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Synthesis and Biological Characterization of the Histone Deacetylase Inhibitor Largazole and C7- Modified Analogues

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Largazole **4a** and analogues with modifications at the C7 position, as well as 2,4′-bithiazole **5a**, have been synthesized using an acyclic cross-metathesis of the corresponding depsipeptide structures assembled by N−C6(O) or C15(O)-N lactam formation. Similar to the parent system **4a**, the series of largazole depsipeptides **4b**−**d**, but not 2,4′-bithiazole **5a**, showed a marked inhibition of recombinant HDAC1 and selectivity over HDAC4, as well as strong pro-apoptotic effects on the NB4 leukemia cell line, but they failed to induce differentiation to mature granulocytes. Functional assays of the analogues correlated with the in vitro activities, as shown by increased H3 and α-tubulin acetylation levels and $p21^{WAF1/CIP1}$ up-regulation in NB4 cells. The activity of the natural product HDACi largazole **4a** is not significantly altered by the presence of groups of different size (H, Et, Ph) at C7 on the dihydrothiazole ring.

Introduction

Global changes of gene expression are constant features of the transformed phenotype. Current knowledge supports the view that these changes are not only due to alterations of the underlying DNA sequence but also to epigenetic changes in the cancer genome.[1,2] Epimutations (primarily methylation of CpG islands on DNA and covalent modifications of lysine, arginine and serine residues on histone proteins, such as acetylation, methylation, phosphorylation, and sumoylation)[3] require enzymatic mechanisms of maintenance and are therefore subjected to pharmacological intervention.[4,5]

In eukaryotic cells, the acetylation status of histones[6,7] is maintained by the opposing catalytic activities of histone deacetylases (HDACs[a]) and histone acetyltransferases (HATs).[8] This epigenetic mark controls transcriptional regulation and gene expression, with acetylation being linked to derepression and deacetylation with a repressed state.[3] Other nonhistone proteins that control fundamental biological processes, such as chromatin remodeling, microtubule stabilization, protein−protein interactions, and small-molecule action, are also modulated by acetyl transfer/deacetylation processes acting on lysine residues. Inhibitors of histone deacetylases (HDACis) have been successfully used to revert hyperacetylation and transcriptional repression of targeted genes.[9−12] Two drugs, suberoyl anilide hydroxamic acid (SAHA, vorinostat) and the depsipeptide FK228 **2** (Figure 1, romidepsin, vide infra), have been approved by the FDA for the treatment of cutaneous T-cell lymphoma, whereas others are undergoing clinical trials alone or in combination with different epigenetic drugs (DNA or histone demethylating agents). Moreover, it is expected that HDACis with selectivity for a particular isoform[13] might offer better therapeutic options.

The 18 known mammalian deacetylase enzymes are divided in two families, the Zn^{2+}-dependent histone deacetylases (11 members, grouped in classes I, II, and IV) and the NAD^{+}-dependent Sir2-like deacetylases or sirtuins (7 members, class III).[14] HDAC deacetylate the ε-acetylamino lysine by a nucleophilic attack of bound water to the Zn^{2+}-activated carbonyl group, generating a tetrahedral zinc alkoxide intermediate stabilized by enzyme residues, which subsequently releases the acetyl group and the lysine products.[15,16] Inhibition strategies have primarily focused on the chelation of Zn^{2+} using a "head group" attached via a connector of variable length and functionality to a cap region able to recognize and interact with the amino acids at the rim of the binding pocket.[17] The existing crystal structures of bacterial HDLP (histone deacetylase-like protein),[18] human HDAC8,[19,20] bacterial HDAH (histone deacetylase-like amidohydrolase),[21] human HDAC7,[22] and human HDAC4[23] validate the canonical mechanism of inhibition and the role of the metal center.

