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# Total synthesis of largazole and analogues: HDAC inhibition, antiproliferative activity and metabolic stability

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ABSTRACT

The total synthesis of largazole and four analogues is reported. All analogues were nanomolar HDAC inhibitors. The antiproliferative activity is driven by lipophilicity and cell permeability. In murine liver homogenates, largazole is rapidly metabolized (half-life $\leqslant$5 min) to the thiol which has a half-life of 51 min.



## 1. Introduction

The N-terminal tails of eukaryotic histone proteins are rich in post-translational modifications such as acetylation, methylation, phosphorylation, ADP-ribosylation, sumoylation and ubiquitination. These dynamic modifications represent a 'histone code' that other proteins can read, write, erase, and modify with functional consequences including chromatin remodeling, transcriptional activation or silencing.[1] Consequently, small molecule inhibitors of histone modifying enzymes are important tools for chemical biology and leads for drug discovery against diseases with mistimed gene transcription or repression.[2] Many of the inhibitors are natural products, a testament to the success of such compounds in possessing unique biological activity and exploring chemical space that is poorly populated by synthetic compounds.[3]

Among the histone modifying enzymes, histone deacetylases (HDACs) catalyze the hydrolysis of acetyllysine residues to lysine. The resulting change in shape, size and charge at physiological pH is a powerful mechanism for cell signaling occurring not only in histones but a large number of nuclear and cytoplasmic proteins.[4] There are 18 human HDACs subdivided into classes according to sequence homology, cellular localization and enzyme mechanism.[5] Classes I (HDACs 1, 2, 3 and 8), II (HDACs 4, 6, 7, 9 and 10) and IV (HDAC 11) hydrolyze the amide bond using a catalytic zinc ion at the active site while class III (sirtuins SIRT1-7) HDACs are metal-free and employ $NAD^+$ as cofactor.

Many synthetic hydroxamic acids inhibit the zinc-dependent HDACs by reversibly binding the metal. Such compounds show promising activity in both in vitro and in vivo disease models. Several examples have reached clinical development with SAHA (marketed by Merck as Zolinza) becoming the first HDAC inhibitor to receive FDA approval for the treatment of cutaneous T-cell lymphoma.[6] The second FDA approved HDAC inhibitor FK228 (marketed by Celgene as Istodax) is very different in structure and comes from a much smaller class of bicyclic depispeptide bacterial natural products comprising FK228, the spiruchostatins and FR901,375 isolated from fermentation extracts of *Chromobacterium violaceum* and *Pseudomonas* sp. (Fig. 1). These natural products are prodrugs that undergo intracellular disulfide reduction to release a zinc-binding thiol at the HDAC active site. Compared to the hydroxamic acid inhibitors, the depsipeptide macrocyclic scaffold provides additional binding interactions to zinc chelation resulting in higher potency and selectivity between HDAC isoforms. We[7] and others[8] have extensively published on total syntheses of the bicyclic depsipeptides and unnatural analogues thereof.

In 2008, the depsipeptide largazole with high antiproliferative activity was isolated from a marine cyanobacterium of the genus *Symploca*.[9] Although the original report claimed 'the 3-hydroxy-7-mercaptohept-4-enoic acid unit is unprecedented in natural

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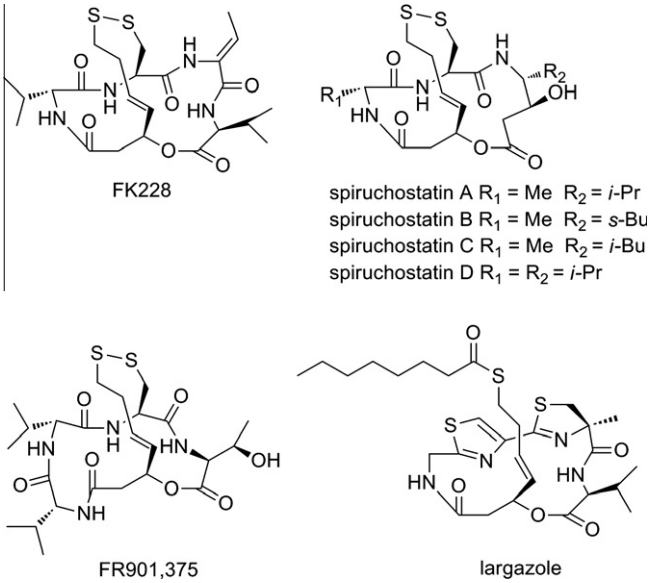



**Figure 1.** Depsipeptide natural product HDAC inhibitors with thiol zinc-binding groups.

**Table 1**
Inhibition of total HDACs in HeLa nuclear cell extracts by SAHA and depsipeptide natural products

| Compound | HDAC inhibition[a] (nM) |
|---|---|
| SAHA | 288 ± 59 |
| FK228 (dithiol) | 24.4 ± 4.0 |
| spiruchostatin A (dithiol) | 5.3 ± 3.3 |
| largazole thiol | 0.043 ± 0.026 |

[a] Data obtained with the Flour-de-Lys assay in our laboratory.

products', this fragment is identical to the zinc-binding thiol in the bicyclic depsipeptides. This suggests that largazole too is a HDAC inhibitor. Indeed, our work on synthetic FK228 analogues had demonstrated that the disulfide linkage can be replaced by an ester which is cleavable intracellularly to release the active thiol.[7d] The largazole hypothesis was soon confirmed and largazole thiol shown to be a potent HDAC inhibitor.[10] Since then, a steady stream of largazole total and analogue syntheses has appeared.[11] There are two main reasons for this burst of activity in a natural product reported only two years ago. Firstly, the pioneering efforts in the synthesis of bicyclic depsipeptide HDAC inhibitors and thiazole/thiazoline containing natural products enabled retrosynthetic routes to be planned with high confidence and successfully executed.[12] Secondly, largazole thiol is an exciting lead as it is a more potent HDAC inhibitor than the bicyclic depsipeptides (Table 1). Here, we report our efforts directed towards largazole and structural analogues and the first pharmacokinetic evaluation of such compounds in terms of metabolic stability.

## 2. Results and discussion

Our initial objective was to complete a total synthesis of largazole to obtain a sample of the natural product and verify the validity of the synthesis route. Once completed, this would then lead to the more illuminating preparation of unnatural analogues. A comparison of the depsipeptides reveals a glycine residue in largazole that corresponds to alanine or valine in FK228 and the spiruchostatins. Largazole analogues with glycine replacements had not been previously explored and we were interested in how analogue **1** (Fig. 2) with a phenylalanine side chain would compare in terms of enzyme and cell growth inhibition.

Further analogues **2**–**4** had the goal of molecular simplification and reduction of molecular weight of largazole. Analogue **2** replaces the valine residue in largazole by the simpler glycine. We had shown[7d] that FK228 analogues with an identical valine to glycine substitution retained potent HDAC and cancer cell growth inhibition. In analogue **3**, the valine residue was replaced by β-alanine, increasing the size of the macrocycle by one atom. Despite the extensive studies with the bicyclic depsipeptides and largazole, such ring expanded analogues were unknown and the effect of this alteration on biological activity could not be predicted. Our next analogue **4** probes the influence of the thiazoline ring in largazole. Both epimers of the thiazoline are HDAC inhibitors as reported by Williams and co-workers[11i] while FK228 contains an achiral dehydrobutyrine instead. These observations suggest that this region of the macrocycle is stereochemically tolerant. Instead of the relatively complex thiazoline or dehydrobutyrine residues in the natural products, analogue **4** contains a structurally simpler achiral α-aminoisobutyric acid (Aib) residue.

### 2.1. Total synthesis of largazole and analogues

The total synthesis of depsipeptide HDAC inhibitors can be broken down to a synthesis of the zinc-binding 3-hydroxy-7-mercaptohept-4-enoic acid unit in enantiopure form, assembly of a linear peptide or depsipeptide with appropriate protecting groups, and macrocylization. We have routinely made the 3-hydroxy-7-mercaptohept-4-enoic acid by an enantioselective aldol reaction directed by the Fujita-Nagao auxiliary[7a] while our experiences[7f] in FK228 total synthesis suggested macrolactamization rather than macrolactonization for ring formation.

Our total synthesis of largazole (Scheme 1) began with the Hantzsch cyclization of Boc-protected glycine **5** (Scheme 1) and methyl bromopyruvate. Conversion of the ester to the nitrile gave thiazole **13**, an intermediate in previous largazole total syntheses.[10,11] In parallel, analogous chemistry starting with phenylalanine led to thiazole **14**. Condensation with (*R*)-methylcysteine[13] hydrochloride salt under Cramer's buffered conditions[11e] provided the thiazole–thiazoline fragments **15** and **16**. These were coupled with amine **17**, an intermediate in our FK228 total synthesis.[7f] The linear depsipeptides were then subjected to deprotection at the N- and C-terminus followed by macrolactamization and thiol deprotection to afford largazole thiol and analogue **1**, respectively. Separately, largazole thiol was acylated to complete a total synthesis of largazole. Overall, largazole was obtained in 0.24% yield in the longest linear sequence.

