The First Biologically Active Synthetic Analogues of FK228, the Depsipeptide Histone Deacetylase Inhibitor

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The FK228 and spiruchostatin bicyclic depsipeptide natural products are among the most potent histone deacetylase (HDAC) inhibitors known. Although FK228 is in advanced clinical trials, the complexity of the natural products has precluded mechanistic studies and the discovery of structure−activity relationships. By total synthesis, we have prepared the first depsipeptide analogues. Our results prove that the dehydrobutyrine residue in FK228 is not essential, and other residues can be substituted without loss of HDAC inhibitory activity. Conformational restriction by the macrocyclic scaffold is important, as a linear peptide was inactive. The intramolecular disulfide formed with a cysteine side chain can be removed provided the zinc-binding thiol is protected to ensure good cellular availability. Like the natural products, the analogues are selective against class I isoforms, with nanomolar inhibition of class I HDAC1 and significantly less potency against class II HDAC6.

Eukaryotic DNA is packaged together with histone and nonhistone proteins into the higher order structure of chromatin. Chromatin proteins feature extensive posttranslational modification[1] including methylation, acetylation, phosphorylation, ubiquitinylation, sumoylation, and poly-ADPribosylation. These dynamic alterations are believed to constitute a 'histone code'[2] that mediates protein−DNA and protein−protein interactions in chromatin and thereby ultimately regulates gene transcription. Consequently, the modulation of the chromatin histone code offers opportunities for targeting diverse disease states at a higher level of intervention than individual signal transduction pathways. Among the enzymes involved in chromatin remodeling, zinc-dependent histone deacetylases (HDACs[a]) are currently at the most advanced stage in drug discovery, with one FDA approval and numerous other compounds in clinical development. These metalloenzymes catalyze the hydrolysis of acetyllysine residues back to lysine. Since the latter, unlike acetyllysine, is protonated at physiological pH, this switch has a dramatic effect on chromatin structure and recruitment of binding partners. Furthermore, reversible lysine acetylation is now recognized[3] as a general posttranslational modification with important functional consequences in nuclear and cytoplasmic proteins unrelated to chromatin, thus increasing the therapeutic importance of this event.

HDAC inhibitor design has concentrated[4] on the class I and class II zinc-dependent enzymes, comprising HDACs 1−11 in the human genome. These hydrolases share a highly conserved catalytic domain containing an active site zinc ion, and the majority of inhibitors fall into five broad structural classes (Figure 1): short chain aliphatic carboxylic acids, hydroxamic acids, benzamides, cyclic peptides, and the depsipeptides. HDAC inhibitors are typically substrate mimics of the linear acetyllysine side chain with a zinc-binding group replacing the scissile acetamide and a 'cap' at the other end extending beyond the enzyme substrate-binding channel. High affinity for the enzymes requires a combination of these complementary interactions. The short chain acids have a carboxylate zinc-binding group, but little in the way of cap interactions, and suffer from relatively weak HDAC inhibition (high micromolar IC_{50}). The benzamides have higher potency (sub-micromolar IC_{50}) and act as competitive reversible inhibitors,[5] with the benzamide apparently functioning as the zinc-binding group. Hydroxamic acids contain a tight-binding zinc chelator, and this alone achieves high activity (sub-micromolar IC_{50}) without the need for significant cap interactions. Within this class, Merck's SAHA (suberoylanilide hydroxamic acid) recently received[6] FDA approval for the treatment of cutaneous T-cell lymphoma. Finally, the cyclic peptides and depsipeptides are complex natural products where the zinc binding group is augmented by a large macrocyclic scaffold. In these compounds, the significant cap interactions can lead to high activity without a strong zinc-binding group. For example, the recently isolated[7] cyclic tetrapeptide azumamide E, with sub-micromolar activity, is the second most potent HDAC inhibitor known with a simple carboxylic acid zinc-binding group. In the case of the depsipeptide FK228, the natural product itself is a prodrug[8] that upon intracellular reduction releases the zinc-binding thiol. While the thiol is weaker in zinc-binding than a hydroxamic acid, additional interactions between the macrocycle and the enzyme 'rim' outside the active site result in the highest potency (nanomolar IC_{50}) among the clinical candidate HDAC inhibitors. Furthermore, as the rim is divergent between HDAC isoforms, FK228 has the potential to discriminate among them and is in fact reported[8] to be significantly more potent against the class I HDAC1 than class II HDAC6. Although the precise targets of class I and class II HDACs

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[a] Abbreviations: Dhb, dehydrobutyrine; EDAC, *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide; HDAC, histone deacetylase; HOBt, 1-hydroxybenzotriazole; MNBA, 2-methyl-6-nitrobenzoic anhydride; SAHA, suberoylanilide hydroxamic acid.

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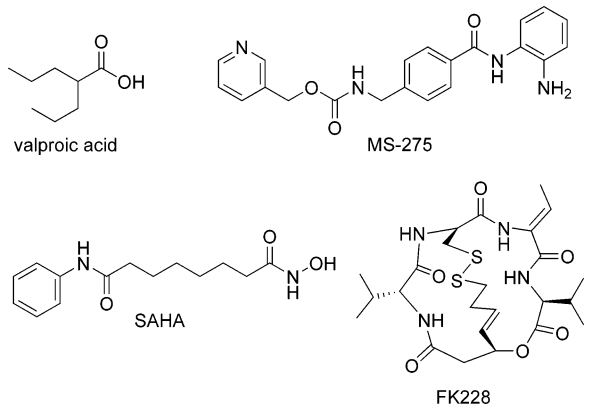


Figure 1. Four examples of clinical candidate HDAC inhibitors.

