_2 (15.0 mL) at ambient temperature was treated with octanoyl chloride (.153 mL, 0.894 mmol) and triethylamine (0.050 mL, 0.357 mmol). The reaction mixture was stirred for 2 h before cooling to 0 °C and quenching with methanol (5.0 mL). The mixture was cooled and concentrated and purified using flash chromatography (0.5% to 4% MeOH in CH_2Cl_2) to obtain the desired octanoyl masked thiol (54.0 mg, 49%): ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 8.67 (d, *J* = 5.2 Hz, 1H), 7.91 (s, 1H), 7.45 (d, *J* = 4.8 Hz, 1H), 6.89 (d, *J* = 8.8 Hz, 1H), 6.57–6.60 (m, 1H), 5.85 (dt, *J* = 14.8, 7.2 Hz, 1H), 5.62–5.73 (m, 2H), 5.10 (dd, *J* = 18.0, 8.0 Hz, 1H), 4.56 (dd, *J* = 8.8, 3.6 Hz, 1H), 4.42 (dd, *J* = 17.6, 3.6 Hz, 1H), 4.05 (d, *J* = 11.6 Hz, 1H), 3.40 (d, *J* = 11.6 Hz, 1H) 2.74–2.90 (m, 3H), 2.49 (t, *J* = 7.2 Hz, 2H), 2.28–2.34 (m, 1H), 2.12–2.19 (m, 1H), 1.78 (s, 3H), 1.58–1.63 (m, 2H), 1.24–1.26 (m, 10H), 0.84 (t, *J* = 6.8 Hz, 3H), 0.74 (d, *J* = 6.8 Hz, 3H), 0.65 (d, *J* = 6.8 Hz, 3H); ^{13}C-NMR (CD_3OD, 100 MHz) δ 199.4, 173.9, 170.2, 169.6, 168.2, 158.6, 149.0, 141.7, 132.0, 128.5, 121.5, 117.9, 84.3, 72.4, 57.7, 43.4, 42.7, 42.7, 39.1, 33.4, 32.0, 31.4, 28.6, 28.5, 27.4, 25.3, 23.6, 22.2, 18.1, 16.1, 13.0; IR (neat) 2959, 2929, 2856, 1740, 1684, 1553, 1420, 1234, 987; HRMS (ESI) *m/z* calcd for $C_{31}H_{45}N_4O_5S_2$ $[M+H]^+$ 617.2826, found 617.2827; $[\alpha]_D$ = +94.3 (*c* 0.62, $CHCl_3$).

(*S*,*E*)-Methyl 3-((*S*)-2-((*R*)-2-(6-(((*tert*-butoxycarbonyl)amino)methyl)pyridin-2-yl)-4-methyl-4,5-dihydrothiazole-4-carboxamido)-3-methylbutanamido)-7-(tritylthio)hept-4-enoate (41)—To a solution of Boc protected amine **28** (500 mg, 0.793 mmol) in CH_2Cl_2 (30 mL) at 0 °C was treated with TFA (3.0 mL). After 2 h, the reaction mixture was concentrated under vacuum, taken back up in toluene (15 mL), and concentrated again. In another flask, acid **32** (278 mg, 0.793 mmol), PyBOP (822 mg, 1.58 mmol), and DIPEA (0.41 mL, 2.37 mmol) were combined in CH_2Cl_2 (35 mL). The freshly deprotected amine was added via CH_2Cl_2 (10 mL) to the flask containing the activated acid at ambient temperature. After 3 h, the resulting mixture was concentrated under reduced

pressure. The crude residue was purified using flash chromatography (20% to 90% ethyl acetate in hexanes) to obtain the desired amide (570 mg, 84%): ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 8.00 (d, *J* = 7.6 Hz, 1H), 7.73 (t, *J* = 7.6 Hz, 1H), 7.34–7.37 (m, 7H), 7.30 (d, *J* = 8.8 Hz, 1H), 7.15–7.26 (m, 9H), 6.59 (d, *J* = 10.0 Hz, 1H), 5.47 (dtd, *J* = 15.6, 6.4, 0.8 Hz, 1H), 5.36 (dd, *J* = 15.2, 6.0 Hz, 1H), 4.70–4.74 (m, 1H), 4.46 (d, *J* = 5.2 Hz, 2H), 4.17 (dd, *J* = 8.8, 6.0 Hz, 1H), 3.67 (d, *J* = 11.6 Hz, 1H), 3.61 (s, 3H), 3.28 (d, *J* = 11.6 Hz, 1H), 2.57 (d, *J* = 5.6 Hz, 2H), 2.08–2.16 (m, 3H) 2.01 (q, *J* =6.8 Hz, 2H), 1.56 (s, 3H), 1.45 (s, 9H), 0.82 (d, *J* = 6.8 Hz, 3H), 0.79 (d, *J* = 6.8 Hz, 3H); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 174.7, 171.5, 171.1, 169.7, 157.7, 156.0, 149.8, 144.8, 137.3, 130.6, 129.5, 127.8, 126.5, 123.8, 120.1, 85.6, 79.6, 66.5, 58.2, 51.7, 47.4, 45.4, 40.6, 38.7, 31.4, 31.3, 31.1, 28.4, 24.7, 19.3, 17.9; IR (neat) 2969, 1654, 1508, 1444, 1365, 1169, 1031, 751; HRMS (ESI) *m/z* calcd for $C_{48}H_{57}N_5NaO_6S_2$ $[M+Na]^+$ 886.3648, found 886.3639; $[\alpha]_D$ = −30.8 (*c* 0.67, $CHCl_3$).

(*S*,*E*)-Methyl 3-((*S*)-2-((*R*)-2-(2-(((*tert*-butoxycarbonyl)amino)methyl)pyridin-4-yl)-4-methyl-4,5-dihydrothiazole-4-carboxamido)-3-methylbutanamido)-7-(tritylthio)hept-4-enoate (42)—To a solution of Boc protected amine **28** (500 mg, 0.793 mmol) in CH_2Cl_2 (30 mL) at 0 °C was treated with TFA (3.0 mL). After 2 h, the reaction mixture was concentrated under vacuum, taken back up in toluene (15 mL), and concentrated again. In another flask, acid **36** (278 mg, 0.793 mmol), PyBOP (822 mg, 1.58 mmol), and DIPEA (0.41 mL, 2.37 mmol) were combined in CH_2Cl_2 (35 mL). The freshly deprotected amine was added via CH_2Cl_2 (10 mL) to the flask containing the activated acid at ambient temperature. After 3 h, the resulting mixture was concentrated under reduced pressure. The crude residue was purified using flash chromatography (20% to 100% ethyl acetate in hexanes) to obtain the desired amide (630 mg, 92%): ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 8.61 (dd, *J* = 4.8, 0.8 Hz, 1H), 7.59–7.61 (m, 2H), 7.15–7.38 (m, 16H), 6.59 (d, *J* = 8.8 Hz, 1H), 5.47 (dtd, *J* = 15.6, 6.4, 0.8 Hz, 1H), 5.35 (dd, *J* = 15.2, 6.0 Hz, 1H), 4.69–4.75 (m, 1H), 4.48 (d, *J* = 5.2 Hz, 2H), 4.15 (dd, *J* = 8.8, 6.4 Hz, 1H), 3.79 (d, *J* = 11.6 Hz, 1H), 3.61 (s, 3H), 3.37 (d, *J* = 11.2 Hz, 1H), 2.57 (d, *J* = 5.2 Hz, 2H), 2.08–2.17 (m, 3H), 2.01 (q, *J* = 6.8 Hz, 2H), 1.55 (s, 3H), 1.45 (s, 9H), 0.82 (d, *J* = 6.4 Hz, 3H), 0.79 (d, *J* = 6.8 Hz, 3H); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 174.1, 171.6, 169.5, 167.2, 158.8, 155.9, 149.7, 144.8, 140.2, 130.6, 129.5, 129.5, 127.8, 126.5, 120.3, 120.0, 85.3, 79.6, 66.5, 58.4, 51.7, 47.3, 45.7, 41.7, 38.6, 31.4, 31.1, 31.0, 28.4, 24.4, 19.3, 17.9; IR (neat) 2969, 1654, 1511, 1443, 1390, 1366, 1168, 846, 750; HRMS (ESI) *m/z* calcd for $C_{48}H_{57}N_5NaO_6S_2$ $[M+Na]^+$ 886.3648, found 886.3661; $[\alpha]_D$ = −29.9 (*c* 0.64, $CHCl_3$).