The synthesis of analogues **2**–**4** was achieved by the same route. The union of glycine and β-alanine versions of amine **17** with thiazole–thiazoline fragment **15** (Scheme 2) followed by macrocyclization afforded analogues **2** and **3**. The synthesis of analogue **4** proceeded via the coupling of thiazole **9** with α-aminoisobutyric acid to give **28**, which was carried forward as in the earlier syntheses (Scheme 3).

### 2.2. HDAC inhibition and antiproliferative activity

We confirmed that our synthetic largazole inhibits HDAC activity in cells by monitoring histone H4K8(ac) accumulation in three cancer cell lines (Fig. 3). As expected, largazole thiol was relatively inactive due to its poor cell permeability.

Largazole and its free thiol were assayed for inhibition of HDAC enzyme activity and growth of the MCF7 breast cancer cell line (Table 2). Largazole thiol was a highly potent picomolar HDAC

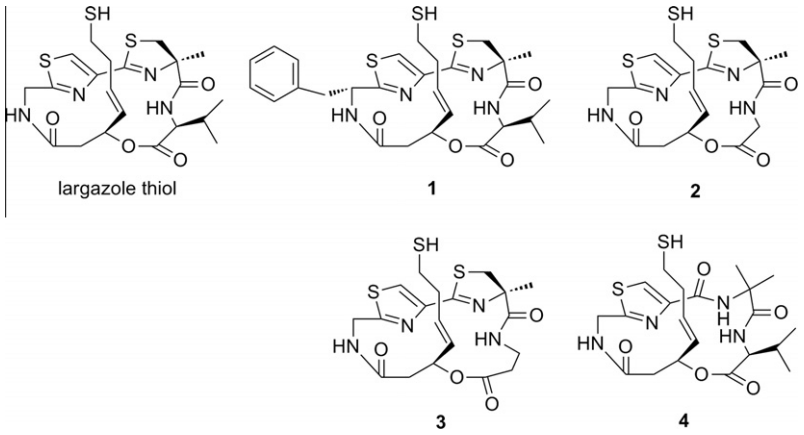
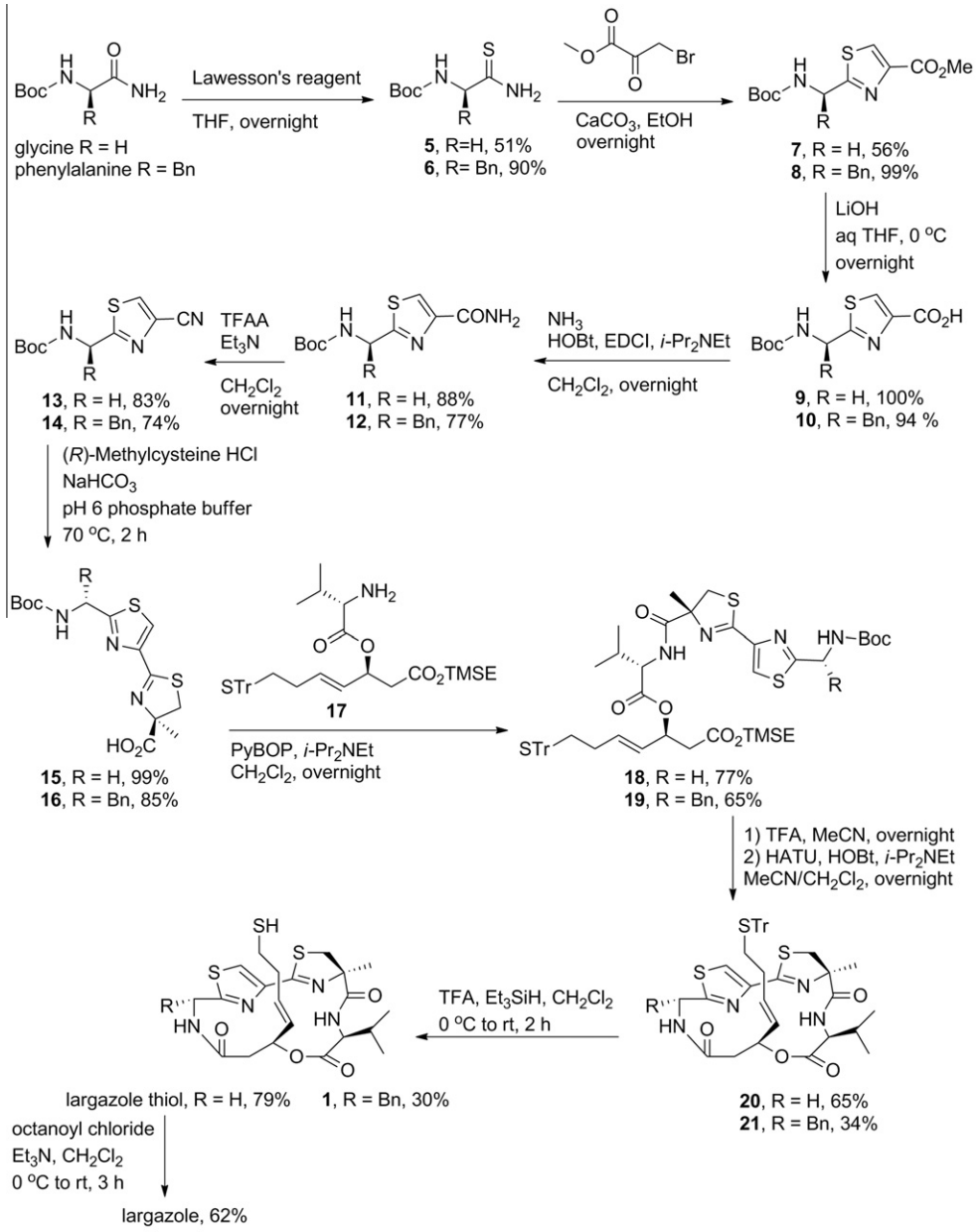
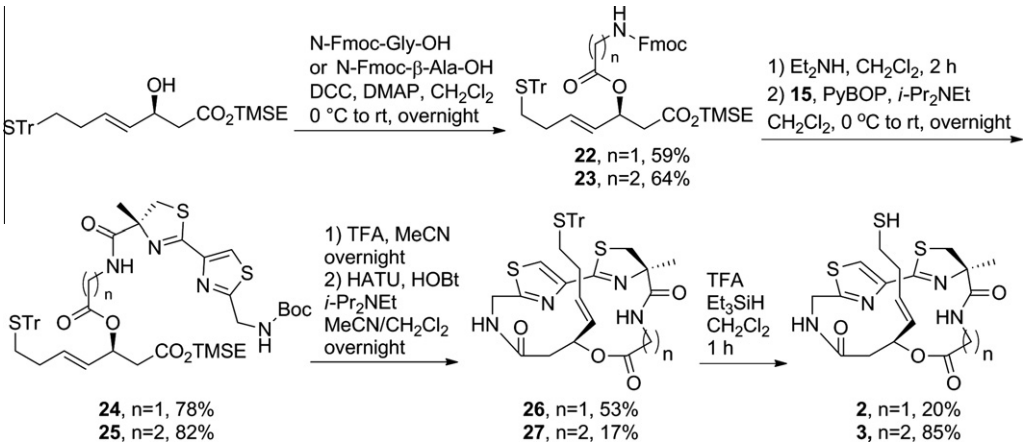


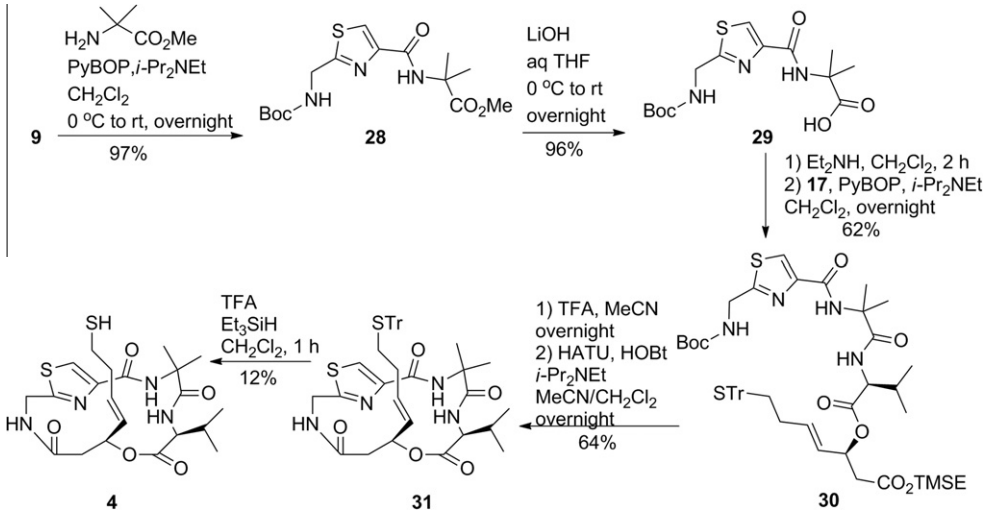

**Figure 2.** Structures of largazole thiol and synthetic analogues **1**–**4**.




**Scheme 1.** Total synthesis of largazole and benzyl analogue **1**.




**Scheme 2.** Synthesis of largazole analogues **2** and **3**.




**Scheme 3.** Synthesis of largazole analogue **4**.

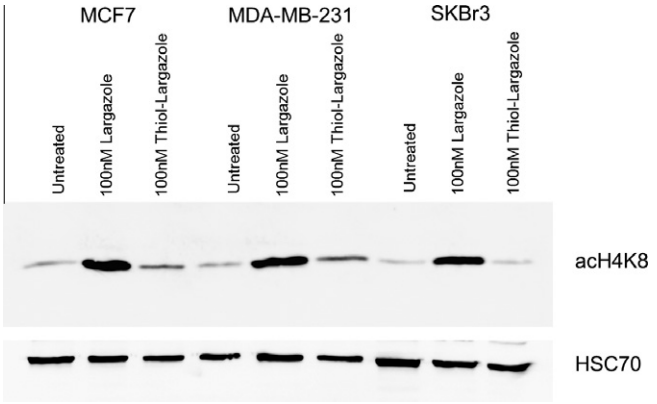



**Figure 3.** Western blotting of H4K8(ac) in MCF7, MDA-MB-231 and SKBr3 cell lines with DMSO control, largazole and largazole thiol at 100 nM.

inhibitor with an approximately 10,000 fold reduction in cellular activity as first reported by Luesch and co-workers.[9,10] Conversely, the natural product largazole had potent antiproliferative activity and some enzyme inhibition that we attribute to chemical hydrolysis back to largazole thiol under the assay conditions.