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[a] Abbreviations: HDACs, histone deacetylases; HATs, histone acetyltransferases; SAHA, suberoylanilide hydroxamic acid; HDLP, histone deacetylase-like protein; HDAH, histone deacetylase-like amidohydrolase; MDA-MB-231, invasive transformed human mammary epithelial cells; U2OS, transformed fibroblastic osteosarcoma cells; NMuMG, nontransformed mouse mammary gland epithelial cell line; NIH3T3, mouse embryonic fibroblast cell line; NB4, acute promyelocytic leukemia cell line; ATRA, *all-trans*-retinoic acid; PML/RARα, promyelocytic leukemia/retinoic receptor alfa fusion protein; FACS, fluorescence-activated cell sorting; HMTs, histone methyl transferases; HDMs, histone demethylases; DNMTs, DNA methyl transferases; TRAIL, tumour necrosis factor-related apoptosis inducing ligand; AML, acute myelogenous leukemia; HCT116, human colorectal carcinoma cell line; HT29, human colon adenocarcinoma grade II cell line; IMR-32, human neuroblastoma cell line; HME, human mammary epithelial cell line; A432, human epidermoid carcinoma cell line.

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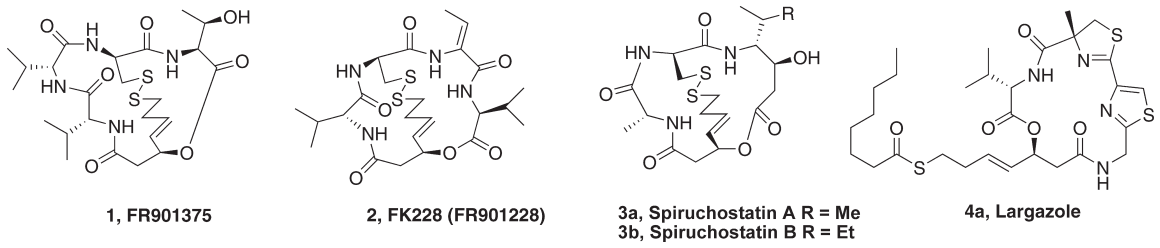


Figure 1. Sulfur-containing natural HDAC inhibitors with depsipeptide scaffolds.

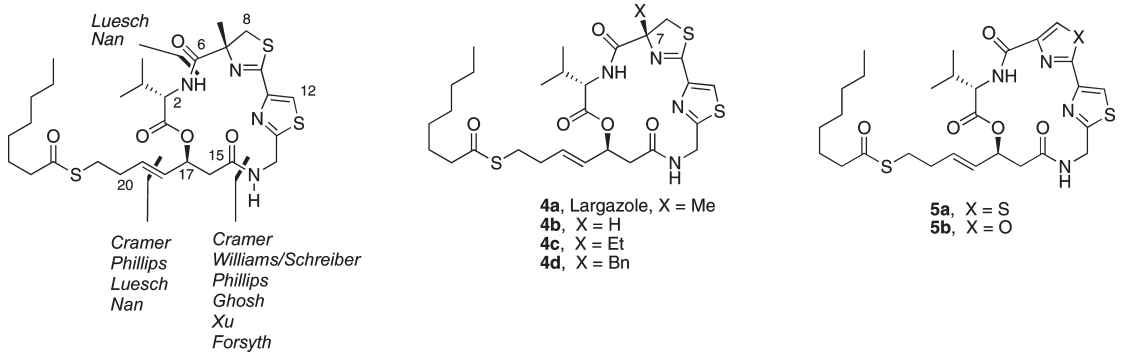


Figure 2. Summary of largazole synthesis and analogues synthesized in this study.

In this regard, Nature has selected unique Zn^{2+}-binding motifs in a collection of validated HDACis.[24] Among these motifs, (3*S*,4*E*)-3-hydroxy-7-mercapto-4-heptenoic acid is found as part of a depsipeptide scaffold in a small group of HDACis[24] which comprise FR901375 **1**,[25] FK228 **2**,[26,27] spiruchostatins **3a** and **3b**,[28] and largazole **4a**.[29] The relative position of the cysteine and the thiol of (3*S*,4*E*)-3-hydroxy-7-mercapto-4-heptenoic acid determines the size of the bicyclic ring system present in **1**−**3**, presumably formed by oxidative intramolecular disulfide formation.