Trityl protected peptide isostere pyridyl “IN” macrocycle (43)—To a solution of methyl ester **41** (530 mg, 0.613 mmol) in THF/H_2O (15 mL, 2:1, v/v) at ambient temperature was added lithium hydroxide monohydrate (77.2 mg, 1.84 mmol). After 2 h, the reaction was cooled to 0 °C and acidified to a pH of ~3 using 1.0 M HCl. The mixture was extracted with CH_2Cl_2 (3 × 15 mL), dried over Na_2SO_4, and concentrated under vacuum. The freshly furnished acid was taken up in CH_2Cl_2 (20 mL) and cooled to 0 °C. The flask was treated with TFA (2.0 mL) and warmed to ambient temperature. After 2 h, the mixture was concentrated under reduced pressure, taken back up in toluene (25 mL), and concentrated again. The crude TFA salt was taken up in acetonitrile (20 mL) and was treated with DIPEA (0.68 mL, 3.90 mmol). The mixture was allowed to stir for 30 min. In another

flask containing acetonitrile (610 mL) was added HATU (488 mg, 1.28 mmol), HOBt (173 mg, 1.28 mmol), and DIPEA (0.68 mL, 3.90 mmol) at room temperature. The flask containing the peptide was added to the flask containing the coupling reagents dropwise over 12 h using a syringe pump. After another 12 h, the reaction mixture was concentrated under reduced pressure. The crude residue was taken up in CH_2Cl_2 (15 mL) and the resulting solid was removed using filtration. The filtrate was concentrated and purified using flash chromatography (1% to 6% MeOH in DCM) to obtain the macrocycle (146 mg, 33%): ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.81 (t, *J* = 7.6 Hz, 1H), 7.62 (d, *J* = 8.0 Hz, 1H), 7.16–7.37 (m, 16H), 6.92 (d, *J* = 6.4 Hz, 1H), 6.73 (d, *J* = 10.8 Hz, 1H), 6.42 (d, *J* = 8.4 Hz, 1H), 5.47 (dt, *J* = 15.6, 6.4 Hz, 1H), 5.34 (dd, *J* = 15.6, 5.6 Hz, 1H), 5.03 (dd, *J* = 18.0, 7.2 Hz, 1H), 4.83–4.90 (m, 1H), 4.60 (dd, *J* = 10.8, 3.2 Hz, 1H), 4.24 (dd, *J* = 17.6, 2.0 Hz, 1H), 3.95 (d, *J* = 12.0 Hz, 1H), 3.45 (d, *J* = 12.0 Hz, 1H), 2.67 (dd, *J* = 14.0, 3.6 Hz, 1H), 2.48–2.52 (m, 1H), 2.44 (dd, *J* = 14.4, 10.8 Hz, 1H), 2.16 (t, *J* = 8.0 Hz, 2H), 1.99–2.06 (m, 2H), 1.87 (s, 3H), 0.74 (d, *J* = 6.8 Hz, 3H), 0.32 (d, *J* = 6.8 Hz, 3H); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 173.8, 172.8, 169.8, 169.8, 157.5, 149.2, 144.8, 138.0, 130.3, 129.6, 129.5, 127.8, 126.6, 124.6, 122.9, 84.8, 66.5, 57.8, 48.3, 44.3, 43.7, 41.4, 31.4, 31.3, 31.1, 24.4, 19.3, 15.3; IR (neat) 2930, 1681, 1644, 1561, 1489, 1274, 1185, 1030, 842; HRMS (ESI) *m/z* calcd for $C_{42}H_{45}N_5NaO_3S_2$ $[M+Na]^+$ 754.2862, found 754.2872; $[\alpha]_D$ = +58.0 (*c* 0.10, $CHCl_3$).

Trityl protected peptide isostere pyridyl "OUT" macrocycle (44)—To a solution of methyl ester **42** (480 mg, 0.555 mmol) in THF/H_2O (15 mL, 2:1, v/v) at ambient temperature was added lithium hydroxide monohydrate (69.9 mg, 1.67 mmol). After 2 h, the reaction was cooled to 0 °C and acidified to a pH of ~3 using 1.0 M HCl. The mixture was extracted with CH_2Cl_2 (3 × 15 mL), dried over Na_2SO_4, and concentrated under vacuum. The freshly furnished acid was taken up in CH_2Cl_2 (20 mL) and cooled to 0 °C. The flask was treated with TFA (2.0 mL) and warmed to ambient temperature. After 2 h, the mixture was concentrated under reduced pressure, taken back up in toluene (25 mL), and concentrated again. The crude TFA salt was taken up in acetonitrile (20 mL) and was treated with DIPEA (0.61 mL, 3.52 mmol). The mixture was allowed to stir for 30 min. In another flask containing acetonitrile (550 mL) was added HATU (439 mg, 1.16 mmol), HOBt (157 mg, 1.16 mmol), and DIPEA (0.61 mL, 3.52 mmol) at room temperature. The flask containing the peptide was added to the flask containing the coupling reagents dropwise over 12 h using a syringe pump. After another 12 h, the reaction mixture was concentrated under reduced pressure. The crude residue was taken up in CH_2Cl_2 (15 mL) and the resulting solid was removed using filtration. The filtrate was concentrated and purified using flash chromatography (1% to 6% MeOH in DCM) to obtain the macrocycle (128 mg, 32%): ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 8.64 (dd, *J* = 4.8, 0.8 Hz, 1H), 7.79 (d, *J* = 0.8 Hz, 1H), 7.16–7.32 (m, 16H), 6.29–6.35 (m, 2H), 6.12 (d, *J* = 10.0 Hz, 1H), 5.54–5.57 (m, 2H) 4.81–4.86 (m, 1H), 4.67 (dd, *J* = 17.2, 6.4 Hz, 1H), 4.40 (dd, *J* = 10.0, 4.4 Hz, 1H), 4.15 (dd, *J* = 17.2, 5.6 Hz, 1H), 3.80 (d, *J* = 11.6 Hz, 1H), 3.49 (d, *J* = 11.6 Hz, 1H), 2.72 (dd, *J* = 14.8, 4.8 Hz, 1H), 2.51 (dd, *J* = 14.8, 5.6 Hz, 1H), 2.34–2.41 (m, 1H), 2.23 (t, *J* = 7.2 Hz, 2H), 1.92–2.12 (m, 2H), 1.70 (s, 3H), 0.84 (d, *J* = 6.8 Hz, 3H), 0.60 (d, *J* = 6.8 Hz, 3H); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 173.1, 170.4, 170.1, 170.1, 159.1, 150.4, 144.6, 139.7, 130.3, 130.1, 129.4, 127.9, 126.7, 120.7, 117.9, 84.5, 66.7, 59.0, 47.9, 45.1, 44.1, 41.3, 31.3, 31.2,

23.8, 23.8, 19.4, 16.5; IR (neat) 2969, 2925, 1708, 1644, 1561, 1409, 1274, 1185, 1030, 842; HRMS (ESI) *m/z* calcd for $C_{42}H_{45}N_5NaO_3S_2$ $[M+Na]^+$ 754.2862, found 754.2867; $[\alpha]_D$ = +32.3 (*c* 0.13, $CHCl_3$).