**Table 2**
HDAC and MCF7 growth inhibition by largazole, largazole thiol and analogues **1**–**4**

| Compound | HDAC inhibition (nM) | MCF7 inhibition (nM) |
|---|---|---|
| Largazole | 572 ± 29 | 5 ± 1 |
| Largazole thiol | 0.043 ± 0.026 | 277 ± 130 |
| **1** | 17.2 ± 2.3 | 377 ± 62 |
| **2** | 0.17 ± 0.05 | 2458 ± 1135 |
| **3** | 3.15 ± 0.35 | >10,000 |
| **4** | 0.99 ± 0.07 | 5902 ± 1698 |

The novel analogue **1** was a significantly weaker HDAC inhibitor than largazole thiol. Nevertheless, in the growth inhibition assay the two compounds had similar activity. This interesting result shows that enzyme and cell activity are not always correlated in the largazole series. It is tempting to suggest that the unexpectedly high cell activity of **1** is due to the introduction of a lipophilic group that aids passive membrane absorption.

Analogue **2** is a subnanomolar HDAC inhibitor indicating that a valine to glycine substitution at this position is not detrimental, matching the result we observed with FK228. Increasing the macrocyle ring size in analogue **3** was also tolerated, leading to a 20-fold decrease in HDAC activity compared to **2** but still providing a single digit nanomolar inhibitor. However, both **2** and **3** in particular are poor in growth inhibition compared to largazole. The Aib containing analogue **4** shows the same pattern-nanomolar HDAC

inhibition while much weaker in growth inhibition. Taken together, these results indicate that molecular simplification of the largazole scaffold is possible without losing HDAC inhibitory activity. As analogues **2**–**4** have a reduced lipophilicity compared to largazole thiol or **1**, the decreased growth inhibitory activity may reflect poorer cell uptake.

### 2.3. Metabolic stability

The therapeutic potential of largazole and related analogues will be determined not only by potency or selectivity but equally by pharmacokinetic properties. Surprisingly, no discussion of the latter is present in the extensive literature on largazole.[9–11] We have investigated the stability of largazole and our analogues in the presence of mouse liver homogenate to determine their susceptibility to metabolism.

Largazole itself was highly unstable with a half-life $\leqslant$5 min. The metabolism consists of thioester hydrolysis to largazole thiol and reveals the potential disadvantage of ester prodrug thiol HDAC inhibitors compared to the bicyclic disulfides such as the clinically approved FK228. For in vivo applications, careful choice of the ester group to slow down hydrolysis will be necessary. Once the ester is cleaved, largazole thiol is relatively stable in murine liver homogenate with a half-life of 51 min at 37 °C. Analogue **2** was similar in stability with a half-life of 32 min. In both cases, one major metabolite of unknown structure was observed. Analogues **3**–**4** had a complex and rapid metabolism with a half-life $\leqslant$5 min.

## 3. Conclusions

We report the total synthesis of largazole and four novel largazole analogues. Although the analogues are less active than largazole thiol itself as HDAC inhibitors, they shed further light on the SAR of this natural product. The extraordinary potency of largazole thiol in enzyme assays is not matched in cell growth inhibitory activity, whether as the free thiol or as the ester natural product. In cell assays the bicyclic depsipeptide natural products are superior despite being significantly poorer in HDAC inhibition. These results imply that largazole has some liabilities related to cell uptake, stability, nonspecific binding or other aspects that lead to its reduced activity in cell-based assays.

Our unnatural analogues **1**–**4** show that the glycine, valine and thiazoline residues in largazole can all be replaced while retaining nanomolar HDAC inhibition. Nevertheless, the analogues are less potent compared to largazole thiol and indicate that the SAR of this natural product is more subtle and less predictable than FK228. Meanwhile, the cell growth inhibition appears to track with lipophilicity. Analogue **1**, the weakest HDAC inhibitor in the series, had the best cell growth inhibition and this is likely due to its increased lipophilicity. Analogues **2**–**4**, while simpler in structure than largazole thiol, are more polar and this has a deleterious effect on growth inhibition.

Largazole was found to be rapidly degraded by mouse liver homogenate to the thiol, which in turn had a half-life of 51 min at 37 °C. Our results highlight the importance of cell activity and ADMET properties such as metabolism rather than in vitro enzyme inhibition in the further development of largazole as a therapeutic lead.

## 4. Experimental

### 4.1. Compound synthesis

#### 4.1.1. (*R*)-*tert*-Butyl 1-(4-carbamoylthiazol-2-yl)-2-phenylethylcarbamate (12)

HOBt (1.41 g, 9.82 mmol, 3 equiv), EDCI (1.89 g, 9.82 mmol, 3 equiv) and Hünig's base (3 mL, 13.09 mmol, 4 equiv) were added to a solution of **10** (1.14 g, 3.27 mmol, 1 equiv) in anhydrous $CH_2Cl_2$ (70 mL). At 0 °C, ammonia solution (0.5 M in dioxane) (65 mL, 32.74 mmol, 10 equiv) was added slowly. The solution was stirred overnight under argon. The aqueous phase was extracted with $CH_2Cl_2$ (3 × 100 mL) and washed with brine (2 × 100 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated. Flash chromatography ($CH_2Cl_2$/MeOH 95:5) afforded **12** as a salmon solid (884 mg, 77%): mp 48–52 °C; $[\alpha]_D^{25}$ +12.0 (*c* 0.26, $CHCl_3$); IR 3300, 1675, 1520, 1368, 1168 $cm^{-1}$; $^1H$ NMR (300 MHz, MeOD) $\delta$ 7.30–7.18 (m, 5H), 4.29 (dd, *J* = 9.0, 5.7 1H), 3.11 (dd, *J* = 13.7, 5.3 Hz, 1H), 2.81 (dd, *J* = 13.7, 9.3 Hz, 1H), 1.36 (s, 9H); $^{13}C$ NMR (75 MHz, MeOD) $\delta$ 177.2, 157.6, 138.7, 130.4, 129.4, 127.7, 80.6, 57.1, 39.4, 28.7; $ES^+$ MS *m/z* 370 ($[M+Na]^+$); HRMS (ESI) *m/z* calcd. for $C_{17}H_{21}N_3Na_1O_3S_1$ $(M+Na)^+$ 370.1196, found 370.1195.

#### 4.1.2. (*R*)-*tert*-Butyl 1-(4-cyanothiazol-2-yl)-2-phenylethylcarbamate (14)

Triethylamine (0.63 mL, 4.54 mmol, 2 equiv) and trifluoroacetic anhydride (0.34 mL, 2.49 mmol, 1.1 equiv) were added to a solution of **12** (789 mg, 2.27 mmol, 1 equiv) in anhydrous $CH_2Cl_2$ (40 mL) at 0 °C under argon. The solution was stirred overnight and the solvent removed. The residue was purified by flash chromatography (hexane/EtOAc 8:2) to give **14** as a white solid (593 mg, 74%): mp 126–130 °C; $[\alpha]_D^{25}$ +1.6 (*c* 0.24, $CH_3OH$); IR 2979, 1705, 1508, 1365, 1255, 1164, 703 $cm^{-1}$; $^1H$ NMR (300 MHz, MeOD) $\delta$ 8.37 (s, 1H), 7.31–7.21 (m, 5H), 5.12 (dd, *J* = 10.2, 4.9 Hz, 1H), 3.43 (dd, *J* = 13.9, 5.3 Hz, 1H), 3.04 (dd, *J* = 13.6, 10.2 Hz, 1H), 1.35 (s, 9H); $^{13}C$ NMR (75 MHz, $CDCl_3$) $\delta$ 174.4, 154.8, 135.6, 130.3, 129.2, 128.7, 127.1, 126.5, 113.8, 80.6, 53.7, 41.0, 28.1; $ES^+$ MS *m/z* 352 ($[M+Na]^+$), 681 ($[2\ M+Na]^+$); HRMS (ESI) *m/z* calcd. for $C_{17}H_{19}N_3Na_1O_2S_1$ $(M+Na)^+$ 352.1090, found 352.1094.