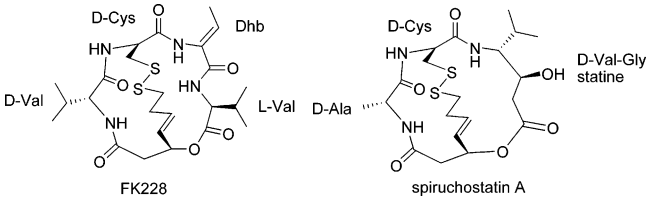


Figure 2. Comparison between FK228 and spiruchostatin A.

remain uncertain, the evidence suggests[9] that class I selectivity is useful for anticancer agents, and that inhibition of specific class II HDACs may have undesirable consequences[10] such as the promotion of cardiac hypertrophy.

Despite their high potency and class I selectivity, the depsipeptides[11,12] comprising FK228 and the spiruchostatins suffer from there being only six examples of these products isolated from extracts of bacterial fermentation. Opportunities for further modification of the natural products are extremely limited, while their daunting structures are considered intractable starting points for medicinal chemistry. Although within this class FK228 is in advanced clinical trials, there are absolutely no related analogues or structure−activity relationships, raising the issue of whether the natural products are the optimized human therapeutic within this class. Four academic groups including our own have reported[13] the synthesis of depsipeptides, but these endeavors were restricted to the natural products. Here, we demonstrate that the systematic total synthesis of depsipeptide analogues can be achieved in a practical manner and provide valuable insights into the importance of the manifold functional groups embedded within FK228. These studies will facilitate the identification of unnatural second generation congeners with improved druglike properties or selectivity against individual HDACs.

Results and Discussion

A comparison between the structures of FK228 and spiruchostatin A (Figure 2) reveals that these depsipeptide natural products share an invariant disulfide moiety, formed by the intramolecular bridging between D-cysteine and a β-hydroxy-γ,δ-unsaturated-ε-thioacid. The differences lie in the rest of the depsipeptide backbone. Of particular note are the presence of an unsaturated dehydrobutyrine (Dhb) residue in FK228, and a D-Val-Gly statine dipeptide isostere in spiruchostatin A. For our first analogue, we designed a hybrid combining features of both natural products: It would retain the D-Ala in spiruchostatin A, and a D-Val-Gly dipeptide instead of the spiruchostatin statine unit.

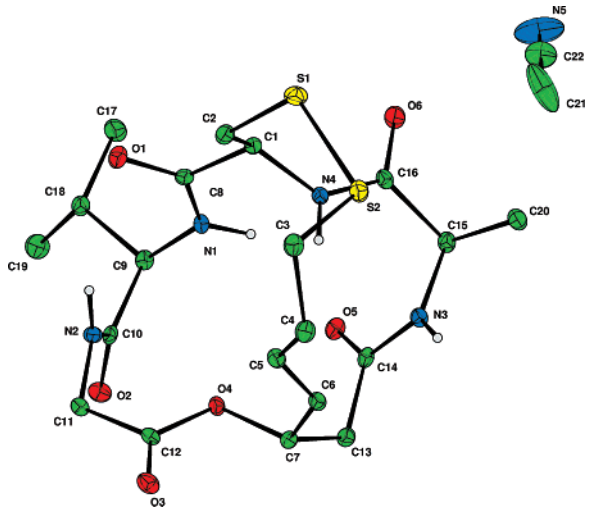


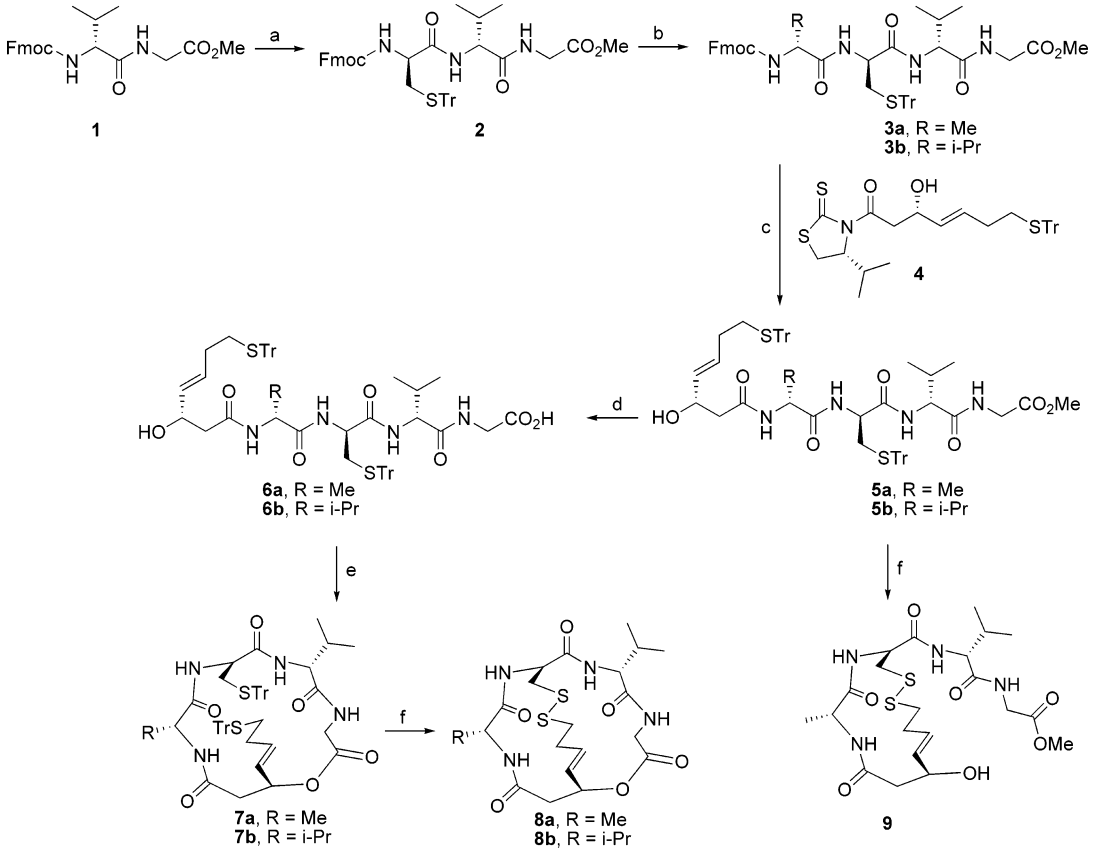
Figure 3. X-ray structure of **8a**, with thermal ellipsoids drawn at the 35% probability level. The structure includes one solvate molecule of acetonitrile.