Peptide isostere pyridyl "IN" thiol (10)—To a solution of trityl protected thiol **43** (150 mg, 0.205 mmol) in CH_2Cl_2 (27.5 mL) at 0 °C was treated with TFA (1.12 mL) and triisopropylsilane (0.089 mL, 0.410 mmol). The reaction mixture was warmed to ambient temperature and stirred for 2 h. The mixture was concentrated and purified using flash chromatography (1% to 6% MeOH in CH_2Cl_2) to obtain the desired thiol (94.6 mg, 94%): ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 7.85 (t, *J* = 7.6 Hz, 1H), 7.65 (d, *J* = 7.6 Hz, 1H), 7.40 (d, *J* = 8.0 Hz, 1H), 7.02 (d, *J* = 5.6 Hz, 1H), 6.74 (d, *J* = 10.8 Hz, 1H), 6.56 (d, *J* = 8.0 Hz, 1H), 5.60 dtd, *J* = 15.2, 6.8, 1.2 Hz, 1H), 5.49 (dd, *J* = 15.6, 6.0 Hz, 1H), 5.08 (dd, *J* = 17.6, 7.2 Hz, 1H), 4.89–4.96 (m, 1H), 4.63 (dd, *J* = 10.8, 2.8 Hz, 1H), 4.32 (dd, *J* = 17.6, 1.6 Hz, 1H), 3.98 (d, *J* = 11.6 Hz, 1H), 3.49 (d, *J* = 11.6 Hz, 1H), 2.69 (dd, *J* = 14.0, 3.2 Hz, 1H), 2.49–2.55 (m, 4H), 2.31 (q, *J* = 6.8 Hz, 2H), 1.91 (s, 3H), 1.38 (t, *J* = 8.0 Hz, 1H), 0.74 (d, *J* = 6.8 Hz, 3H), 0.31 (d, *J* = 6.8 Hz, 3H); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 174.5, 173.4, 170.1, 169.9, 157.7, 148.6, 138.2, 131.2, 129.1, 125.1, 123.2, 84.1, 57.8, 48.6, 44.3, 43.7, 41.2, 36.3, 31.2, 24.0, 24.0, 19.3, 15.2; IR (neat) 2926, 2864, 1667, 1577, 1500, 1293, 1181, 1049; HRMS (ESI) *m/z* calcd for $C_{23}H_{32}N_5O_3S_2$ $[M+H]^+$ 490.1947, found 490.1946; $[\alpha]_D$ = +105 (*c* 0.08, $CHCl_3$).

Peptide isostere pyridyl "OUT" thiol (12)—To a solution of trityl protected thiol **44** (115 mg, 0.157 mmol) in CH_2Cl_2 (22.0 mL) at 0 °C was treated with TFA (0.90 mL) and triisopropylsilane (0.065 mL, 0.314 mmol). The reaction mixture was warmed to ambient temperature and stirred for 2 h. The mixture was concentrated and purified using flash chromatography (1% to 6% MeOH in CH_2Cl_2) to obtain the desired thiol (68.4 mg, 89%): ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 8.66 (d, *J* = 3.6 Hz, 1H), 7.76 (s, 1H), 7.35 (d, *J* = 4.4 Hz, 1H), 7.01 (d, *J* =8.4 Hz, 1H) 6.86–6.88 (m, 1H), 6.19 (d, *J* = 10.4 Hz, 1H), 5.52–5.53 (m, 2H), 4.89–4.91 (m, 1H), 4.85 (d, *J* = 17.6 Hz, 1H), 4.46 (dd, *J* = 10.4, 4.0 Hz, 1H), 4.36 (d, *J* = 17.2 Hz, 1H), 3.84 (d, *J* = 12.0 Hz, 1H), 3.48 (d, *J* = 12.0 Hz, 1H), 2.44–2.72 (m, 5H), 2.30 (q, *J* = 6.4 Hz, 2H), 1.78 (s, 3H), 1.31 (t, *J* = 7.2 Hz, 1H), 0.80 (d, *J* = 6.8 Hz, 3H), 0.49 (d, *J* = 6.8 Hz, 3H); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 173.3, 171.2, 159.6, 150.1, 140.3, 130.8, 128.9, 121.0, 117.8, 84.8, 58.6, 47.9, 44.0, 40.0, 36.2, 31.2, 24.0, 23.9, 23.7, 19.5, 16.1; IR (neat) 2965, 2930, 1655, 1534, 1410, 1201, 1142, 1026; HRMS (ESI) *m/z* calcd for $C_{23}H_{32}N_5O_3S_2$ $[M+H]^+$ 490.1947, found 490.1952; $[\alpha]_D$ = +129 (*c* 0.08, $CHCl_3$).

Peptide isostere pyridyl "IN" (9)—To a solution of peptide pyridine "IN" thiol **10** (25.0 mg, 0.051 mmol) in THF (1.0 mL) at ambient temperature was treated with *N*-octanoyl–imidazole (19.8 mg, 0.102 mmol), imidazole (6.9 mg, 0.102 mmol), and DMAP (0.6 mg, 0.005 mmol). The reaction mixture was warmed to 50 °C and stirred for 6 h. The mixture was cooled and concentrated and purified using flash chromatography (0.5% to 6% MeOH in CH_2Cl_2) to obtain the desired octanoyl masked thiol (20.6 mg, 66%): ^{1}H-NMR (CD_3OD, 400 MHz) δ 8.08 (t, *J* = 7.6 Hz, 1H), 7.84 (d, *J* = 7.6 Hz, 1H), 7.66 (d, *J* = 7.6 Hz, 1H), 7.00 (d, *J* = 10.8 Hz, 1H), 5.52–5.64 (m, 2H), 4.92 (d, *J* = 17.6 Hz, 1H), 4.77–4.83 (m, 1H), 4.53–4.57 (m, 1H), 4.38 (d, *J* = 17.2 Hz, 1H), 3.93 (d, *J* = 11.6 Hz, 1H), 3.67 (d, *J* = 12.0

Hz, 1H), 2.89 (t, *J* = 7.2 Hz, 2H), 2.68 (dd, *J* = 14.0, 11.2 Hz, 1H), 2.53 (dd, *J* = 14.0, 4.0 Hz, 1H), 2.52 (t, *J* = 7.2 Hz, 2H), 2.34–2.42 (m, 1H), 2.23–2.30 (m, 2H), 1.89 (s, 3H), 1.59–1.63 (m, 2H), 1.26–1.29 (m, 10H), 0.87 (t, *J* = 6.8 Hz, 3H), 0.75 (d, *J* = 7.2 Hz, 3H), 0.35 (d, *J* = 6.8 Hz, 3H); ^{13}C-NMR (CD_3OD, 100 MHz) δ 203.4, 178.9, 177.9, 175.9, 174.4, 161.2, 151.4, 143.9, 143.8 135.0, 132.5, 129.7, 127.3, 87.3, 61.6, 47.5, 47.3, 47.1, 43.8, 35.8, 35.3, 34.9, 32.5, 32.4, 31.6, 29.3, 26.5, 26.1, 22.4, 18.5, 16.9; IR (neat) 2957, 2928, 2855, 1678, 1530, 1403, 965; HRMS (ESI) *m/z* calcd for $C_{31}H_{46}N_5O_4S_2$ $[M+H]^+$ 616.2986, found 616.2988; $[\alpha]_D$ = +107 (*c* 0.23, $CHCl_3$).