#### 4.1.3. (*R*)-2-(2-((*R*)-1-(*tert*-Butoxycarbonylamino)-2-phenyl ethyl) thiazol-4-yl)-4-methyl-4,5-dihydrothiazole-4-carboxylic acid (16)

To a solution of **14** (329 mg, 1.04 mmol, 1 equiv) and (*R*)-methylcysteine hydrochloride (338 mg, 1.96 mmol, 1.9 equiv) in anhydrous methanol (20 mL), were added $NaHCO_3$ (444 mg, 5.24 mmol, 5 equiv) and 6.46 mL of a solution of phosphate buffer (pH 6). The reaction mixture was degassed by bubbling briefly argon through it and stirred at 70 °C for 2 h. To the cold reaction mixture were added water (20 mL) and saturated $NaHCO_3$ solution (20 mL). The solution was washed with EtOAc (3 × 20 mL). The aqueous layer was acidified to pH 1–2 with a solution of saturated $KHSO_4$ and extracted with EtOAc (3 × 20 mL). The combined organic layers were washed with brine (4 × 50 mL), dried over $MgSO_4$, concentrated and purified by flash chromatography ($CH_2Cl_2$/MeOH/AcOH 94:5:1) to give **16** as a white solid (399 mg, 85%): mp 60–70 °C. $[\alpha]_D^{25}$ −13.8 (*c* 0.25, $CH_3OH$); IR 2363, 2336, 1709, 1527, 673 $cm^{-1}$; $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$ 7.98 (s, 1H), 7.31–7.12 (m, 5H), 5.31–5.24 (m, 2H), 3.90 (d, *J* = 11.7 Hz, 1H), 3.39 (d, *J* = 11.7 Hz, 1H), 3.32 (d, *J* = 6.2 Hz, 2H), 1.70 (s, 3H), 1.41 (s, 9H); $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$ 175.6, 172.6, 165.0, 155.0, 147.9, 136.1, 129.4, 128.5, 126.9, 122.0, 84.2, 80.3, 53.7, 41.2, 40.9, 28.2, 24.1; $ES^+$ MS *m/z* 470 ($[M+Na]^+$).

#### 4.1.4. (*S*,*E*)-2-(Trimethylsilyl)ethyl 3-((*S*)-2-((*R*)-2-(2-((*R*)-1-(*tert*-butoxycarbonylamino)-2-phenylethyl)thiazol-4-yl)-4-methyl-4,5-dihydrothiazole-4-carboxamido)-3-methylbutanoyl-oxy)-7-(tritylthio)hept-4-enoate (19)

To a suspension of **16** (36 mg, 0.08 mmol, 1.1 equiv) and **17** (obtained by deprotection of 63 mg, 0.07 mmol, 1 equiv of the Fmoc-carbamate) in anhydrous $CH_2Cl_2$ (5 mL) and anhydrous acetonitrile (2 mL) was added PyBOP (44 mg, 0.08 mmol, 1.1 equiv) at

0 °C and Hünig's base (32 μL, 0.18 mmol, 2.5 equiv). The solution was warmed to rt and stirred overnight. The solvent was then removed and flash chromatography (hexane/EtOAc 8:2) gave **19** as an oil (55 mg, 65%): $[\alpha]_D^{25}$ −24.4 (*c* 0.25, $CHCl_3$); IR 2359, 2340, 1731, 1504, 1168, 673 $cm^{-1}$; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 7.82 (s, 1H), 7.36–7.11 (m, 20H), 5.68–5.59 (m, 2H), 5.35 (dd, *J* = 15.4, 7.5 Hz, 1H), 5.26 (br s, 1H), 5.15 (br s, 1H), 4.46 (dd, *J* = 9.0, 4.6 Hz, 1H), 4.12 (m, 2H), 3.76 (dd, *J* = 11.4, 8.1 Hz), 3.31–3.28 (m, 3H), 2.66 (dd, *J* = 15.6, 7.7 Hz, 1H), 2.53 (dd, *J* = 15.6, 5.65 Hz, 1H), 2.16–2.10 (m, 3H), 2.03 (q, *J* = 6.7 Hz, 2H), 1.57 (s, 3H), 1.38 (s, 9H), 0.94 (m, 2H), 0.81 (d, *J* = 6.9 Hz, 3H), 0.72 (d, *J* = 6.8 Hz, 3H), 0.00 (s, 9H); $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$ 174.4, 170.3, 169.5, 163.4, 154.9, 148.8, 144.7, 136.1, 133.8, 133.8, 129.5, 128.5, 127.8, 126.9, 126.5, 120.6, 85.1, 80.2, 71.6, 66.5, 63.0, 56.7, 53.6, 41.3, 41.1, 39.6, 31.2, 31.1, 31.0, 28.2, 24.7, 19.0, 17.3, 17.2, −1.5; $ES^+$ MS *m/z* 1069 ($[M+Na]^+$).

#### 4.1.5. (5*R*,8*S*,11*S*)-8-Isopropyl-5-methyl-11(2-4-tritylsulfanyl-but-1-enyl)-10-oxa-3,17-dithia-7,14,19,20-tetraaza-tricyclo [14.2.1.1∗2,5∗]icosa-1(18),2(20),16(19)-triene-6,9,13-trione (21)

Acyclic precursor **19** (58 mg, 0.05 mmol, 1 equiv) was dissolved in $CH_2Cl_2$ (2.5 mL), cooled to 0 °C and treated with TFA (0.11 mL). The reaction mixture was warmed to rt, stirred overnight, the solvents evaporated and the crude amino acid coevaporated with toluene to remove residual TFA. The crude amino acid was taken up in $CH_2Cl_2$ (2.5 mL) and added dropwise to a stirred solution of $i$Pr$_2$NEt (0.05 mL, 0.30 mmol, 6 equiv) in dry acetonitrile (50 mL). The mixture was stirred for 10 min and a solution of HATU (40 mg, 0.10 mmol, 2 equiv) and HOBt (12 mg, 0.10 mmol, 2 equiv) in acetonitrile (2.5 mL) was added dropwise. The solution was stirred overnight. The solvent was then removed and flash chromatography ($CH_2Cl_2$/MeOH 99:1) gave **21** as an oil (14 mg, 34%): $[\alpha]_D^{25}$ −31.3 (*c* 0.37, $CHCl_3$); $^1H$ NMR (400 MHz, $CDCl_3$) of the major compound $\delta$ 7.80 (s, 1H), 7.42–7.18 (m, 20H), 7.10 (d, *J* = 9.1 Hz, 1H), 5.78–5.70 (m, 1H), 5.67–5.64 (m, 1H), 5.58–5.54 (m, 1H), 5.34 (dd, *J* = 15.5, 7.1 Hz, 1H), 4.57 (dd, *J* = 3.2, 9.2 Hz, 1H), 4.09 (d, *J* = 11.5 Hz, 1H), 3.30 (d, *J* = 11.6 Hz, 1H), 3.23 (d, *J* = 7.3 Hz, 1H), 3.17 (d, *J* = 7.3 Hz, 1H), 2.67–2.54 (m, 2H), 2.18–2.16 (m, 1H), 2.09–2.05 (m, 4H), 1.89 (s, 3H), 0.63 (d, *J* = 6.8 Hz, 3H), 0.46 (d, *J* = 6.9 Hz, 3H); $^{13}C$ NMR (100 MHz, $CDCl_3$) of the major compound $\delta$ 173.7, 171.6, 170.3, 169.3, 169.1, 157.0, 145.2, 135.8, 133.6, 130.0, 129.8, 129.2, 128.9, 128.2, 128.1, 127.9, 127.0, 84.9, 72.6, 67.0, 58.0, 52.9, 43.5, 41.9, 40.8, 34.5, 31.8, 31.5, 24.3, 19.2, 16.8; $ES^+$ MS *m/z* 851 ($[M+Na]^+$); HRMS (ESI) *m/z* calcd. for $C_{47}H_{48}N_4Na_1O_4S_3$ $(M+Na)^+$ 851.2730, found 851.2730.