Several reports on the reactivity of **2** have confirmed earlier suggestions that depsipeptides **1**−**3** are pro-drugs, which in the reducing environment of the cells release the Zn-chelating group via glutathione disulfide exchange reactions.[30] It has been demonstrated that the disulfide bond of FK228 is cleaved in the cells by the reducing activity of glutathione because both the active thiol[31] and the glutathione conjugates of FK228 were identified using mass spectrometry analysis of blood samples.[32] Upon bioreduction, depsipeptides **1** and **3** must likewise release the Zn^{2+}-binding butenyl thiol.[33]

Largazole **4** was isolated in 2008 from a marine cyanobacterium of the genus *Symploca* collected from Key Largo in Florida.[29] Spectroscopic analysis and chemical degradation studies established the stereostructure of this depsipeptide including the absolute configuration (2*S*,7*R*,17*S*) of the three stereogenic centers (Figure 2). Differently from **1**−**3**, largazole **4a** features a 4-methylthiazoline-4-carboxylate flanked by L-valine and by a thiazole unit, and the latter is bound to the lactone-forming (3*S*,4*E*)-3-hydroxy-7-mercapto-4-heptenoic acid, which is capped with an *n*-octanoyl group that presumably increases its physiological stability. Bioactivation of largazole **4a** must involve the action of esterases/lipases to release the thiol, thus resembling the remaining members of the series (Figure 1) in its physiological mode of action against HDAC.

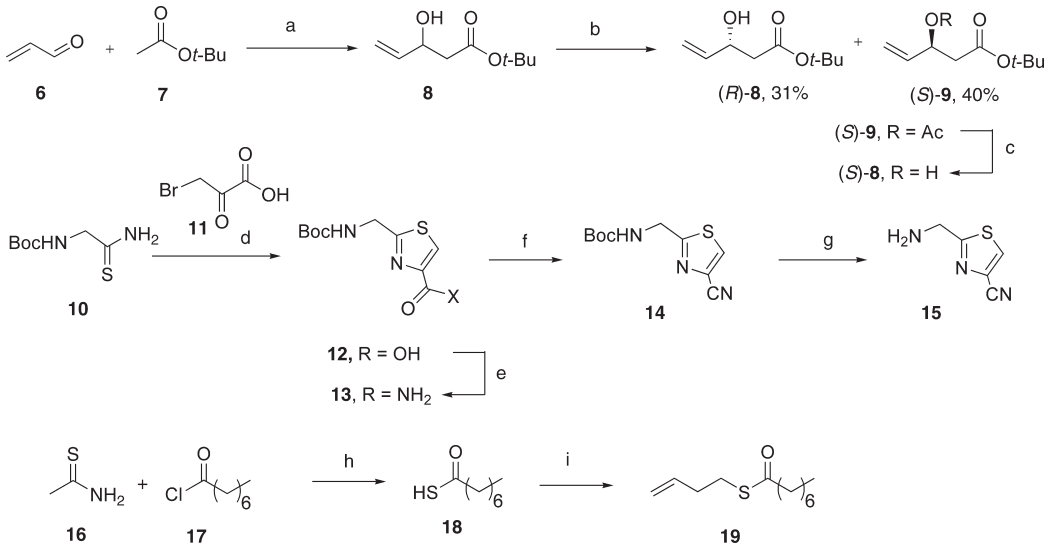
Preliminary biological assays of **4a** revealed a potent inhibition of tumor growth in several cancer cell lines, and most importantly a high selectivity for the inhibition of transformed cells (i.e., the invasive transformed human mammary epithelial cells MDA-MB-231 and the fibroblastic osteosarcoma cells U2OS) versus normal cells (NMuMG and NIH3T3, respectively).[29]

An intensive synthetic program started in several laboratories following the disclosure of the largazole structure and the reports of its extraordinary potency and selectivity.[24] This has resulted in the publication to date of nine syntheses of largazole and some analogues.[34−44] Figure 2 depicts the synthetic strategies used in the preparation of the natural product. These mainly differ in the position of the ring formation by macrolactamization and also in the use of the intact (3*S*,4*E*)-3-hydroxy-7-mercapto-4-heptenoate side chain or a shorter terminal alkene, which in the latter case is followed by a cross-metathesis as late stage reaction. The synthetic approaches have also provided a series of analogues, which have contributed to gather SAR information and discover more potent and selective congeners, as recently reviewed.[24]