The synthesis of the analogue began with the preparation of tetrapeptide **3a** (Scheme 1) by standard methods. The peptide was then condensed with β-hydroxy amide **4**, obtained by the asymmetric aldol reaction described[13c] in our spiruchostatin A total synthesis, to give **5a**. Hydrolysis of the methyl ester afforded *seco*-acid **6a**, and the crucial macrolactonization[14] was achieved under either Steglich (dicyclohexyl carbodiimide) or Yamaguchi conditions (2,4,6-trichlorobenzoyl chloride) to furnish macrolide **7a** in modest yields of 36% or 11%, respectively. Subsequently, we switched to Shiina's reagent,[15] 2-methyl-6-nitrobenzoic anhydride (MNBA), used[13e] by Doi and Takahashi in their spiruchostatin A total synthesis. This reagent effects cyclization under mild conditions (room temperature), and we find it is the method of choice for these depsipeptides. In the present case, **7a** was obtained in 70% yield, and intramolecular disulfide bond formation then led to bicyclic analogue **8a**. Overall, this route was reliable and scalable, and an experimental procedure is provided for the preparation of **8a** on 200 mg scale. Other analogues were prepared in a similar manner (procedures in Supporting Information) and the compounds examined in three assays (Table 1): (1) growth inhibition of MCF7 breast cancer cells as an indicator of in vitro cell activity; (2) inhibition of total HDAC activity from HeLa cell nuclear extracts as an indicator of in vitro enzyme activity; (3) inhibition of HDAC1 and HDAC6 as a measure of class I/class II selectivity (all enzyme assays for disulfides performed in the presence of excess dithiothreitol (DTT) to ensure reduction to the dithiol).

Importance of the Dehydrobutyrine Residue. In our first analogue **8a**, we excised the unsaturated dehydrobutyrine (Dhb) residue present in FK228, a potential site for covalent modification by nucleophilic Michael addition. While the spiruchostatins do not contain this residue, their statine unit can potentially undergo elimination to generate a similarly reactive α,β-unsaturated ester. Since **8a** is devoid of either feature, it should clarify the relevance of an electrophilic reaction site in depsipeptides.

Analogue **8a** proved to be a potent growth inhibitor of MCF7 cells. Mechanistic studies confirmed the effect was due to HDAC inhibition, as **8a** increased cellular histone acetylation levels and activated the promoter for the $p21^{cip1/waf1}$ cell cycle inhibitor when linked to a luciferase reporter gene. Analogue **8a** is similar in potency to FK228 in inhibition of total HDACs from HeLa cells and in fact slightly more potent against

Scheme 1. Synthesis of Depsipeptide Analogues **8a**, **8b**, and **9**[a]



[a] Reagents: (a) i. Et_2NH; ii. Fmoc-D-Cys(Tr), EDAC, HOBt, i-Pr_2NEt; (b) i. Et_2NH; ii. Fmoc-D-Ala (**8a**) or Fmoc-D-Val (**8b**), EDAC, HOBt, i-Pr_2NEt; (c) i. Et_2NH; ii. **4**, cat. DMAP; (d) LiOH; (e) MNBA, DMAP; (f) I_2.

Table 1. IC_{50} Values (nM) for Inhibition of (a) MCF7 Breast Cancer Cells, (b) Total HDACs from HeLa Cell Nuclear Extract, and (c) HDAC1 and HDAC6[a]

	MCF7 growth inhibition	HeLa nuclear HDACs	HDAC1	HDAC6
SAHA	500	288 ± 59	775 ± 167	196 ± 3
FK228	0.75 ± 0.2	15 ± 9	3.97 ± 0.30	787 ± 24
8a	37 ± 4	15 ± 7	1.60 ± 0.03	881 ± 138
8b	5.1 ± 0.2	7.2 ± 2.9	1.60 ± 0.04	897 ± 40
9	> 1,000	> 3,200	nd	nd
15a	> 1,000	7 ± 2	17.5 ± 1.9	4922 ± 686
15b	55 ± 11	242	>100	>10000

[a] For comparison, data with the hydroxamic acid SAHA and FK228 are shown. nd = not determined.

HDAC1. These results validate our hypothesis that the depsipeptide scaffold is amenable to synthetic reengineering without loss of activity. Although **8a** features three amino acid changes relative to FK228, it remains a nanomolar HDAC inhibitor. Furthermore, the results clearly demonstrate the unsaturated Dhb residue within FK228 is not a prerequisite for HDAC inhibition and can be replaced by other chemically robust and more drug-friendly side chains.