#### 4.1.6. (5*R*,8*S*,11*S*)-8-Isopropyl-11-(2-4-mercapto-but-1-enyl)-5-methyl-10-oxa-3,17-dithia-7,14,19,20-tetraaza-tricyclo[14.2.1.1∗.2,5∗]icosa-1(18),2(20),16(19)-triene-6,9,13-trione (1)

At 0 °C, under argon, trifluoroacetic acid (0.05 mL, 0.64 mmol, 64 equiv) and triisopropylsilane (7 μL, 0.03 mmol, 3 equiv) were added to a solution of **21** (14 mg, 0.01 mmol, 1 equiv) in $CH_2Cl_2$ (1.5 mL). The solution was warmed to rt and stirred for 4 h. The solvent was then removed and the compound was purified by flash chromatography (hexane/EtOAc 7:3) to give **1** as a yellowish oil (3 mg, 30%): $[\alpha]_D^{25}$ −31.6 (*c* 0.13, $CHCl_3$); $^1H$ NMR (400 MHz, $CD_3OD$) of the major compound $\delta$ 8.14 (s, 1H), 7.33–7.23 (m, 5H), 5.82–5.75 (m, 1H), 5.62–5.54 (m, 3H), 4.50–4.47 (m, 1H), 3.94 (d, *J* = 11.6 Hz, 1H), 3.40 (d, *J* = 11.5 Hz, 1H), 3.20–3.17 (m, 1H), 3.04–2.97 (m, 1H), 2.55–2.52 (m, 4H), 2.36–2.31 (m, 2H), 2.09–2.03 (m, 1H), 1.82 (s, 3H), 0.67 (d, *J* = 6.9 Hz, 3H), 0.44 (d, *J* = 6.9 Hz, 3H); $^{13}C$ NMR (100 MHz, $CDCl_3$) of the major compound $\delta$ 173.1, 171.7, 169.4, 168.2, 167.8, 135.4, 132.8, 129.5, 128.9, 128.5, 127.6, 127.2, 73.3, 58.0, 52.6, 42.9, 41.6, 36.3, 33.8, 29.7, 23.8, 22.7, 18.6, 16.6; $ES^+$ MS *m/z* 609 ($[M+Na]^+$); HRMS (ESI) *m/z* calcd. for $C_{28}H_{34}N_4Na_1O_4S_3$ $(M+Na)^+$ 609.1634, found 609.1624.

#### 4.1.7. (*S*,*E*)-2-(Trimethylsilyl)ethyl 3-(2-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)acetoxy)-7-(tritylthio)hept-4-enoate (22)

The protected β-hydroxy ester (200 mg, 0.39 mmol, 1 equiv) and *N*-Fmoc-glycine (126 mg, 0.42 mmol, 1.1 equiv) were dissolved in anhydrous $CH_2Cl_2$ (7 mL). DCC (96 mg, 0.46 mmol, 1.2 equiv) and DMAP (4.7 mg, 0.04 mmol, 0.1 equiv) were added to the reaction mixture cooled to 0 °C. The reaction mixture was stirred overnight. The precipitate was filtered and solvents were evaporated. The crude was purified by flash chromatography (EtOAc/hexane 5:95) to give **22** as a colorless oil (180 mg, 59%): $[\alpha]_D^{23}$ −9.9 (*c* 0.54, $CHCl_3$); IR 3368, 2964, 2918, 2843, 1735, 1520, 1448, 1247, 1172, 1051, 858, 839, 733, 692 $cm^{-1}$; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 7.78 (d, *J* = 7.5 Hz, 2H), 7.60 (d, *J* = 7.0 Hz, 2H), 7.15–7.46 (m, 19H), 5.57–5.76 (m, 2H), 5.39 (dd, *J* = 15.6, 7.0 Hz, 1H), 5.24 (br s, 1H), 4.39 (d, *J* = 7.0 Hz, 2H), 4.24 (t, *J* = 7.0 Hz, 1H), 4.16 (t, *J* = 8.5 Hz, 2H), 3.96 (d, *J* = 5.5 Hz, 2H), 2.67 (dd, *J* = 15.8, 8.3 Hz, 1H), 2.55 (dd, *J* = 15.8, 5.3 Hz, 1H), 2.20 (t, *J* = 7.5 Hz, 2H), 2.08 (t, *J* = 7.0 Hz, 2H), 0.97 (t, *J* = 8.5 Hz, 2H), 0.04 (s, 9H); $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$ 169.7, 168.9, 156.1, 144.8, 143.8, 141.3, 133.6, 129.6, 127.9, 127.7, 127.6, 127.1, 126.6, 125.1, 120.0, 71.9, 67.2, 66.6, 63.1, 47.1, 42.8, 39.6, 31.3, 31.1, 17.3, −1.5; $ES^+$ MS *m/z* 820 ($[M+Na]^+$); HRMS (ESI) *m/z* calcd. for $C_{48}H_{51}NNaO_6SSi$ $(M+Na)^+$ 820.3099, found 820.3090.

#### 4.1.8. (*S*,*E*)-2-(Trimethylsilyl)ethyl 3-(2-((*R*)-2-(2-((*tert*-butoxy carbonylamino)methyl)thiazol-4-yl)-4-methyl-4,5-dihydro thiazole-4-carboxamido)acetoxy)-7-(tritylthio)hept-4-enoate (24)

Carbamate **22** (170 mg, 0.21 mmol, 1 equiv) was deprotected by stirring for 2 h in anhydrous acetonitrile (5 mL) and diethylamine (110 μl, 1.07 mmol, 5 equiv) followed by concentration in vacuo. To a solution of **15** (84 mg, 0.24 mmol, 1.1 equiv) in anhydrous $CH_2Cl_2$ (5 mL) cooled at 0 °C, PyBOP (151 mg, 0.26 mmol, 1.2 equiv) and Hünig's base (112 μL, 0.64 mmol, 3 equiv) were added. After 10 min, the previous deprotected amine (123 mg, 0.21 mmol, 1 equiv) in solution in anhydrous $CH_2Cl_2$ (5 mL) was added dropwise. The reaction mixture was stirred at rt overnight and concentrated in vacuo. The crude material was purified by flash chromatography (EtOAc/hexane 3:7) to afford **24** as a colorless oil (153 mg, 78%): $[\alpha]_D^{24}$ −40.3 (*c* 0.27, $CHCl_3$); IR 3342, 2971, 2952, 1727, 1671, 1599, 1512, 1444, 1365, 1244, 1168, 858, 835, 741, 695 $cm^{-1}$; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 7.91 (s, 1H), 7.47–7.34 (m, 6H), 7.34–7.12 (m, 9H), 5.73–5.54 (m, 2H), 5.36 (dd, *J* = 15.6, 7.0 Hz, 1H), 5.26 (br s, 1H), 4.64 (d, *J* = 6.0 Hz, 2H), 4.22–4.09 (m, 2H), 4.02 (dd, *J* = 7.5, 5.5 Hz, 2H), 3.76 (d, *J* = 11.5 Hz, 1H), 3.34 (d, *J* = 11.5 Hz, 1H), 2.66 (dd, *J* = 15.8, 8.3 Hz, 1H), 2.53 (dd, *J* = 15.8, 5.3 Hz, 1H), 2.18 (t, *J* = 7.0 Hz, 2H), 2.12–1.96 (m, 2H), 1.60 (s, 3H), 1.48 (s, 9H), 1.07–0.88 (m, 2H), 0.04 (s, 9H); $ES^+$ MS *m/z* 937 ($[M+Na]^+$); HRMS (ESI) *m/z* calcd. for $C_{47}H_{58}N_4NaO_7S_3Si$ $(M+Na)^+$ 937.3129, found 937.3136.

#### 4.1.9. (5*R*,11*S*)-5-Methyl-11-((*E*)-4-(tritylthio)but-1-en-1-yl)-10-oxa-3,17-dithia-7,14,19,20-tetraazatricyclo[14.2.1.12,5]icosa-1(18),2(20),16(19)-triene-6,9,13-trione (26)

Acyclic precursor **24** (138 mg, 0.15 mmol) was dissolved in $CH_2Cl_2$ (7 mL), cooled to 0 °C and treated with TFA (1.5 mL). The reaction mixture was warmed to rt and stirred overnight. The reaction mixture was concentrated in vacuo and then coevaporated with toluene to remove residual TFA. The crude amino acid (108 mg, 0.15 mmol, 1 equiv) was taken up in anhydrous $CH_2Cl_2$ (13 mL) and added dropwise to a vigorously stirred solution of Hünig's base (158 μL, 0.90 mmol, 6 equiv), HOBt (41 mg, 0.30 mmol, 2 equiv) and HATU (115 mg, 0.30 mmol, 2 equiv) in anhydrous acetonitrile (110 mL). The reaction mixture was stirred overnight, and then concentrated in vacuo. The crude material was purified by flash chromatography (hexane/EtOAc 10:1 then 100% EtOAc) to afford

**26** as a pale yellow solid (55.4 mg, 53%): mp 136–138 °C; $[\alpha]_D^{24}$ −51.2 (*c* 0.22, $CH_3OH$); IR 3628, 2923, 1734, 1670, 1541, 1507, 1262, 840 $cm^{-1}$; $^1H$ NMR (400 MHz, $CD_3OD$) $\delta$ 8.02 (s, 1H), 7.40–7.29 (m, 6H), 7.29–7.10 (m, 9H), 5.74–5.53 (m, 2H), 5.41 (dd, *J* = 15.6, 7.0 Hz, 1H), 4.97 (d, *J* = 17.6 Hz, 1H), 4.34 (d, *J* = 17.6 Hz, 1H), 4.06 (d, *J* = 18.1 Hz, 1H), 3.97 (d, *J* = 11.5 Hz, 1H), 3.73 (d, *J* = 18.1 Hz, 1H), 3.31 (d, *J* = 11.5 Hz, 1H), 2.97 (dd, *J* = 17.3, 11.3 Hz, 1H), 2.63 (dd, *J* = 17.1, 2.0 Hz, 1H), 2.17 (t, *J* = 6.0 Hz, 2H), 2.02 (q, *J* = 6.5 Hz, 2H), 1.74 (s, 3H); $^{13}C$ NMR (100 MHz, $CD_3OD$) $\delta$ 176.2, 172.8, 169.7, 168.0, 166.8, 148.4, 146.5, 134.4, 130.9, 129.5, 129.0, 127.9, 126.7, 85.4, 73.8, 67.9, 44.6, 42.7, 42.3, 40.2, 32.5, 32.4, 25.5; $ES^+$ MS *m/z* 719 ($[M+Na]^+$); HRMS (ESI) *m/z* calcd. for $C_{37}H_{36}N_4NaO_4S_3$ $(M+Na)^+$ 719.1791, found 719.1795.