Peptide isostere pyridyl "OUT" (11)—To a solution of peptide pyridine "OUT" thiol **12** (8.0 mg, 0.016 mmol) in CH_2Cl_2 (0.2 mL) at ambient temperature was treated with octanoyl chloride (.005 mL, 0.032 mmol), DIPEA (0.011 mL, 0.064 mmol), and DMAP (0.1 mg, 0.008 mmol). The reaction mixture was stirred for 2 h before cooling to 0 °C and quenching with methanol (5.0 mL). The mixture was cooled and concentrated and purified using flash chromatography (0.5% to 6% MeOH in CH_2Cl_2) to obtain the desired octanoyl masked thiol (5.4 mg, 55%): ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 8.65 (dd, *J* = 4.8, 0.8 Hz, 1H), 7.88 (d, *J* = 0.8 Hz, 1H), 7.33 (dd, *J* = 4.8, 1.6 Hz, 1H), 6.27–6.33 (m, 2H), 6.12 (d, *J* = 10.0 Hz, 1H), 5.61–5.72 (m, 2H), 4.90–4.95 (m, 1H), 4.75 (dd, *J* = 16.8, 6.8 Hz, 1H), 4.53 (dd, *J* = 17.2, 6.0 Hz, 1H), 4.46 (dd, *J* = 10.4, 4.0 Hz, 1H), 3.83 (d, *J* = 12.0, 1H), 3.48 (d, *J* = 11.6 Hz, 1H), 2.86 (t, *J* = 7.2 Hz, 2H), 2.71 (dd, *J* = 15.6, 4.4 Hz, 1H), 2.56 (dd, *J* = 15.6, 7.6 Hz, 1H), 2.43–2.51 (m, 2H), 2.26–2.33 (m, 1H), 1.77 (s, 3H), 1.56–1.64 (m, 2H), 1.24–1.29 (m, 10H), 0.85 (t, *J* = 6.8 Hz, 3H), 0.82 (d, *J* = 6.8 Hz, 3H), 0.52 (d, *J* = 6.8 Hz, 3H); ^{13}C-NMR (CD_3OD, 100 MHz) δ 199.5, 173.4, 171.7, 170.9, 170.4, 158.3, 148.5, 142.1, 130.9, 128.5, 121.3, 118.9, 84.5, 58.1, 43.7, 43.4, 42.9, 39.2, 32.0, 31.4, 31.1, 28.6, 28.5, 27.7, 25.4, 22.3, 22.2, 18.9, 15.8, 12.9; IR (neat) 2956, 2927, 2855, 1679, 1649, 1519, 1403, 1031, 965; HRMS (ESI) *m/z* calcd for $C_{31}H_{46}N_5O_4S_2$ $[M+H]^+$ 616.2981, found 616.2981; $[\alpha]_D$ = +40.6 (*c* 0.35, $CHCl_3$).

HDAC Biochemical Assay

Compounds **1**-**12** were tested against HDAC1-9 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 and pH 7.4. Additional 200 μM TCEP was added for HDAC6) and followed by fluorogenic release of 7-amino-4-methylcoumarin from 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%). IC_{50} is 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 Km=[substrate].

797 and 10326 Cell Viability

For dose-response cellular viability assays, 797 and 10326 cells were seeded onto separate 384-well tissue culture-treated plates at a density of 2.0×10^4 cells/well in a volume of 50 μL/well. Addition of compound was performed with a JANUS Workstation (PerkinElmer Life and Analytical Sciences) using a 384-well pinhead tool that is calibrated to deliver 100 nL drug/well. Final DMSO concentration in the well was 0.02%. After 48 hours of incubation with compound, cells were analyzed for cell viability using the ATPLite (Perkin Elmer) luminescent assay kit per the manufacturer's instructions. Luminescence was read on an EnVision 2104 Multilabel Plate Reader (PerkinElmer Life and Analytical Sciences). Replicate measurements were analyzed with respect to dose and estimates of IC_{50} were calculated by logistic regression (GraphPad Prism). Standard error of the LogIC50 values were performed using GraphPad according to the following equation:

$$\mathrm{SE(Pi)}=\mathrm{sqrt}[(\mathrm{SS}/\mathrm{DF}) * \mathrm{Cov(i,i)}]$$

where:

Pi: i-th adjustable(non-constant) parameter SS: sum of squared residuals

DF: degrees of freedom (the number of data points minus number of parameters fit by regression)

Cov(i,i): i-th diagonal element of covariance matrix

sqrt(): square root

Computational Details

An adapted strategy was used to investigate the conformational space of Largazole and the pyridyl derivatives described here in the free thiol form and bound to HDACs 1, 6, and 8.[10] Monte Carlo conformational searches were used to generate the conformational ensemble of the ligands in solution. Then, molecular dynamics (MD) simulations were used to sample the available conformations of the bound ligands. The bound conformations were compared to the conformational ensemble in solution via heavy atom root-mean-squared distance (rmsd) and the energy difference between the closest conformation and the global minimum was recorded.

Molecular Dynamics

Initial coordinates for the protein structures were derived from previously developed homology models for HDACs 11[13,21,22] and 6[15] and the recently published HDAC8-Largazole crystal structure[14] (pdb code 3RQD). For MD simulations the ionizable residues were set to most likely ionization states at pH 7. The **6, 8, 10** and **12** were modeled with the pyridyl nitrogen protonated. The 15 inhibitor-enzyme complexes were solvated with a periodic box of TIP3P[23] water molecules extending 10 Å from any protein atoms. The crystallographic potassium ions were kept for the HDAC8 complexes and sodium ions were added to neutralize the system.

The ff03[24,25] version of the all-atom AMBER force field and GAFF[26] were employed to model the protein structures and inhibitors, respectively. Atom-centered partial charges were fit using the RESP method.[27] Energy minimizations were used first to relax only solvent molecules and counterions before relaxing the full complexes to remove any bad contacts in the initial geometries. The MD simulations were performed with the PMEMD module in the AMBER 12 suite of programs. All bonds containing hydrogen were constrained with the SHAKE[28] algorithm and the timestep was chosen to be 2 fs. A nonbonded cutoff 10.0 Å was chosen, and the nonbonded pair list was updated every 25 steps. The temperature was kept constant at 300 K using Langevin dynamics with a collision frequency of 1.0 ps^{-1}, and the pressure was kept constant at 1 atm with isotropic position scaling. Periodic boundary conditions were employed to simulation a continuous system with contributions from long range interactions included with the Particle-Mesh-Ewald (PME) method.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

HDACi Histone Deacetylase Inhibitor

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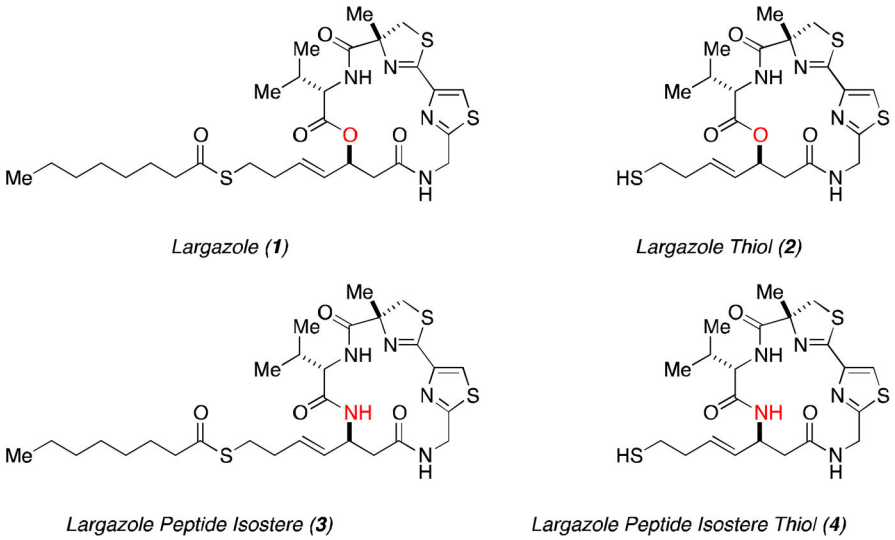


Figure 1.
Structure of Largazole and Largazole thiol and their peptide isosteres.