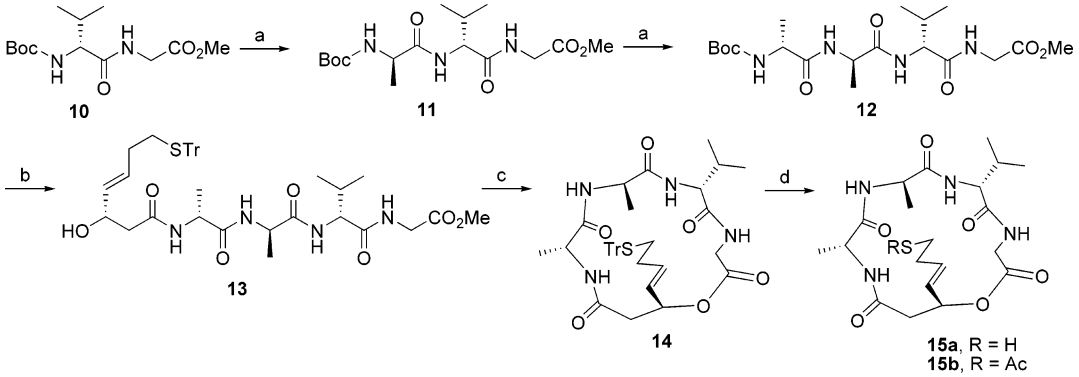
Structural Insights into Bicyclic Depsipeptides. We have solved the X-ray crystal structure of **8a** (Figure 3). The molecule is folded in a compact manner with a rigid backbone and two intramolecular hydrogen bonds and is similar to the reported[11c] X-ray structure of FK228. The intramolecular hydrogen bonding in these depsipeptides probably helps reduce the polar surface area and improve membrane permeability,[16] while also accounting for the relatively high stability in vivo and oral bioavailability[17] compared to linear peptides.

Improving the Activity of 8a. In **8a**, the R side chain was chosen as alanine to correspond with spiruchostatin A. In FK228, this position is occupied by a valine side chain. To determine the relative importance of this difference, we prepared valine analogue **8b** by the same route. Analogue **8b** was more potent than **8a** in growth and HDAC inhibition. In these assays, the analogue is close in potency to the parent FK228 and suggests that a bulky side chain is preferable for R.

Importance of the Macrolide Ring. The disulfide bond cyclization of linear hydroxy ester **5a** afforded monocyclic analogue **9**. This compound would presumably undergo reduction to provide a zinc-binding thiol in the same way as **8a/b**, but in the context of a linear peptide rather than a cyclic depsipeptide. Analogue **9** was essentially inactive in cell growth or HDAC inhibition. While growth inhibition is influenced by bioavailability, the enzyme assay indicates **9** is inherently inactive toward HDACs. Apparently, linear peptides are conformationally too flexible to bind with high affinity to the enzyme despite the presence of a zinc-binding group. The macrocyclic scaffold is hence an important contributor to HDAC inhibition by depsipeptides.

Importance of the Disulfide Bridge. The depsipeptides are unique among HDAC inhibitors in acting by a prodrug mechanism. Upon intracellular cleavage of the disulfide, one thiol, with the four-carbon spacer, is believed to act as the zinc-binding group. The role of the other thiol derived from a cysteine residue is unknown. It is possible that it is a reactive nucleophilic center or engages in other interactions with the enzyme. To shed light on this issue, we prepared analogue **15a** (Scheme 2) whereby the central cysteine was replaced by alanine, giving a monocyclic scaffold.

Scheme 2. Synthesis of Depsipeptide Analogues **15a** and **15b**[a]



[a] Reagents: (a) i. TFA; ii. Boc-D-Ala, EDAC, HOBt, i-Pr_2NEt; (b) i. TFA; ii. **4**, cat. DMAP; (c) i. LiOH; ii. MNBA, DMAP; (d) **15a**: TFA, Et_3SiH; **15b**: i. TFA, Et_3SiH; ii. AcCl, Et_3N.

While **15a** was weakly active in cell growth inhibition, it was a potent HDAC inhibitor. Furthermore, the in vitro enzyme assay did not require treatment with DTT like FK228 or **8a/b** to ensure disulfide cleavage. This strongly suggests that the cysteine thiol in the natural products is expendable and that the lack of cellular activity with **15a** is due to poor bioavailability or uptake. We then converted **15a** to the acetate **15b**. Interestingly, ester **15b** was the opposite of **15a** biologically, inhibiting cell growth but only marginally active in the HDAC assay. We believe **15b** is taken up by cells and hydrolyzed by esterases to generate **15a**, which is active intracellularly. In the enzyme assay, **15b** lacking a good zinc-binding group should be relatively inactive. A low sub-micromolar level of activity was observed, and we attribute this to the presence of impurities such as DTT in the enzyme preparation that are capable of hydrolyzing **15b** back to **15a**.

Taken together, our results indicate clear roles for the two sulfur atoms in bicyclic depsipeptide HDAC inhibitors. One functions as a zinc-binding group within the enzyme active site upon disulfide cleavage and is necessary for activity. The second thiol serves an equally important role in packaging the depsipeptides for cellular uptake. With FK228, there is mass spectrometric evidence[18] that the cysteine thiol can form glutathione conjugates or add intramolecularly in a Michael reaction to the Dhb residue. Our observations with **15** demonstrate that the cysteine can be dispensed with, provided the zinc-binding thiol is protected in a prodrug manner. In the same way, others have reported[19] simple thiol HDAC inhibitors that are active in cell assays, provided the thiol is masked as an ester.

HDAC Isoform Selectivity. Isoform selectivity is likely to be an important challenge for future HDAC inhibitors. In preliminary experiments, we examined our analogues against class I HDAC1 and class II HDAC6. Like FK228, analogues **8a/b** display similarly impressive fold selectivity between these two enzymes. Although these analogues feature conservative structural alterations, this confirms that the intrinsic discriminatory power of the depsipeptide scaffold was not disrupted. The more radical substitution in **15a** led to slightly lower HDAC1/HDAC6 selectivity. Thus, while the cysteine thiol is not needed for HDAC inhibition, its absence reduced this selectivity. As further analogues are explored in a wider panel of HDACs, compounds with new selectivity patterns will undoubtedly be discovered.