We wish to report on the preparation of largazole and analogues substituted at the C7 position (**4c**−**d**), including the parent dihydrothiazole (**4b**) and the oxidized thiazole and oxazole counterparts (**5**) (Figure 2) as well as their biological characterization in the NB4 leukemia cell line. The free thiols of **4b** and **5a** have already been reported by Williams, Schreiber et al.[41] While this work was in progress, the synthesis of the C7-demethyl-largazole **4b** and its thiazole analogue **5a** was reported by Nan et al.[43]

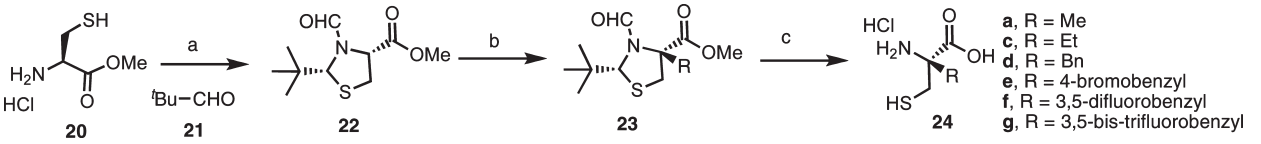
Our first approach to largazole analogues (see Supporting Information (SI)) envisioned the ring closure by lactonization using the Mitsunobu activation of the hydroxyl group in the acyclic precursor. Although this strategy had been successfully implemented for the synthesis of FK228 **2**,[45] in the case of largazole **4a**, it faced unsurmountable difficulties. Other authors have also noted the lack of reactivity of similar systems.[42] A timely study of Ganesan et al.[46] on the formation of depsipeptides by either lactamization or lactonization revealed that the latter fails with bulky amino acids (such as valine) at the C-terminus. As a consequence, the strategy was

Scheme 1[a]



[a] Reagents and conditions: (a) LDA, THF, 97%; (b) vinylacetate, Amano lipase, 4 Å MS, pentane, 30 °C, (***S***)-**9**, 40%, (***R***)-**8**, 31%; (c) K_2CO_3, THF, 76%; (d) bromopyruvic acid **11**, $CaCO_3$, EtOH, 99%; (e) NH_3, HOBt, DCC, DMF, 71%; (f) $(CF_3CO)_2O$, Et_3N, CH_2Cl_2, 44%; (g) TMSI, CH_3CN, 30 min, 99%; (h) NaOH, benzene, 52%; (i) 4-bromo-1-butene, K_2CO_3, acetone, 99%.

Scheme 2[a]



[a] Reagents and conditions: (a) (1) pivalaldehyde **21**, Et_3N, pentane, 99%, (2) HCOOH, HCOONa, Ac_2O, 0 °C, 67%; (b) RX (MeI, EtI, $ArCH_2Br$), LDA, DMPU, THF, −90 °C (**a**, 74%; **c**, 34%; **d**, 59%; **e**, 63%; **f**, 50%; **g**, 63%); (c) 5M HCl, 110 °C (**a**, 99%; **c**, 77%; **d**, 75%; **e**, 33%; **f**, 18%).

modified and macrocyclic lactam formation was selected for ring closure. Within this retrosynthetic analysis, the acyclic cross metathesis[47] would follow the macrolactamization to complete the side chain as the last step of the sequence. The analogues of largazole with different substituents at C7 (**4b**−**d**) would accordingly be made starting from the corresponding dihydrothiazoles.[48] Compounds **5** would in turn be acquired from either incorporation of the heterocyclic components in the depsipeptide ring or by oxidation of the corresponding dihydroderivatives.