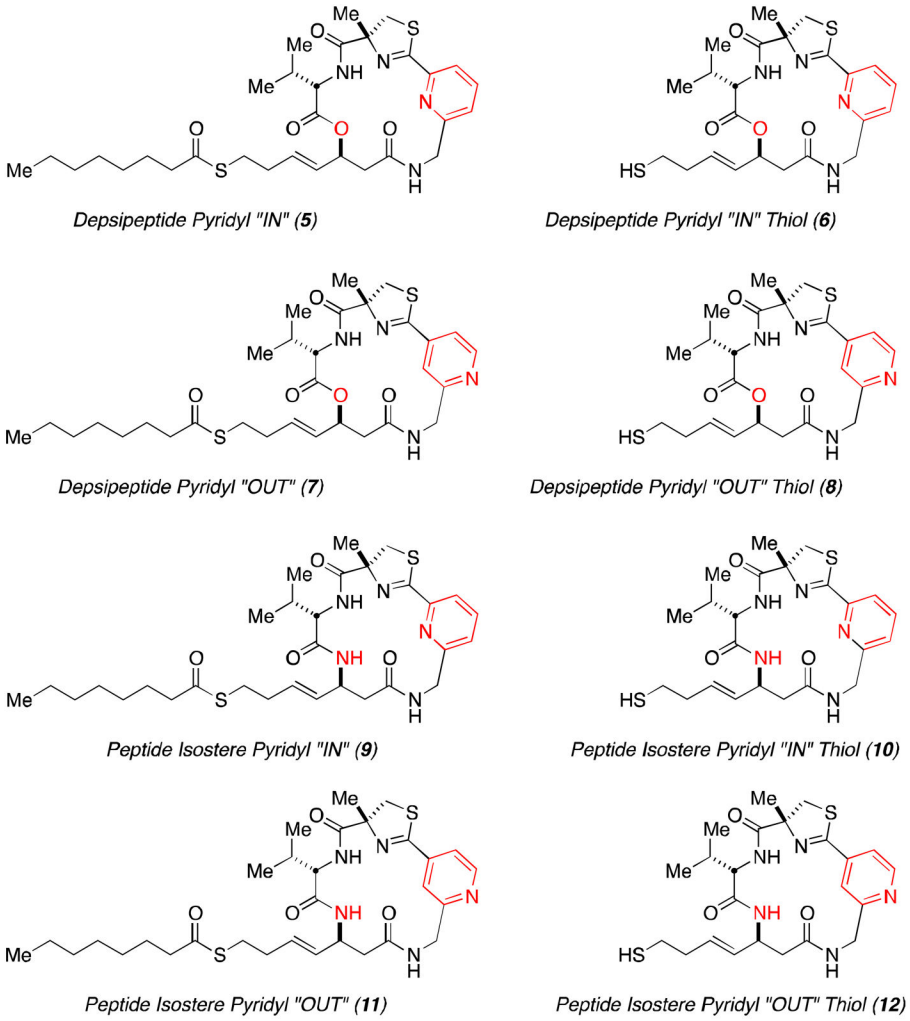


Figure 2.
Pyridine containing library based on Largazole.

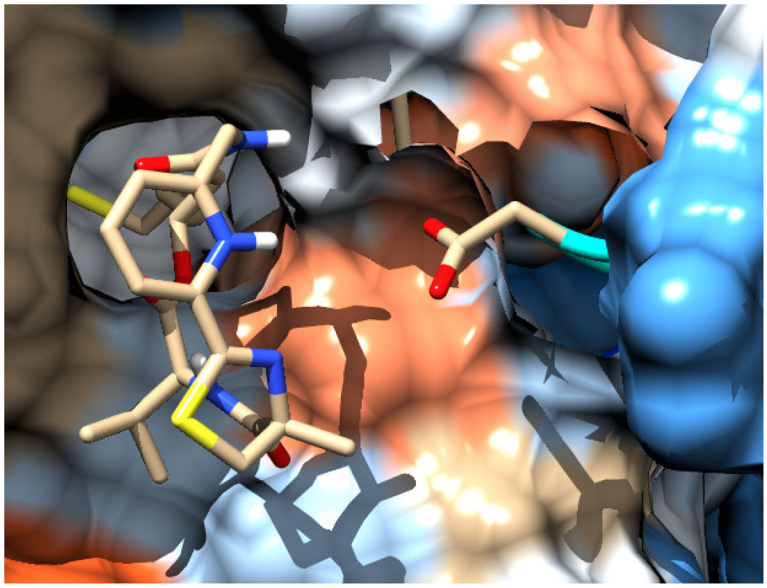


Figure 3.
Model of pyridyl Largazole thiol analogue bound to HDAC1

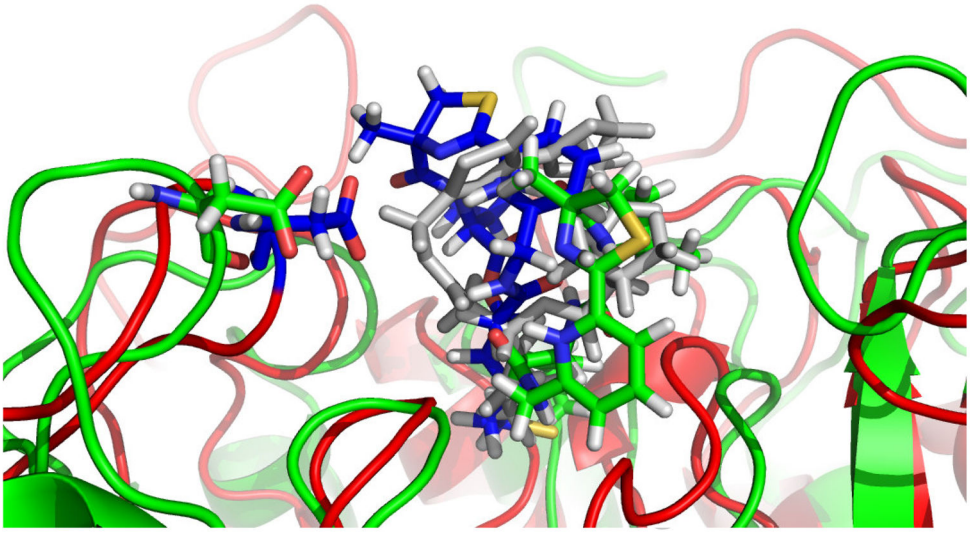


Figure 4.

Snapshots from the MD simulations of **6** (blue, with protein in red) and **10** (green) in HDAC1. Position of **2** is shown in grey for reference purposes

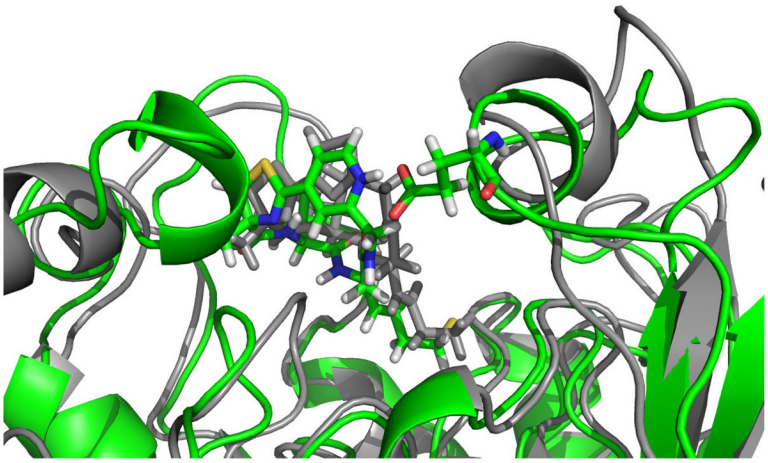


Figure 5.