Summary. We have demonstrated the first successful and practical route toward synthetic analogues of the depsipeptide HDAC inhibitors. Our initial results elucidate the relative importance of several structural features: (1) the dehydrobutyrine side chain within FK228, or the statine within spirucho-statins as a latent precursor to an unsaturated unit, is not essential for potent HDAC inhibition; (2) excising the macrocyclic ester bond to give a linear peptide results in loss of activity, suggesting that conformational restriction of the scaffold is important; (3) the cysteine residue within depsipeptides is an ingenious solution by Nature to protect the zinc-binding thiol as a disulfide prodrug with higher bioavailability and is expendable if other means of masking the thiol are employed; (4) the other hydrophobic residues in the depsipeptide can be altered without loss of HDAC inhibitory activity; (5) analogues were identified that rival or surpass FK228 in potency as HDAC inhibitors, with similar selectivity against HDAC1 versus HDAC6.

These studies illustrate the powerful impact of total synthesis programs upon the investigation of biologically active natural products. The above conclusions would be almost impossible to derive from the natural products alone. As our investigations mature, we anticipate the discovery of second-generation unnatural depsipeptides with novel patterns of isoform selectivity. Such compounds will be valuable molecular probes of individual HDAC function, as well as potential therapeutics for indications where a global HDAC inhibitor is unsuitable.

Experimental Section

Analogue Synthesis. General. Chemicals and reagents were purchased from commercial suppliers and used without further purification. Anhydrous dichloromethane was freshly distilled from calcium hydride and tetrahydrofuran from sodium wire with benzophenone as indicator. All other anhydrous solvents were purchased as Aldrich sure-seal bottles. Analytical TLC was carried out on precoated plastic plates, normal phase Merck 60 F_{254} silica plates. Visualization was carried out either with short-wave UV or staining with aqueous potassium permanganate or phosphomolybdic acid. Column chromatography was performed using silica (Apollo, 70−230 mesh). Melting points were taken on a hot stage apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM spectrometer at 300 or 400 and 75 or 100 MHz, respectively. Chemical shifts are given in ppm. Characteristic splitting patterns due to spin spin coupling are expressed as follows: br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. All coupling constants are measured in hertz. Low-resolution mass spectra were obtained with a Micromass platform single quadrupole mass spectrometer using acetonitrile as delivery eluent. Infrared spectra were obtained using a Perkin-Elmer spectrometer with Golden-Gate attachment.

[(*R*)-2-(9*H*-Fluoren-9-ylmethoxycarbonylamino)-3-methyl-butyrylamino]-acetic Acid Methyl Ester (1). At 0 °C to a stirred solution of Fmoc-D-valine (2.70 g, 7.96 mmol) in CH_2Cl_2 (50 mL) were added EDAC·HCl (1.83 g, 9.55 mmol), HOBt (1.30 g,

9.55mmol), and i-Pr_2NEt (3.8 mL, 27.86 mmol). After the mixture was stirred for 5 min, glycine methyl ester hydrochloride (1.0 g, 7.96 mmol) was then added and the reaction mixture warmed to rt and stirred for 4 h, diluted with CH_2Cl_2 (50 mL), washed with water (25 mL), 10% HCl (25 mL), 5% $NaHCO_3$ (25 mL), and satd NaCl (25 mL) solutions, dried (Na_2SO_4), filtered, and concentrated to give an off white solid which was recrystallized from CH_3CN to give **1** as a white solid (2.81 g, 86%): mp = 148−150 °C; $[\alpha]^{22}_D$ −7.32 (*c* 0.50, $CHCl_3$); IR ν_{max} 3287, 1750, 1690, 1649, 1535 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.5 Hz, 2H), 7.57 (d, *J* = 7.0 Hz, 2H), 7.39 (t, *J* = 7.0 Hz, 2H), 7.28 (m, 2H), 6.54 (s, 1H), 5.44 (s, 1H), 4.43−4.38 (m, 2H), 4.21 (t, *J* = 7.0 Hz, 1H), 4.01−3.96 (m, 3H), 3.72 (s, 3H), 2.16 (m, 1H), 0.96 (t, *J* = 9.0 Hz, 6H); ^{13}C NMR (100 MHz, 1:1 $CDCl_3/CD_3OD$) δ 172.6, 170.0, 156.8, 143.6, 141.1, 127.5, 126.8, 124.8, 119.7, 66.7, 60.1, 51.8, 47.0, 40.7, 30.8, 18.7, 17.4; LRMS (ES+) *m/z* 842.8 (30%, $[2_M + Na]^+$), 432.9 (100%, $[M + Na]^+$).