#### 4.1.10. (5*R*,11*S*)-11-((*E*)-4-Mercaptobut-1-en-1-yl)-5-methyl-10-oxa-3,17-dithia-7,14,19,20-tetra-azatricyclo[14.2.1.12,5]icosa-1(18),2(20),16(19)-triene-6,9,13-trione (2)

*S*-Trityl macrocycle **26** (45.3 mg, 65 μmol, 1 equiv) was dissolved in $CH_2Cl_2$ (8 mL) and cooled to 0 °C and successively treated with $Et_3SiH$ (21 μL, 130 μmol, 2 equiv) and TFA (320 μL, 6.93 mmol, 64 equiv). The reaction mixture was warmed to rt and stirred for 2 h, before being concentrated and purified by flash chromatography with EtOAc as eluent to provide **2** as a white solid (6 mg, 20%): mp 102–104 °C; $[\alpha]_D^{24}$ +9.7 (*c* 0.24, $CH_3OH$); IR 3380, 2964, 2930, 2843, 1750, 1678, 1591, 1512, 1262, 1187, 1043 $cm^{-1}$; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 7.74 (s, 1H), 7.09 (br s, 1H), 6.41 (br s, 1H), 5.94–5.71 (m, 2H), 5.52 (dd, *J* = 15.5, 7.3 Hz, 1H), 5.23 (dd, *J* = 17.4, 8.9 Hz, 1H), 4.27 (dd, *J* = 17.3, 4.0 Hz, 1H), 4.21–4.07 (m, 2H), 3.88 (dd, *J* = 18.8, 3.1 Hz, 1H), 3.25 (d, *J* = 11.3 Hz, 1H), 2.92 (dd, *J* = 16.7, 11.0 Hz, 1H), 2.69 (d, *J* = 15.2 Hz, 1H), 2.58 (q, *J* = 7.4 Hz, 2H), 2.37 (q, *J* = 6.7 Hz, 2H), 1.84 (s, 3H), 1.39 (t, *J* = 7.8 Hz, 1H); $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$ 173.8, 169.5, 167.7, 167.5, 166.2, 135.4, 133.1, 128.8, 124.6, 84.3, 72.5, 43.7, 42.2, 41.3, 40.2, 36.2, 25.3, 23.8; $ES^+$ MS *m/z* 477 ($[M+Na]^+$); HRMS (ESI) *m/z* calcd. for $C_{18}H_{22}N_4NaO_4S_3$ $(M+Na)^+$ 477.0695, found 477.0702.

#### 4.1.11. (*S*,*E*)-2-(Trimethylsilyl)ethyl 3-(3-(((9H-fluoren-9-yl) methoxy) carbonylamino)propanoy-loxy)-7-(tritylthio)hept-4-enoate (23)

The protected β-hydroxy ester (208 mg, 0.40 mmol, 1 equiv) and *N*-Fmoc-β-alanine (137 mg, 0.44 mmol, 1.1 equiv) were dissolved in anhydrous $CH_2Cl_2$ (8 mL), cooled to 0 °C and DCC (99 mg, 0.48 mmol, 1.2 equiv) and DMAP (4.9 mg, 0.04 mmol, 0.1 equiv) were then added. The reaction mixture was stirred overnight. The precipitate was filtered and solvents were evaporated. The crude was purified by flash chromatography (EtOAc/hexane 5:95) to give **23** as a colorless oil (207 mg, 64%): $[\alpha]_D^{23}$ −9.9 (*c* 0.56, $CHCl_3$); IR 3376, 3058, 2949, 1724, 1504, 1455, 1244, 1164, 1081, 1066, 1036, 990, 975, 862, 835, 737, 703 $cm^{-1}$; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 7.75 (d, *J* = 7.5 Hz, 2H), 7.59 (d, *J* = 7.0 Hz, 2H), 7.52–7.34 (m, 19H), 5.70–5.53 (m, 2H), 5.48 (br s, 1H), 5.38 (dd, *J* = 15.6, 7.0 Hz, 1H), 4.34 (d, *J* = 7.0 Hz, 2H), 4.28–4.07 (m, 3H), 3.40–3.50 (m, 2H), 2.69–2.46 (m, 4H), 2.19 (t, *J* = 7.0 Hz, 2H), 2.07 (t, *J* = 6.5 Hz, 2H), 0.95 (t, *J* = 8.5 Hz, 2H), 0.01 (s, 9H); $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$ 171.2, 170.1, 156.3, 144.8, 144.0, 141.3, 133.1, 129.6, 128.0, 127.9, 127.7, 127.0, 126.6, 125.1, 119.9, 70.9, 66.8, 66.6, 63.1, 47.2, 39.7, 36.7, 34.8, 31.3, 31.2, 17.3, −1.5; $ES^+$ MS *m/z* 834 ($[M+Na]^+$); HRMS (ESI) *m/z* calcd. for $C_{49}H_{53}NNaO_6SSi$ $(M+Na)^+$ 834.3255, found 834.3270.

#### 4.1.12. (*S*,*E*)-2-(Trimethylsilyl)ethyl 3-(3-((*R*)-2-(2-((*tert*-butoxy carbonylamino)methyl)thiazol-4-yl)-4-methyl-4,5-dihydro thiazole-4-carboxamido)propanoyloxy)-7-(tritylthio)hept-4-enoate (25)

Carbamate **23** (185 mg, 0.23 mmol, 1 equiv) was dissolved in anhydrous acetonitrile (5 mL) and diethylamine (118 μl, 1.14 mmol, 5 equiv) was added to the reaction mixture. After stirring for 2 h, the reaction mixture was concentrated in vacuo.

To a solution of **15** (90 mg, 0.25 mmol, 1.1 equiv) in anhydrous $CH_2Cl_2$ (5 mL) cooled at 0 °C, PyBOP (162 mg, 0.28 mmol, 1.2 equiv) and Hünig's base (120 μL, 0.69 mmol, 3 equiv) were added. After 15 min, the previous deprotected amine (135 mg, 0.23 mmol, 1 equiv) in anhydrous $CH_2Cl_2$ (5 mL) was added dropwise. The reaction mixture was stirred at rt overnight and concentrated in vacuo. The crude material was purified by flash chromatography (EtOAc/hexane 3:7) to afford **25** as a colorless oil (175 mg, 82%): $[\alpha]_D^{25}$ −39.8° (*c* 0.61, $CHCl_3$); IR 3361, 2967, 2941, 2926, 1724, 1659, 1520, 1440, 1368, 1259, 1168, 1032, 866, 835, 745, 699 $cm^{-1}$; $^1H$ NMR (300 MHz, $CDCl_3$) $\delta$ 7.97 (br s, 1H), 7.39 (d, *J* = 7.5 Hz, 6H), 7.33–7.12 (m, 9H), 5.57 (dt, *J* = 14.6, 7.5 Hz, 2H), 5.35 (dd, *J* = 15.1, 7.0 Hz, 1H), 5.28 (br s, 1H), 4.63 (d, *J* = 5.5 Hz, 2H), 4.21–4.06 (m, 2H), 3.76 (d, *J* = 11.5 Hz, 1H), 3.43–3.59 (m, 2H), 3.33 (d, *J* = 11.5 Hz, 1H), 2.62 (dd, *J* = 15.8, 7.8 Hz, 1H), 2.51 (dt, *J* = 10.3, 5.5 Hz, 3H), 2.18 (t, *J* = 7.5 Hz, 2H), 2.08–1.95 (m, 2H), 1.57 (s, 3H), 1.48 (s, 9H), 1.04–0.87 (m, 2H), 0.03 (s, 9H); $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$ 174.5, 170.8, 169.9, 165.5, 154.3, 144.8, 132.9, 129.6, 128.1, 127.9, 126.6, 122.1, 85.0, 80.5, 77.2, 70.8, 66.6, 63.0, 42.3, 41.3, 39.7, 34.9, 34.2, 31.3, 31.2, 28.3, 24.8, 22.6, 17.3, −1.5; $ES^+$ MS *m/z* 951 ($[M+Na]^+$); HRMS (ESI) *m/z* calcd. for $C_{48}H_{60}N_4NaO_7S_3Si$ $(M+Na)^+$ 951.3286, found 951.3287.