Snapshots from the MD simulations of **2** (grey) and **12** (green) in HDAC6. Position of **2** is shown in grey for reference purposes

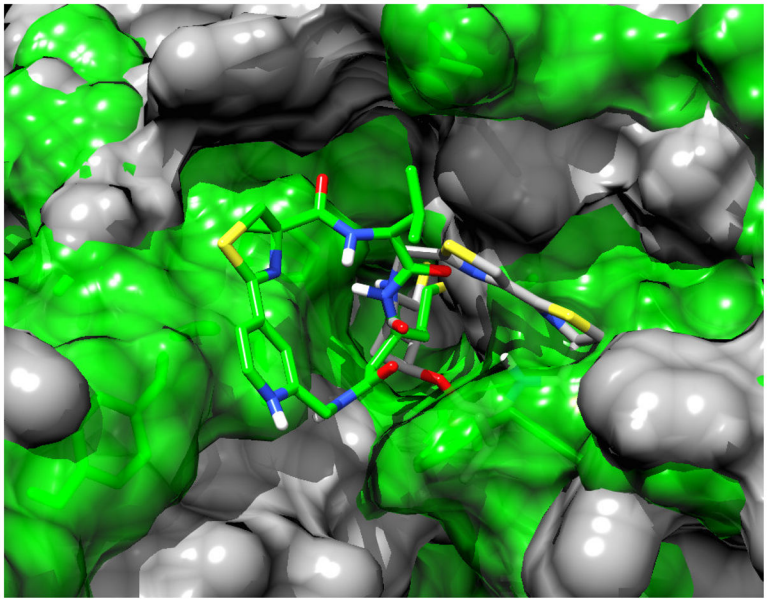
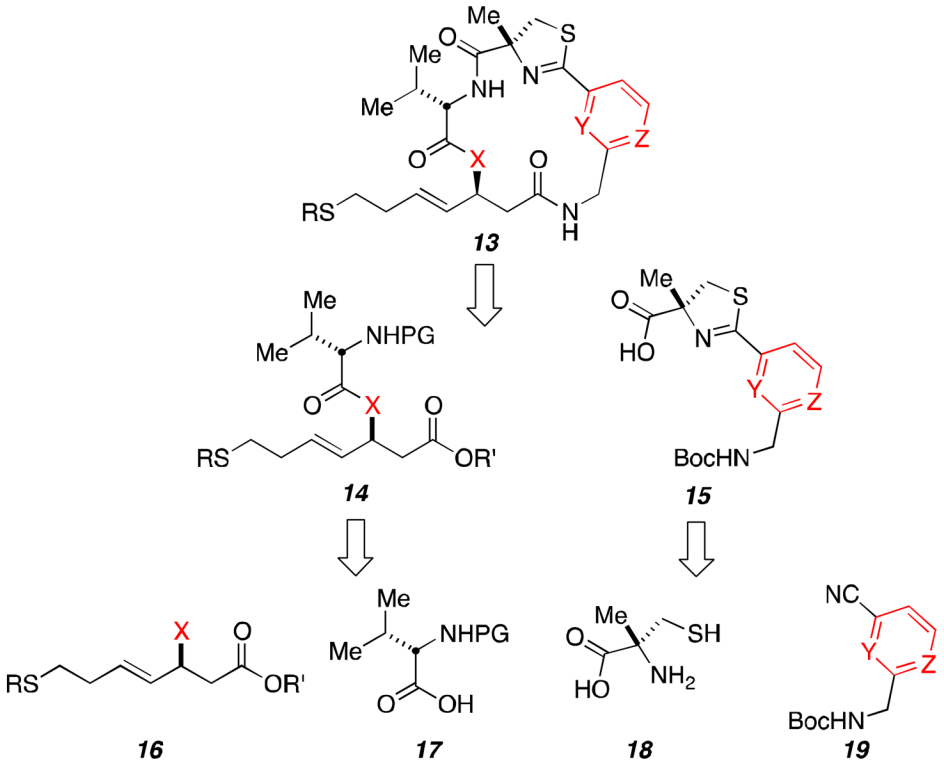
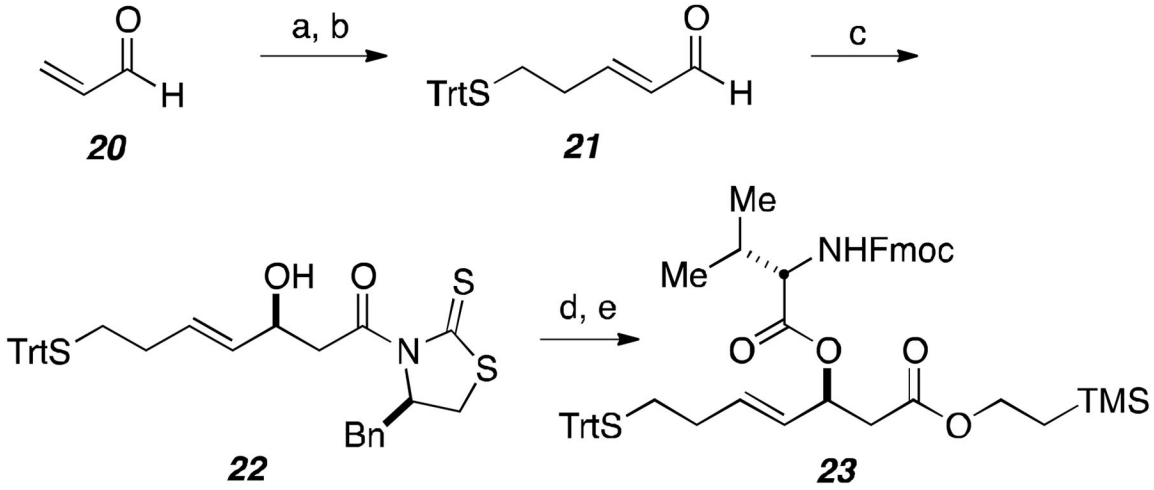


Figure 6.
Snapshots from the MD simulations of **2** (grey) and **12** (green) in HDAC8.

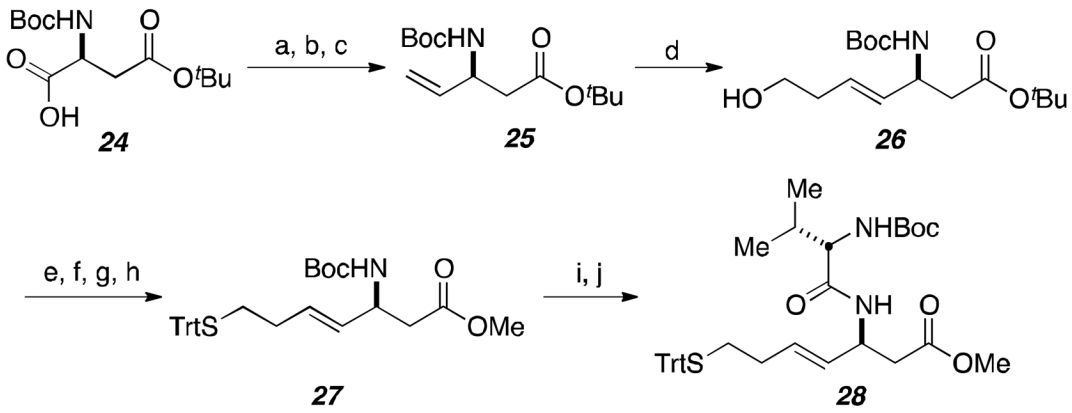


Scheme 1.
Retrosynthetic analysis of library.



Scheme 2.