{(*R*)-2-[(*S*)-2-(9*H*-Fluoren-9-ylmethoxycarbonylamino)-3-(tritylsulfanyl)-propionylamino]-3-methyl-butyrylamino}-acetic Acid Methyl Ester (2). To a stirred solution of **1** (1.0 g, 2.44 mmol) in CH_3CN (48.5 mL) at rt was added Et_2NH (2.5 mL). After being stirred for 3 h at rt, the reaction mixture was diluted with hexane (100 mL) and concentrated to give the crude amine as a colorless oil. At 0 °C to a stirred solution of Fmoc-D-cysteine(STrt)-OH (1.70 g, 2.9 mmol) in CH_3CN (25 mL) were added EDAC·HCl (561.0 mg, 2.93 mmol), HOBt (396 mg, 2.93 mmol), and i-Pr_2NEt (1.31 mL, 7.32 mmol). After the mixture was stirred for 5 min, the crude amine was then added, the reaction mixture warmed to rt and stirred for 4 h, solvent removed, the residue dissolved in CH_2Cl_2 (100 mL), washed with water (25 mL), 10% HCl (25 mL), 5% $NaHCO_3$ (25 mL), and satd NaCl (25 mL) solutions, dried (Na_2SO_4), and filtered, and solvent removed to give an off white solid which was recrystallized from CH_3CN to give **2** as a white solid (1.46 g, 79%): $[\alpha]^{22}_D$ −2.35 (*c* 0.50, $CHCl_3$); IR ν_{max} 3267, 1646, 1543 cm^{-1}; ^{1}H NMR 400 MHz (400 MHz, $CDCl_3$) δ 7.76 (t, *J* = 7.0 Hz, 2H), 7.54 (d, *J* = 6.5 Hz, 2H), 7.39−7.21 (m 19H), 6.87 (s, 1H), 6.23 (d, *J* = 8.0, 2H), 5.04 (d, *J* = 7.0, 1H), 4.37 (d, *J* = 7.0, 2H), 4.29 (dd, *J* = 8.6, 5.0, 1H), 4.17 (t, *J* = 6.5, 1H), 3.96 (m, 1H), 3.72 (dd, *J* = 18.1, 5.0, 1H), 3.65 (s, 3H), 3.60 (m, 1H), 2.70 (d, *J* = 6.5, 2H), 2.30 (m, 1H), 1.70 (s, 1H), 0.87 (dd, *J* = 13.0, *J* = 7.0 Hz, 6H); ^{13}C NMR (100 MHz, 2:1 $CDCl_3/CD_3OD$) δ 171.5-(C), 170.8(C), 170.1(C), 156.2(C), 144.2(C), 143.6(C), 143.5(C) 141.2(C), 129.4(CH), 128.0(CH), 127.6(CH) 127.0(CH), 126.9(CH), 124.9(CH), 119.8(CH), 67.1(CH), 67.0(CH_2), 58.2(CH), 53.9(CH), 52.0(CH_3), 46.9(CH), 40.5(CH_2), 33.6(CH_2), 30.2(CH), 18.9, 17.2-(CH_3); LRMS (ES+) *m/z* 777.8 (100%, $[M+Na]^+$); Anal. Calcd for $C_{45}H_{45}N_3O_6S$: 71.50; H, 6.00; N, 5.56. Found C, 71.42; H, 5.99; N, 5.55.

((*R*)-2-{(*S*)-2-[(*R*)-2-(9*H*-Fluoren-9-ylmethoxycarbonylamino)-propionylamino]-3-tritylsulfanylpropionylamino}-3-methyl-butyrylamino)-acetic Acid Methyl Ester (3a). To a stirred solution of **2** (900 mg, 1.19 mmol) in CH_3CN/ CH_2Cl_2 (1:1, 60 mL) at rt was added Et_2NH (3 mL). After being stirred for 3 h at rt, the reaction mixture was diluted with heptane (60 mL) and concentrated to give the crude amine as a colorless oil. At 0 °C to a stirred solution of Fmoc-D-alanine (529 mg, 1.7 mmol) in CH_2Cl_2 (30 mL) were added EDAC·HCl (326 mg, 1.7 mmol), HOBt (230 mg, 1.7 mmol), and i-Pr_2NEt (627 μL, 3.6 mmol). After the mixture was stirred for 5 min, a solution of the crude amine in CH_2Cl_2 (20 mL) was then added and the reaction mixture warmed to rt and stirred for 18 h. Then the reaction mixture was washed with water (15 mL), 10% HCl (15 mL), 5% $NaHCO_3$ (15 mL), and satd NaCl (15 mL) solutions, dried (Na_2SO_4), and filtered and the solvent removed to give an off white solid which was recrystallized from CH_3CN to give **3a** as a white solid (810 mg, 0.98 mmol, 82%): mp = 195−197 °C; $[\alpha]^{22}_D$ +5.1 (*c* 0.50, $CHCl_3$); IR ν_{max} 3267, 3054, 1744, 1706, 1635, 1531 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.5 Hz, 2H), 7.52 (d, *J* = 7.0 Hz, 2H), 7.39 (m, 7H), 7.26−7.15 (m, 13H), 6.79 (s, 1H), 6.62 (s, 1H), 5.47 (s, 1H), 4.43−4.28 (m, 4H), 4.13 (m, 1H), 4.01 (m, 1H), 3.88 (s, 2H), 3.63 (s, 3H), 2.81 (m, 1H), 2.52 (m, 1H), 2.26, (m, 1H), 1.30 (d, *J* = 6.0 Hz, 3H), 0.94 (d, *J* = 6.0 Hz, 3H), 0.89 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.7, 171.1, 170.2, 170.1, 156.1, 144.4, 143.9, 143.8, 141.4 × 2, 129.6, 128.3, 127.9, 127.2, 127.1, 125.1, 120.1, 67.3, 58.5, 52.7, 52.3, 50.8, 47.2, 41.0, 33.5, 30.4, 19.3, 19.0, 17.7; LRMS (ES+) *m/z* 849.2 (100%, $[M + Na]^+$), 865.1 (20%, $[M + K]^+$); HRMS (ES+) $C_{48}H_{50}N_4O_7SNa$ Calcd 849.3292, found 849.3286.