#### 4.1.13. (5*R*,12*S*)-5-Methyl-12-((*E*)-4-(tritylthio)but-1-en-1-yl)-11-oxa-3,18-dithia-7,15,20,21-tetraazatricyclo[15.2.1.12,5] henicosa-1(19),2(21),17(20)-triene-6,10,14-trione (27)

Acyclic precursor **25** (152 mg, 0.16 mmol) was dissolved in $CH_2Cl_2$ (7.6 mL), cooled to 0 °C and treated with TFA (1.6 mL). The reaction mixture was warmed to rt, stirred overnight, concentrated in vacuo and then coevaporated with toluene to remove residual TFA. The crude amino acid (120 mg, 0.17 mmol, 1 equiv) was then taken up in anhydrous $CH_2Cl_2$ (14 mL) and added dropwise to a vigorously stirred solution of Hünig's base (172 μL, 0.99 mmol, 6 equiv), HOBt (45 mg, 0.33 mmol, 2 equiv) and HATU (125 mg, 0.33 mmol, 2 equiv) in anhydrous acetonitrile (120 mL). The reaction mixture was stirred overnight, concentrated in vacuo and the crude material was purified by flash chromatography (hexane/EtOAc 10:1 then 100% EtOAc) to afford **27** as a colourless oil (20 mg, 17%): $[\alpha]_D^{24}$ +37.5 (*c* 0.01, $CH_3OH$). IR 2960, 2926, 2850, 1739, 1671, 1463, 1266, 1085, 1028, 805 $cm^{-1}$; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 7.71 (s, 1H), 7.36–7.16 (m, 15H), 6.96 (br s, 1H), 6.09 (dd, *J* = 15.6, 7.5 Hz, 1H), 5.73–5.59 (m, 1H), 5.55 (td, *J* = 7.2, 3.7 Hz, 1H), 4.99 (dd, *J* = 16.8, 7.8 Hz, 1H), 4.03 (d, *J* = 11.5 Hz, 1H), 3.94 (dd, *J* = 17.1, 4.0 Hz, 1H), 3.49 (d, *J* = 4.0 Hz, 2H), 3.33 (d, *J* = 11.5 Hz, 1H), 2.78–2.61 (m, 2H), 2.56–2.42 (m, 2H), 2.39–2.22 (m, 2H), 2.18–1.95 (m, 2H), 1.68 (s, 3H); $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$ 174.1, 171.5, 169.7, 167.5, 162.0, 148.0, 144.7, 132.0, 129.9, 129.6, 127.9, 126.7, 124.1, 84.6, 73.0, 66.9, 42.7; 42.3, 40.9, 35.4, 33.8, 31.4, 31.3, 25.5; $ES^+$ MS *m/z* 733 ($[M+Na]^+$); HRMS (ESI) *m/z* calcd. for $C_{38}H_{38}N_4NaO_4S_3$ $(M+Na)^+$ 733.1947, found 733.1947.

#### 4.1.14. (5*R*,12*S*)-12-((*E*)-4-Mercaptobut-1-en-1-yl)-5-methyl-11-oxa-3,18-dithia-7,15,20,21-tetra-azatricyclo[15.2.1.12,5] henicosa-1(19),2(21),17(20)-triene-6,10,14-trione (3)

S-Trityl macrocycle **27** (16.1 mg, 23 μmol, 1 equiv) was dissolved in $CH_2Cl_2$ (5.5 mL) and cooled to 0 °C. The mixture was successively treated with $Et_3SiH$ (10 μL, 130 μmol, 2.7 equiv) and TFA (320 μL, 6.93 mmol, 64 equiv). The reaction mixture was warmed to rt and stirred for 2 h, before being concentrated and purified by flash chromatography with EtOAc as eluent to provide **3** as a white solid (9 mg, 85%): mp 80–82 °C; $[\alpha]_D^{24}$ +1.6 (*c* 0.36, $CH_3OH$); IR 3353, 2926, 2854, 1739, 1671, 1542, 1179, 1039 $cm^{-1}$; $^1H$ NMR (300 MHz, $CDCl_3/CD_3OD$) $\delta$ 7.78 (br s, 1H), 7.65 (s, 1H), 7.19

(br s, 1H), 5.65 (t, $J$ = 5.0 Hz, 2H), 5.43 (m, 1H), 4.94 (td, $J$ = 16.6, 3.8 Hz, 1H), 4.24 (d, $J$ = 16.6 Hz, 1H), 3.92 (d, $J$ = 11.5 Hz, 1H), 3.39 (q, $J$ = 5.0 Hz, 2H), 3.24 (m, $J$ = 11.5 Hz, 1H), 2.72 (dd, $J$ = 15.8, 6.8 Hz, 1H), 2.58–2.34 (m, 5H), 2.34–2.15 (m, 2H), 1.65 (s, 3H); $^{13}$C NMR (300 MHz, $CDCl_3/CD_3OD$) $\delta$ 174.4, 171.3, 170.8, 167.8, 163.3, 147.5, 131.2, 129.9, 124.1, 84.1, 72.6, 42.1, 41.9, 40.5, 35.9, 34.8, 33.1, 25.1, 23.4; ES$^+$ MS $m/z$ 491 ([M+Na]$^+$); HRMS (ESI) $m/z$ calcd. for $C_{19}H_{24}N_4NaO_4S_3$ (M+Na)$^+$ 491.0852, found 491.0850.

#### 4.1.15. Methyl 2-(2-((*tert*-butoxycarbonylamino)methyl)thiazole-4-carboxamido)-2-methylpro-panoate (28)

To a solution of **9** (200 mg, 0.77 mmol, 1.1 equiv) in $CH_2Cl_2$ (10 mL) cooled at 0 °C, PyBOP (549 mg, 0.93 mmol, 1.1 equiv) and Hünig's base (405 μL, 2.33 mmol, 3 equiv) were added. After 10 min, 2-aminoisobutyric acid methyl ester hydrochloride (108 mg, 0.70 mmol, 1 equiv) in solution in $CH_2Cl_2$ (10 mL) was added dropwise. The reaction mixture was stirred at rt overnight and concentrated in vacuo. The crude material was purified by flash chromatography (EtOAc/hexane 3:7) to afford **28** as a white solid (244 mg, 97%): mp 91.7–93.6 °C; IR 3338, 2986, 2933, 1746, 1712, 1667, 1538, 1448, 1368, 1285, 1247, 1149 cm$^{-1}$; $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 8.00 (s, 1H), 7.74 (br s, 1H), 5.28 (br s, 1H), 4.60 (d, $J$ = 5.9 Hz, 2H), 3.77 (s, 3H), 1.67 (s, 6H), 1.48 (s, 9H); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ 174.7, 169.2, 160.2, 150.0, 149.8, 123.6, 80.5, 56.4, 52.7, 42.3, 28.3, 25.0; ES$^+$ MS $m/z$ 380 ([M+Na]$^+$); HRMS (ESI) $m/z$ calcd. for $C_{15}H_{23}N_3NaO_5S$ (M+Na)$^+$ 380.1251, found 380.1249.

#### 4.1.16. 2-(2-((*tert*-Butoxycarbonylamino)methyl)thiazole-4-carboxamido)-2-methylpropanoic acid (29)

To a solution of **28** (242 mg, 0.68 mmol, 1 equiv) in 8 mL of THF/water (3:1) at 0 °C was added LiOH (49 mg, 2.03 mmol, 3 equiv). The reaction mixture was stirred at rt overnight. The solution was diluted with water (3 mL) and acidified to pH 1–2 with a saturated $KHSO_4$ solution. This aqueous layer was extracted twice with EtOAc (10 mL). The combined organic layers were washed with brine, dried over $MgSO_4$ and concentrated in vacuo to provide **29** as a yellow solid (225 mg, 96%): mp 171–173 °C; IR 3342, 2930, 2850, 2363, 2340, 1712, 1656, 1542, 1455, 1376, 1278, 1251, 1160 cm$^{-1}$; $^1$H NMR (400 MHz, $CD_3OD$) $\delta$ 8.10 (s, 1H) 4.55 (s, 2H) 1.66 (s, 6H) 1.50 (s, 9H); $^{13}$C NMR (100 MHz, $CD_3OD$) $\delta$ 177.8, 173.0, 162.7, 158.5, 150.9, 124.9, 81.1, 57.7, 43.3, 28.8, 25.3; ES$^+$ MS $m/z$ 366 ([M+Na]$^+$); HRMS (ESI) $m/z$ calcd. for $C_{14}H_{21}N_3NaO_5S$ (M+Na)$^+$ 366.1094, found 366.1094.

#### 4.1.17. (*S*,*E*)-2-(Trimethylsilyl)ethyl 3-((*S*)-2-(2-(2-((*tert*-butoxycarbonylamino)methyl)thiazole-4-carboxamido)-2-methylpropanamido)-3-methylbutanoyloxy)-7-(tritylthio)hept-4-enoate (30)

Carbamate **17** (371 mg, 0.44 mmol) was dissolved in anhydrous acetonitrile (4 mL) and diethylamine (229 μl, 2.21 mmol). After stirring for 2 h, the reaction mixture was concentrated in vacuo. The free amine was purified by flash chromatography (EtOAc/hexane 1:1).