The synthesis of homochiral (***S***)-**8** followed well-established procedures[49] that are amenable to the preparation of multigram quantities (Scheme 1). Condensation of acrolein **6** with the enolate derived from *tert*-butylacetate **7** provided allylic alcohol **8**,[49] which upon treatment with vinylacetate and Amano lipase underwent kinetic resolution producing (*S*)-allyl acetate **9** readily separable from enantioenriched (***R***)-**8**. Saponification of (***S***)-**9** afforded (*S*)-*tert*-butyl 3-hydroxypent-4-enoic acid (***S***)-**8**[37] (Scheme 1).

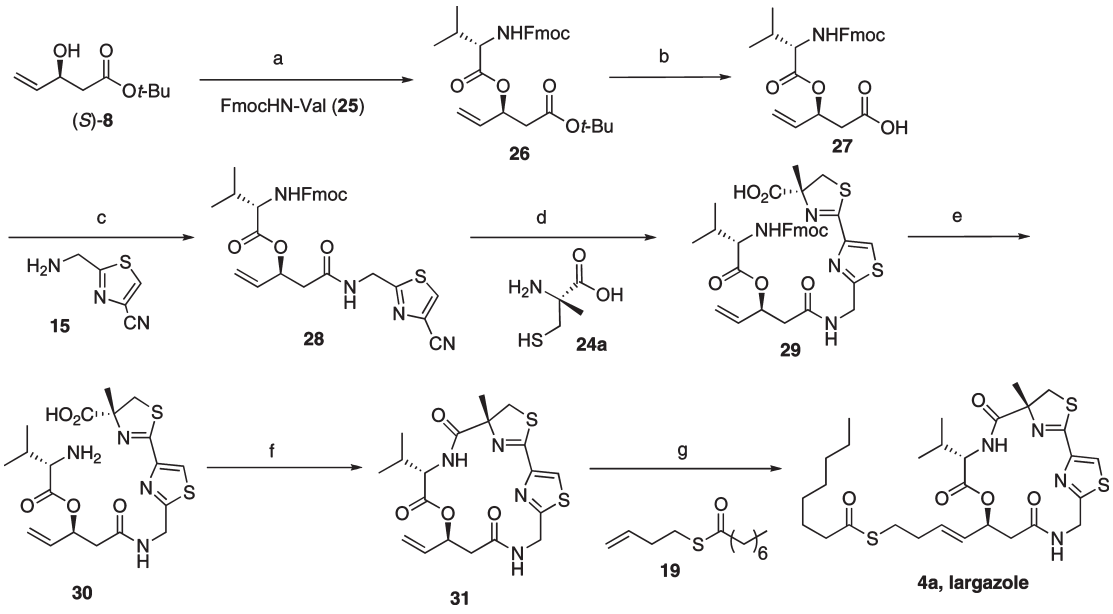
The second component for the synthesis of the bisthiazole unit was derived from 2-aminomethyl-thiazole-4-carbonitrile **15** (Scheme 1). Addition of bromopyruvic acid **11** to thioamide **10** afforded thiazole C4-carboxylic acid **12**, which was converted into nitrile **14** in two steps: formation of amide **13**[38,50,51] from acid **12** and ammonia in the presence of hydroxybenzotriazole (HOBt) and dicyclohexylcarbodiimide (DCC) and its dehydration using trifluoroacetic anhydride and base (44% yield). The *tert*-butoxycarbonyl (Boc) group of **14** was released with trimethylsilyl iodide (TMSI) in acetonitrile as described to provide **15**.[38]

The octanoyl but-3-en-1-thiol **19** required for the olefin cross-metathesis was acquired following the precedents of Leusch et al.[34] Thioacetamide **16** was added to octanoyl chloride **17** to produce thioacid **18**, which was alkylated with 4-bromo-1-butene to afford terminal alkene **19** (Scheme 1).

For the preparation of the α-alkyl cysteines **24**, we followed the procedure described by Pattenden.[48] Treating L-cysteine **20** with pivalaldehyde **21** induced the formation of the thiazoline, and this was followed by formamide formation to afford **22** as a single diastereomer (Scheme 2). The deprotonation and reaction of the enolate of **22** (LDA, THF/DMPU, −90 °C) with the electrophile afforded the alkyl and benzyl derivatives with high stereoselectivity as a result of its capture opposite the bulky *tert*-butyl substituent. The yields of the substituted compounds are moderate at best but in line with those described. Unfortunately, the severe acidic reaction conditions required for the deprotection and hydrolysis of the thiazoline (5 M HCl, 110 °C) are incompatible with the halogenated benzyl derivatives, and the yields for **e** and **f** are very low. Therefore, only the remaining cysteine derivatives were advanced further in the synthetic sequence.