((*R*)-2-{(*S*)-2-[(*R*)-2-((*E*)-(*S*)-3-Hydroxy-7-tritylsulfanyl-hept-4-enoylamino)-propionylamino]-3-tritylsulfanyl-propionylamino}-3-methyl-butyrylamino)-acetic Acid Methyl Ester (5a). To a stirred solution of **3a** (760 mg, 0.92 mmol) in CH_2Cl_2/CH_3CN (3:2, 150 mL) at rt was added Et_2NH (7.5 mL). After being stirred for 5 h at rt, the reaction mixture was diluted with heptane (60 mL) and solvent removed and CH_2Cl_2 (50 mL) added, filtered, and concentrated to give the crude amine as a colorless oil. At 0 °C to a stirred solution of the crude amine in CH_2Cl_2 (30 mL) was added a solution of **4** (672 mg, 1.20 mmol, prepared according to the procedure in Yurek-George, A.; Habens, F.; Brimmell, M.; Packham, G.; Ganesan, A. *J. Am. Chem. Soc.* **2004**, *126*, 1030−1031) in CH_2Cl_2 (5 mL) and DMAP (15 mg, 0.12 mmol) at rt. After the mixture was stirred at rt for 12 h, the solvent was removed and the residue was purified by flash chromatography (eluent 10−100% EtOAc/CH_2Cl_2) to give **5a** as a white glass (740 mg, 80%): mp = 191−193 °C; $[\alpha]^{22}_D$ −18.0 (*c* 0.50, $CHCl_3$); IR ν_{max} 3272, 3064, 1758, 1692, 1621 cm^{-1}; ^{1}H NMR (400 MHz, 5% CD_3OD/ $CDCl_3$) δ 7.40 (m, 12H), 7.25 (m, 12H), 7.20 (m, 6H), 6.96 and 6.88 (labile NH, d, *J* = 8.0 Hz, 1H), 5.49 (dt, *J* = 15.0 Hz, 6.5 Hz, 1H), 5.37 (dd, *J* = 15.5 Hz, 6.0 Hz, 1H), 4.32 (m, 2H), 4.22 (d, *J* = 6.0 Hz, 1H), 3.98 (t, *J* = 7.0 Hz, 1H), 3.92 (d, *J* = 18.1 Hz, 1H), 3.72 (d, *J* = 17.6 Hz, 1H), 3.67 (s, 3H), 2.64 (dd, *J* = 13.0, 7.5 Hz, 1H), 2.58 (dd, *J* = 13.0, 7.5 Hz, 1H), 2.56−2.18 (m, 9H), 2.11 (q, *J* = 6.5 Hz, 2H), 1.31 (d, *J* = 7.5 Hz, 3H), 0.91 (t, *J* = 7.0 Hz, 6H); ^{13}C NMR (100 MHz, 9:1 $CDCl_3$/ CD_3OD) δ 173.1, 172.0, 171.4, 170.6, 170.2, 144.9, 144.3, 132.8, 129.9, 129.7, 129.5, 128.2, 128.0, 127.1, 126.7, 69.6, 67.2, 66.7, 59.0, 52.8, 49.6, 43.7, 40.9, 40.8, 33.1, 31.5, 31.3, 30.0, 19.2, 17.5 × 2; LRMS (ES+) *m/z* 1050.4 (100%, $[M + Na]^+$).

(6*R*,9*S*,12*R*,16*S*)-6-Isopropyl-12-methyl-16-((*E*)-4-tritylsulfanyl-but-1-enyl)-9-tritylsulfanylmethyl-1-oxa-4,7,10,13-tetraaza-cyclohexadecane-2,5,8,11,14-pentaone (7a). To a stirred solution of **5a** (700 mg, 0.70 mmol) in THF (15 mL) at 0 °C was added a solution of LiOH (25 mg, 1.05 mmol) in H_2O (2.4 mL). After being stirred for 1 h, the reaction mixture was diluted with H_2O (30 mL), acidified to pH 3−4 with 1 M $KHSO_4$, and extracted with EtOAc (3 × 30 mL). The organic layer was washed with satd NaCl (15 mL), dried (Na_2SO_4), filtered, and concentrated to give a white solid which was triturated with ether, to give the *seco*-hydroxy acid **6a** as a white solid (693 mg, 99%) which was used crude directly in the next step. mp = 191−193 °C; $[\alpha]^{22}_D$ −18.5 (*c* 0.50, $CHCl_3$); IR ν_{max} 3413, 1711, 1678, 1630, 1451 cm^{-1}; ^{1}H NMR (400 MHz, 5% $CD_3OD/CDCl_3$) δ 7.30 (m, 12H), 7.16 (m, 12H), 7.13 (m, 6H), 5.43 (dt, *J* = 15.5 Hz, 6.0 Hz, 1H), 5.30 (dd, *J* = 15.5 Hz, 6.0 Hz, 1H), 4.27 (m, 2H), 4.21 (m, 1H), 3.99 (m, 1H), 3.75 (s, 2H), 3.77 (m, br, 6H), 2.55 (dd, *J* = 12.5 Hz, 6.5 Hz, 1H), 2.44 (dd, *J* = 12.5 Hz, 7.5 Hz, 1H), 2.21 (m, 2H), 2.12 (m, 3H), 2.02 (m, 2H), 1.23 (d, *J* = 7.0 Hz, 3H), 0.83 (d, *J* = 7.0 Hz, 3H), 0.80 (d, *J* = 7.0 Hz, 3H); ^{13}C NMR (100 MHz, 5%$CD_3OD/CDCl_3$) δ 173.0, 172.1, 171.6, 171.5, 170.5, 144.9, 144.3, 132.7, 129.8, 129.6, 129.5, 128.2, 127.9, 127.0, 126.7, 69.5, 67.1, 66.7, 58.7, 52.7, 43.5, 40.9, 33.2, 31.5, 31.3, 30.3, 19.2, 19.1, 17.6; LRMS (ES+) *m/z* 1013.2 (100%, $[M + Na]^+$).