To a solution of **29** (86.2 mg, 0.25 mmol, 1.1 equiv) in anhydrous $CH_2Cl_2$ (5 mL) cooled to 0 °C, PyBOP (162 mg, 0.27 mmol, 1.2 equiv) and Hünig's base (119 μL, 0.69 mmol, 3 equiv) were added. After 10 min, the previous deprotected **17** (141 mg, 0.23 mmol, 1 equiv) in solution in $CH_2Cl_2$ (5 mL) was added dropwise. The reaction mixture was left at rt overnight and concentrated in vacuo. The crude material was purified by flash chromatography (EtOAc/hexane 3:7) to afford **30** as a white solid (134 mg, 62%): mp 61–63 °C; $[\alpha]_D^{25}$ +5.4 ($c$ 0.26, $CHCl_3$); IR 3338, 2960, 2926, 1731, 1682, 1535, 1497, 1444, 1391, 1365, 1251, 1172, 1028, 975 cm$^{-1}$; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 8.10 (s, 1H), 7.81 (s, 1H), 7.54–7.40 (m, 6H), 7.40–7.22 (m, 9H), 7.19 (d, $J$ = 8.5 Hz, 1H), 5.82–5.57 (m, 2H), 5.41 (dd, $J$ = 7.5, 15.6 Hz, 1H), 5.34 (br s, 1H), 4.66 (d, $J$ = 5.5 Hz, 2H), 4.58 (dd, $J$ = 8.5, 4.0 Hz, 1H), 4.30–4.09 (m, 2H), 2.72 (dd, $J$ = 15.8, 7.8 Hz, 1H), 2.58 (dd, $J$ = 15.6, 6.0 Hz, 1H), 2.35–2.15 (m, 3H), 2.09 (q, $J$ = 6.5 Hz, 2H), 1.73 (s, 3H), 1.76 (s, 3H), 1.55 (s, 9H), 1.09–0.79 (m, 8H), 0.10 (s, 9H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 173.9, 170.7, 169.6, 169.4, 160.7, 155.6, 149.8, 144.8, 133.7, 129.5, 127.8, 127.8, 126.6, 123.8, 80.5, 71.6, 66.6, 63.1, 57.6, 57.1, 42.3, 39.7, 31.3, 31.1, 28.3, 25.8, 25.0, 19.0, 17.4, 17.3, −1.5; ES$^+$ MS $m/z$ 965 ([M+Na]$^+$ 100%); HRMS (ESI) $m/z$ calcd. for $C_{50}H_{66}N_4NaO_8S_2Si$ (M+Na)$^+$ 965.3984, found 965.3964.

#### 4.1.18. (7*S*,10*S*,*E*)-7-Isopropyl-4,4-dimethyl-10-(4-(tritylthio)but-1-enyl)-9-oxa-16-thia-3,6,13,18-tetraaza-bicyclo[13.2.1]octadec-1(17)-ene-2,5,8,12-tetraone (31)

Acyclic precursor **30** (218 mg, 0.23 mmol) was dissolved in $CH_2Cl_2$ (11 mL), cooled to 0 °C and treated with TFA (2.3 mL). The reaction mixture was warmed to rt, stirred overnight, concentrated in vacuo and then coevaporated with toluene to remove residual TFA.The crude amino acid (172 mg, 0.23 mmol, 1 equiv) was then taken up in $CH_2Cl_2$ (20 mL) and added dropwise to a vigorously stirred solution of Hünig's base (242 μL, 1.39 mmol, 6 equiv), HOBt (63 mg, 0.46 mmol, 2 equiv) and HATU (176 mg, 0.46 mmol, 2 equiv) in acetonitrile (165 mL). The reaction mixture was stirred overnight, and then concentrated in vacuo. The crude material was purified by flash chromatography (hexane/EtOAc 10:1 to 100% EtOAc) to afford **31** as a pale yellow solid (108 mg, 64%): mp 132–134 °C; $[\alpha]_D^{25}$ +32.3 ($c$ 0.34, $CHCl_3$); IR 3364, 2967, 2926, 2854, 1739, 1678, 1542, 1493, 1444, 1255, 1179, 839, 748, 695 cm$^{-1}$; $^1$H NMR (400 MHz, $CDCl_3/CD_3OD$) $\delta$ 8.32 (s, 1H), 8.05 (d, $J$ = 9.0 Hz, 1H), 7.87 (d, $J$ = 2.5 Hz, 1H), 7.64–7.37 (m, 15H), 6.00–5.82 (m, 2H), 5.82–5.68 (m, 1H), 5.26 (d, $J$ = 17.6 Hz, 1H), 4.78 (dd, $J$ = 9.3, 4.8 Hz, 1H), 4.47 (d, $J$ = 17.1 Hz, 1H), 3.06 (dd, $J$ = 17.1, 9.5 Hz, 1H), 2.90 (d, $J$ = 16.1 Hz, 1H), 2.60–2.40 (m, 3H), 2.40–2.17 (m, 2H), 2.12 (s, 3H), 1.86 (s, 3H), 1.14 (d, $J$ = 7.0 Hz, 3H), 0.97 (d, $J$ = 7.0 Hz, 3H); $^{13}$C NMR (100 MHz, $CDCl_3/CD_3OD$) $\delta$ 175.9, 171.8, 170.7, 167.9, 163.1, 149.9, 145.5, 133.7, 130.3, 128.9, 128.6, 127.4, 124.0, 72.7, 67.5, 60.0, 58.4, 41.1, 40.8, 32.0, 31.9, 31.8, 25.6, 25.3, 19.8, 17.5; ES$^+$ MS $m/z$ 747 ([M+Na]$^+$); HRMS (ESI) $m/z$ calcd. for $C_{40}H_{44}N_4NaO_5S_2$ (M+Na)$^+$ 747.2645, found 747.2641.

#### 4.1.19. (7*S*,10*S*,*E*)-7-Isopropyl-10-(4-mercaptobut-1-enyl)-4,4-dimethyl-9-oxa-16-thia-3,6,13,18-tetraaza-bicyclo[13.2.1]octadec-1(17)-ene-2,5,8,12-tetraone (4)

*S*-Trityl macrocycle **31** (73.1 mg, 0.10 mmol, 1 equiv) was dissolved in $CH_2Cl_2$ (12 mL) and cooled to 0 °C. The mixture was successively treated with $Et_3SiH$ (32 μL, 0.20 mmol, 2 equiv) and TFA (495 μL, 6.93 mmol, 61 equiv). The reaction mixture was warmed to rt and stirred for 2 h, before being concentrated and purified by flash chromatography with EtOAc as eluent to provide **4** as a white solid (6 mg, 12%): mp 56–58 °C; $[\alpha]_D^{25}$ +61.3 ($c$ 0.24, $CHCl_3$); IR 3334, 2960, 2926, 2854, 1734, 1678, 1542, 1463, 1383, 1256, 974 cm$^{-1}$; $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 8.03 (s, 1H), 7.65 (br s, 1H), 6.60–6.39 (m, 2H), 5.84–5.70 (m, 3H), 5.16 (dd, $J$ = 17.3, 7.8 Hz, 1H), 4.64 (dd, $J$ = 9.5, 4.0 Hz, 1H), 4.37 (dd, $J$ = 17.6, 4.0 Hz, 1H), 2.86–2.62 (m, 2H), 2.62–2.46 (m, 2H), 2.46–2.21 (m, 3H), 1.90 (s, 3H), 1.62 (s, 3H), 1.35 (t, $J$ = 7.5 Hz, 1H), 0.91 (d, $J$ = 6.5 Hz, 3H), 0.72 (d, $J$ = 7.0 Hz, 3H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 174.4, 170.0, 168.9, 166.9, 161.0, 149.5, 132.1, 129.0, 123.3, 71.8, 59.6, 57.4, 41.3, 40.8, 36.0, 31.3, 25.8, 24.8, 23.8, 19.1, 16.8; ES$^+$ MS $m/z$ 505 ([M+Na]$^+$); HRMS (ESI) $m/z$ calcd. for $C_{21}H_{30}N_4NaO_5S_2$ (M+Na)$^+$ 505.1550, found 505.1551.

### 4.2. HDAC enzyme assay and MCF7 growth inhibition assay

These were performed according to standard procedures as previously reported.[7d] The HDAC enzyme assay of largazole thiol and analogues **1–4** was performed in the presence of DDT, by incubating 1 mM of compound with 10 mM DTT (10-fold excess) for 1 h prior to the assay.

### 4.3. Metabolic stability studies

Murine liver homogenates were prepared by adding 10 mM Tris buffer (pH 7.4, 1:3 w:v) to the tissue and gently breaking it up with a pestle and mortar. The assay commenced with the addition of compound to preincubated homogenate at 37 °C to give a final drug concentration of 100 μM. Samples (20 μL) were taken at 0, 15, 30, 45, 60 and 120 min, diluted with methanol (60 μL) and stored on ice prior to centrifugation at 10,000g for 5 min at 4 °C to precipitate liver proteins. The supernatant was transferred to a polypropylene vial and stored at −20 °C before analysis.

Samples (2 μL) were analyzed by UPLC/MS employing gradient separation. Mobile phase A consisted of 10% MeOH/90% $dH_2O$ containing 0.05% TFA, and mobile phase B of 90% MeOH/10% $dH_2O$ containing 0.05% TFA. Analysis was performed on a Waters C18 10 cm Acquity column 1.7 μM (10 cm × 2.1 mm, Milford) with a flow rate of 0.3 mL/min. The gradient started with 25% B which increased to 30% over the first 2 min. From 2 to 30 min %B increased from 30% to 80%, from 30 to 32 min %B decreased from 80% to 25% and remained at 25% until the end of the run. Peaks were detected by ESI MS using Selected Ion Recording for the parent ions.

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### Supplementary data

Supplementary data (Synthetic procedures for preparation of known compounds and NMR spectra for novel compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.02.024.

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