With the required building blocks at hand, we proceeded to construct the modified depsipeptide structures using largazole **4a** as target (Scheme 3). Coupling between β-hydroxyester (***S***)-**8** and Fmoc-L-valine **25** followed by hydrolysis of the *tert*-butyl ester **26**[37] provided acid **27**. Condensation with the amino group of 2-aminomethyl-thiazole-4-carbonitrile **15** using the standard activation of the carbonyl group[52,53] faced problems derived from the β-elimination of the acyloxy derivative. A comprehensive survey of reaction conditions

Scheme 3[a]



[a] Reagents and conditions: (a) Fmoc-L-valine **25**, DIC, DMAP, CH_2Cl_2, 97%; (b) TFA, CH_2Cl_2, 99%; (c) PyBOP, DMAP, CH_3CN, 1h, 93%; (d) $NaHCO_3$, DMF/H_2O; (e) piperidine, CH_3CN; (f) HATU, HOBt, DIPEA, CH_2Cl_2, 53% (two steps); (g) alkene **19**, second generation Hoveyda−Grubbs catalyst, DCE, 90 °C, 27%.

including the nature and amount of the activating agent, solvent, amine, and base was required to induce an efficient coupling. Only when **27** was added to **15** (1.5 equiv) previously treated with benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP, 2 equiv) and dimethylaminopyridine (DMAP, 4 equiv) in CH_3CN, high yields of **28** were produced, with only traces of the elimination product due to the excess base employed (low yields were obtained if the amount of DMAP was reduced).

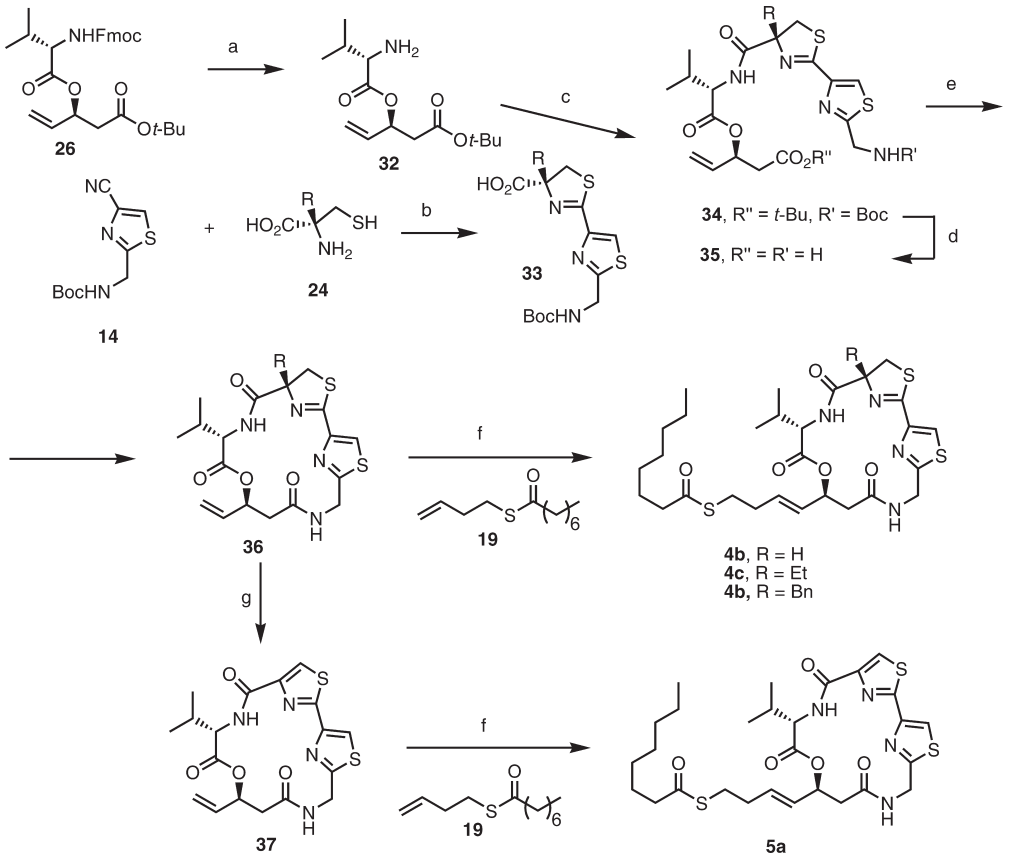
An additional optimization study was conducted for the coupling of the substituted cysteines **24** and nitrile **28**. Using α-methylcysteine **24a** as a model, the different variables (nature and amount of base, temperature, pH and reaction time) were systematically changed. Common undesired reactions such as carbamate hydrolysis and saponification of the ester at the valine residue were noted when the reaction progress was monitored by HPLC-MS. Only when excess α-methylcysteine (1.5 equiv relative to **28**) was used in the presence of $NaHCO_3$ in a DMF/H_2O mixture at 25 °C for 24 h could the Fmoc protected peptide **29** be obtained in 79% yield (Scheme 3).