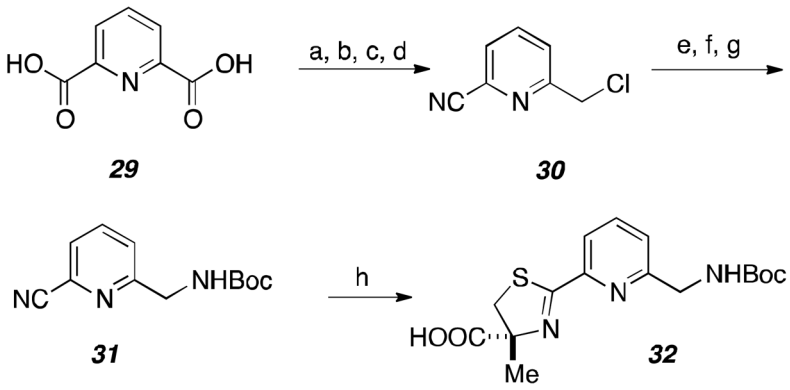
Synthesis of fragment **23** for depsipeptide macrocycle. Reagents and conditions: (a) TrtSH, Et_3N, CH_2Cl_2; (b) (formylmethylene)triphenylphosphorane, PhH, 80 ° C, 77% over 2 steps; (c) (*R*)-1-(4-benzyl-2-thioxothiazolidin-3-yl)ethanone, $TiCl_4$, DIPEA, CH_2Cl_2, −78 ° C, 76%; (d) 2-(trimethylsilyl)ethanol, imidazole, CH_2Cl_2, 83%; (e) N-Fmoc-Val-OH, EDCI, DIPEA, DMAP, CH_2Cl_2, 77%.



Scheme 3.

Synthesis of fragment **28** for peptide macrocycle.

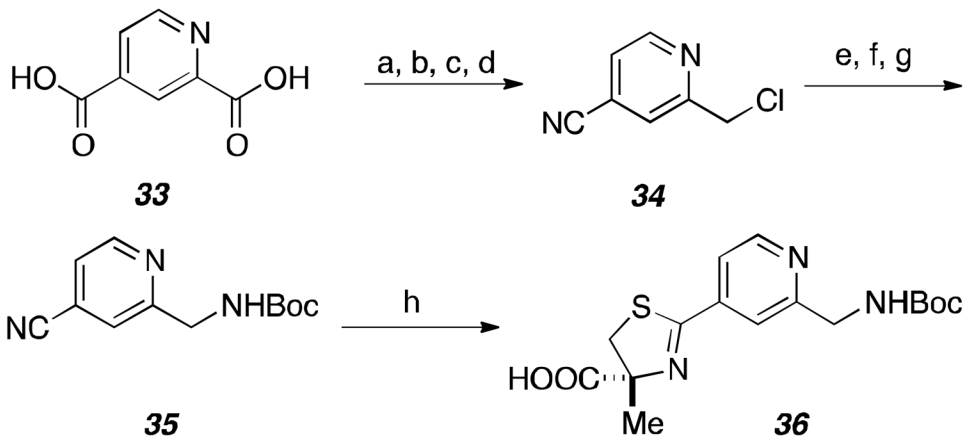
Reagents and conditions: (a) 4-methylmorpholine, isobutyl chloroformate, THF, −40 ° C; $NaBH_4$, MeOH, −20 ° C, 66%; (b) oxalyl chloride, DMSO, DIPEA, CH_2Cl_2, −65 ° C; (c) methyltriphenylphosphonium bromide, KHMDS, THF, −78 ° C, 80% over 2 steps; (d) 3-buten-1-ol, Grubbs catalyst, 2nd generation, CH_2Cl_2, 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 2 steps.



Scheme 4.

Synthesis of the pyridyl "IN" fragment **32**.

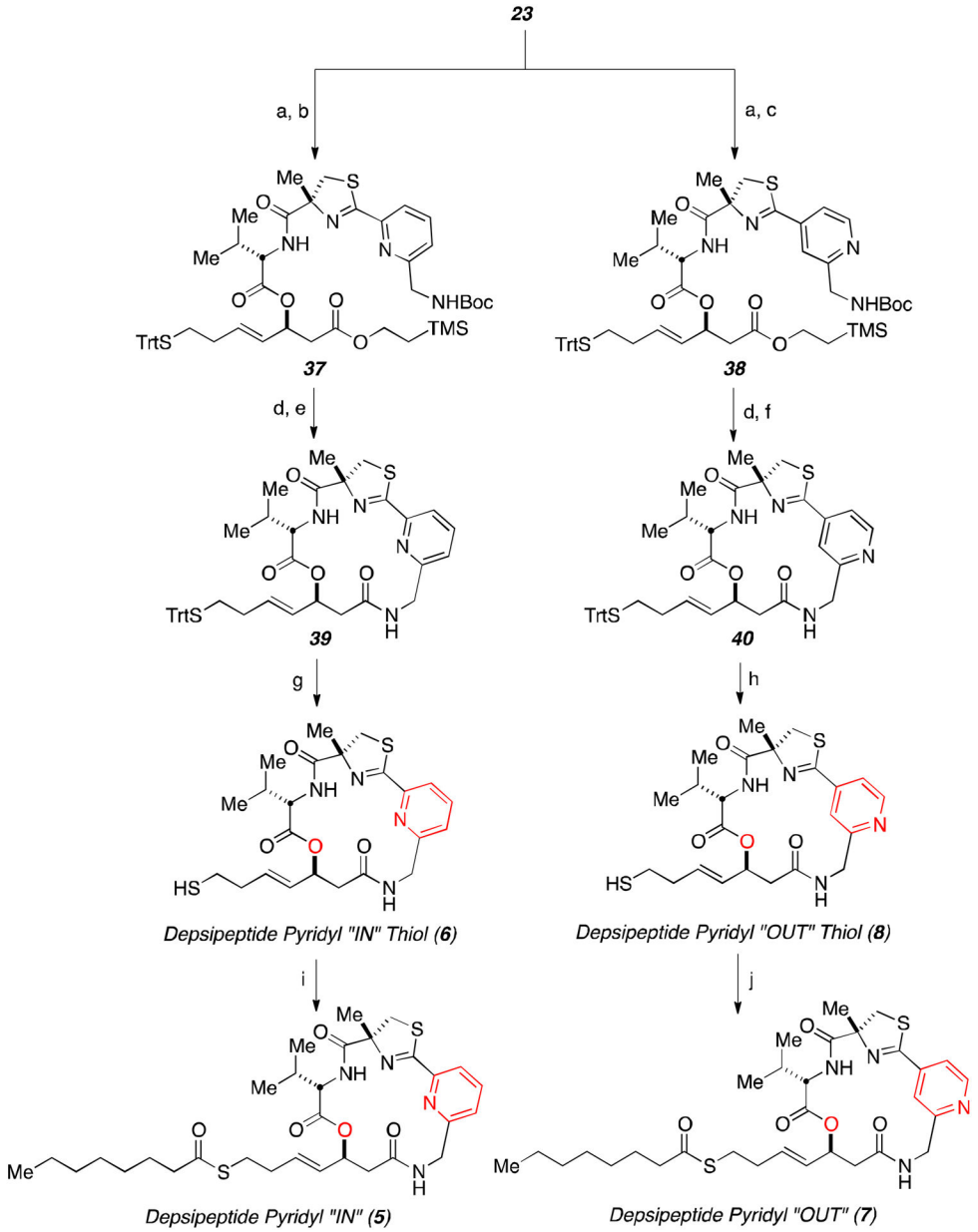
Reagents and Conditions: (a) PTSA, EtOH, 80 ° C, 100%; (b) $NaBH_4$, EtOH, 80 ° C, 53%; (c) NH_4OH, EtOH; (d) $POCl_3$, DMF, 0 ° C; (e) potassium phthalimide, DMF, 69% over three steps; (f) H_2NNH_2-H_2O, MeOH, THF; (g) Boc_2O, Et_3N, CH_2Cl_2, 43% over 2 steps; (h) α-methyl-L-cysteine, $NaHCO_3$, pH 6.0 buffer, MeOH, 70 ° C, 84%.