To a stirred solution of 2-methyl-6-nitrobenzoic anhydride (MNBA) (289 mg, 0.84 mmol) and DMAP (205 mg, 1.68 mmol) in CH_2Cl_2 (160 mL) was added dropwise a solution of crude **6a** (693 mg, 0.70 mmol) in CH_2Cl_2/THF (2:1, 600 mL) over 5 h. After a further 12 h, 1 M HCl (150 mL) was added, and the mixture was extracted with CH_2Cl_2 (3 × 100 mL). The combined organic phase was then washed with satd $NaHCO_3$ (150 mL) followed by brine (80 mL), dried ($MgSO_4$), filtered, and concentrated. The residue was purified by flash chromatography (eluent 50−100% EtOAc/hexanes) to give the depsipeptide **7a** as a white glass (478 mg,

0.49 mmol, 70%): $[\alpha]^{22}_D$ −7.0 (*c* 0.50, $CHCl_3$); IR ν_{max} 1735, 1659, 1526 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.43−7.33 (m, 12H), 7.32−7.13 (m, 20H), 6.77 (d, J = 5.3 Hz, 1H), 6.54 (d, J = 7.3 Hz, 1H), 5.58 (dt, J = 15.1, 6.8 Hz, 1H), 5.44−5.29 (m, 2H), 4.55 (dd, J = 16.8, 9.0 Hz, 1H), 4.46 (dd, J = 9.5, 4.3 Hz, 1H), 3.89 (quin, (dd, J = 7.0 Hz, 1H), 3.77 (dt, J = 8.3, 5.3 Hz, 1H), 3.43 (dd, J = 16.8, 2.8 Hz, 1H), 3.03 (dd, J = 12.6, 8.3 Hz, 1H), 2.61−2.49 (m, 3H), 2.18 (t, J = 7.5 Hz, 2H), 2.11−1.95 (m, 2H), 1.38 (d, J = 7.0 Hz, 3H), 0.94 (d, J = 6.8 Hz, 3H), 0.90 (d, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.0, 170.9, 170.8, 169.8, 169.3, 144.9, 144.3, 133.2, 129.7, 129.5, 128.3, 128.0, 127.2, 126.8, 72.3, 67.3, 66.8, 58.3, 55.9, 50.5, 41.7 × 2, 32.5, 31.3, 31.2, 28.8, 19.8, 19.8, 17.2, 16.7; LRMS (ES+) *m*/*z* 996 (100%, $[M + Na]^+$), 974 (10%, $[M + H]^+$); HRMS (ES+) $C_{58}H_{60}N_4O_6S_2Na$ Calcd 995.3846, found 995.3860.

(*E*)-(1*S*,7*R*,10*S*,21*R*)-7-Isopropyl-21-methyl-2-oxa-12,13-dithia-5,8,20,23-tetraaza-bicyclo[8.7.6]tricos-16-ene-3,6,9,19,22-pentaone (8a). To a vigorously stirring solution of I_2 (1120 mg, 4.42 mmol) in CH_2Cl_2/MeOH (9:1, 1000 mL) was added **7a** (430 mg, 0.44 mmol) in CH_2Cl_2/MeOH (9:1, 500 mL) dropwise over 30 min. After the mixture was stirred for a further 30 min, 0.1 M sodium thiosulfate (300 mL) and satd NaCl (100 mL) were added, and the mixture was extracted with EtOAc (3 × 100 mL). The combined organic extract was dried ($MgSO_4$) and filtered and solvent removed. The residue was purified by flash chromatography (eluent 1−6% MeOH/CH_2Cl_2) to give **8a** (205 mg, 0.42 mmol, 96%) as a white solid. A small amount was recrystallized from CH_3CN to give crystalline colorless blocks: mp >235 °C dec; $[\alpha]^{22}_D$ −98.0 (*c* 0.50, $CHCl_3$); IR ν_{max} 3300, 2477, 1734, 1659, 1526, 1446 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.46 (d, J = 6.5 Hz, 1H), 7.33 (t, J = 5.3 Hz, 1H), 7.28 (d, J = 8.8 Hz, 1H), 6.60 (d, J = 3.8 Hz, 1H), 5.95 (dtd, J = 16.1, 6.5, 2.0 Hz, 1H), 5.80−5.70 (m, 2H), 4.85 (ddd, J = 9.8, 8.5, 3.8 Hz, 1H), 4.21 (qd, J = 7.3, 3.8 Hz, 1H), 4.11 (d, J = 5.3 Hz, 2H), 3.44 (dd, J = 15.6, 10.0 Hz, 1H), 3.20 (dd, J = 10.3, 6.8 Hz, 1H), 3.01−2.95 (m, 2H), 2.92 (dd, J = 15.6, 4.0 Hz, 1H), 2.85−2.58 (m, 5H), 1.49 (d, J = 7.5 Hz, 3H), 0.98 (d, J = 6.5 Hz, 3H), 0.92 (d, J = 6.5 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.3, 171.6, 171.0, 169.4, 168.2, 130.4, 130.3, 69.8, 64.9, 54.5, 52.0, 42.4 × 2, 38.7, 38.6, 32.7, 27.5, 20.8, 20.1, 16.7; LRMS (ES+) *m*/*z* 995 (50%, $[2M + Na]^+$), 509 (80%, $[M + Na]^+$), 487 (100%, $[M + H]^+$); HRMS (ES+) $C_{20}H_{30}N_4O_6S_2Na$ Calcd 509.1499, found 509.1511.

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Supporting Information Available: Synthesis procedures for analogues **8b**, **9**, **15a**, and **15b**, NMR spectra and HRMS data for all novel compounds, and experimental protocols for biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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