Scheme 5.

Synthesis of pyridine “OUT” fragment.

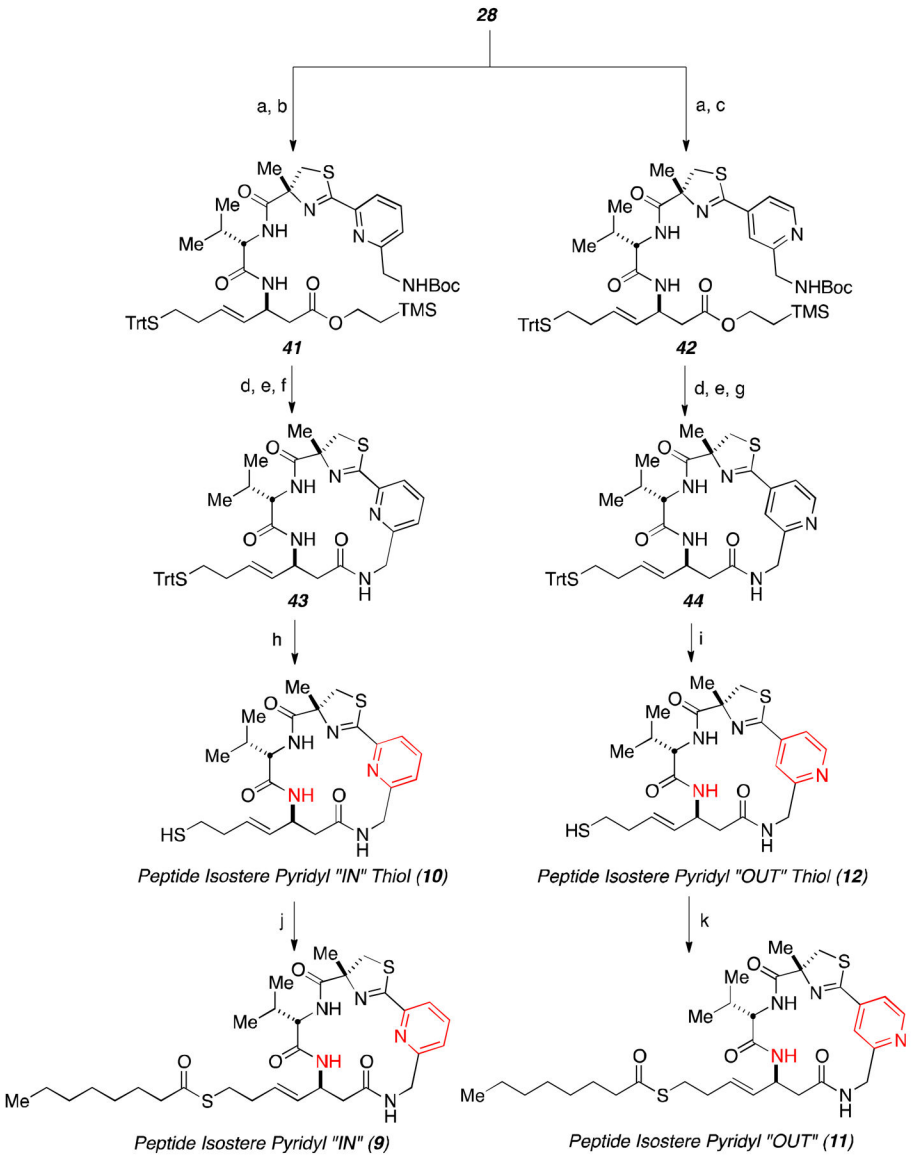
Reagents and conditions: (a) PTSA, EtOH, 80 ° C, 100%; (b) $NaBH_4$, $CaCl_2$, EtOH, 80 ° C, 61%; (c) NH_4OH, EtOH; (d) $POCl_3$, DMF, 0 ° C; (e) potassium phthalimide, DMF, 50% over three steps; (f) H_2NNH_2-H_2O, MeOH, THF; (g) Boc_2O, Et_3N, CH_2Cl_2, 84% over 2 steps; (h) α-methyl-L-cysteine, $NaHCO_3$, pH 6.0 buffer, MeOH, 70 ° C, 85%.



Scheme 6.

Synthesis of depsipeptide containing analogs.

Reagents and conditions: (a) Et_2NH, CH_3CN; (b) **32**, PyBOP, DIPEA, 79% over 2 steps; (c) **36**, PyBOP, DIPEA, 81% over 2 steps; (d) TFA, CH_2Cl_2; (e) HATU, HOBt, DIPEA, CH_3CN, 20% over 2 steps; (f) HATU, HOBt, DIPEA, CH_3CN, 23% over 2 steps; (g) TFA, $^{i}Pr_3SiH$, CH_2Cl_2, 82%; (h) TFA, $^{i}Pr_3SiH$, CH_2Cl_2, 89%; (i) *N*-octanoyl–imidazole, imidazole, DMAP, THF, 50 ° C, 69%; (j) octanoyl chloride, Et_3N, 49%.



Scheme 7.

Synthesis of peptide containing inhibitors.

Reagents and conditions: (a) TFA, CH_2Cl_2; (b) **32**, PyBOP, DIPEA, 84% over 2 steps; (c) **36**, PyBOP, DIPEA, 92% over 2 steps; (d) LiOH, THF, H_2O; (e) TFA, CH_2Cl_2; (f) HATU, HOBt, DIPEA, CH_3CN, 33% over 3 steps; (g) HATU, HOBt, DIPEA, CH_3CN, 32% over 3 steps; (h) TFA, $^{i}Pr_3SiH$, CH_2Cl_2, 94%; (i) TFA, $^{i}Pr_3SiH$, CH_2Cl_2, 89%; (j) *N*-octanoyl–imidazole, imidazole, DMAP, THF, 50 ° C, 66%; (k) octanoyl chloride, Et_3N, 55%.

Table 1

HDAC inhibitory activity of Largazole analogs (IC_{50} nM).

Compound	HDAC1	HDAC2	HDAC3	HDAC6	HDAC8
1	10.09	18.65	9.09	165.6	1068
2	**2.51**	**4.19**	**2.78**	28.11	228.4
3	544.1	825.2	1151	-	-
4	**1.95**	**3.38**	**2.59**	102	255.3
5	203.6	349.5	332.1	-	-
6	**2.68**	**4.39**	**3.07**	48.55	341.3
7	340	655.4	319.5	-	-
8	**2.20**	**4.42**	**2.31**	35.16	101.8
9	340.3	471.8	332.4	-	-
10	42	69.8	42.5	-	-
11	816.9	1240	846.5	-	-
12	13.2	20.77	14.59	2849	1491

Table 2

Activity of Largazole analogs in 797 and 10326 cell lines (IC_{50}s μM)

Compound	797	797 LogIC_{50}	10326	10326 LogIC_{50}
1	0.024	−7.621 ± 0.06565	0.025	−7.606 ± 0.02845
2	0.1	−7.016 ± 0.03453	0.08	−7.083 ± 0.06683
3	0.11	−6.971 ± 0.04341	0.17	−6.763 ± 0.06589
4	1.74	−5.761 ± 0.05449	11.94	−4.923 ± 0.9900
5	0.03	−7.529 ± 0.05220	0.038	−7.422 ± 0.04499
6	0.22	−6.654 ± 0.04604	0.29	−6.526 ± 0.09239
7	0.01	−7.949 ± 0.07215	0.01	−7.873 ± 0.04062
8	0.09	−7.031 ± 0.06228	0.12	−6.913 ± 0.03566
9	0.31	−6.509 ± 0.02559	0.55	−6.263 ± 0.07115
10	-	-	-	-
11	0.07	−7.122 ± 0.05525	0.13	−6.873 ± 0.06520
12	4.25	−5.371 ± 0.04826	8.64	−5.063 ± 0.1964