To effect the macrocycle ring formation,[54] the open chain precursor **30** obtained by deprotection of the amino group of **29** was treated with *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU), HOBt, and di-isopropylethylamine (DIPEA) in CH_2Cl_2 as described previously,[34,36] affording **31** in 53% yield for the three steps. Cross-metathesis between alkene **19** and depsipeptide **31** was finally achieved in 27% yield (13% overall from **25** and **8**) upon treatment with Hoveyda−Grubbs catalysis, following the optimized conditions reported in the literature for these substrates.[38]

The sequence that worked successfully for largazole **4a** was extended to the remaining analogues, but we encountered unexpected difficulties in the condensation reaction of **24** with **28**, and only α-ethylcysteine **24c** (99%), α-benzylcysteine **24d** (90%), and cysteine **24b** (12%) afforded the desired products **29b−d**. The remaining derivatives and also serine showed a very low reactivity. Moreover, the cyclization of the condensation products **30b−d** after deprotection of **29b−d** was likewise inefficient (for R = Bn, H) or took place in low yield (R = Et, 7%). This failure called for a change on the strategy optimized for the synthesis of largazole **4a** and shifted the macrolactamation to the other peptide bond of the molecule (Scheme 4). Deprotection of the carbamate of β-acyloxyester **26** upon treatment with piperidine (97% yield) afforded amine **32**,[37] whereas condensation of nitrile **14** and the substituted cysteines **24** provided the thiazole-dihydrothiazole unit **33**.[38] Dissapointingly, the halogenated benzylcysteines did not undergo condensation with nitrile **14** under the reaction conditions previously optimized for the parent cysteine (**24b**, R = H, 97% yield). For ethylcysteine **24c** and benzylcysteine **24d**, the use of $NaHCO_3$ in a mixture of MeOH and phosphate buffer (**33c**, R = Et, 49%; **33d**, R = Bn, 52%) was critical for the success of the condensation. Serine also failed to react with **14**, despite the modifications on the nature of the base and the reaction temperature or the use of $ZnCl_2$ to activate the nitrile. The HATU-induced condensation of acid **33** and amine **32** provided in almost quantitative yield the open-chain precursor of the depsipeptide skeletons, **34**, which underwent deprotection upon treatment with TFA and Et_3SiH to afford **35**. A subsequent HATU-promoted intramolecular amide formation provided depsipeptides **36**. Cross-metathesis proceeded under the same conditions as for parent **4a** and produced the desired analogues in similar yields. Within the same scheme, application of the oxidative elimination reaction conditions to the dihydrothiazole depsipeptide **36** using 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU) and $BrCCl_3$ in CH_2Cl_2 allowed acquisition of the 2,4′-bithiazole derivative **37** in yields similar to those described for the same compound, and this step was followed by the acyclic cross-metathesis.[41]

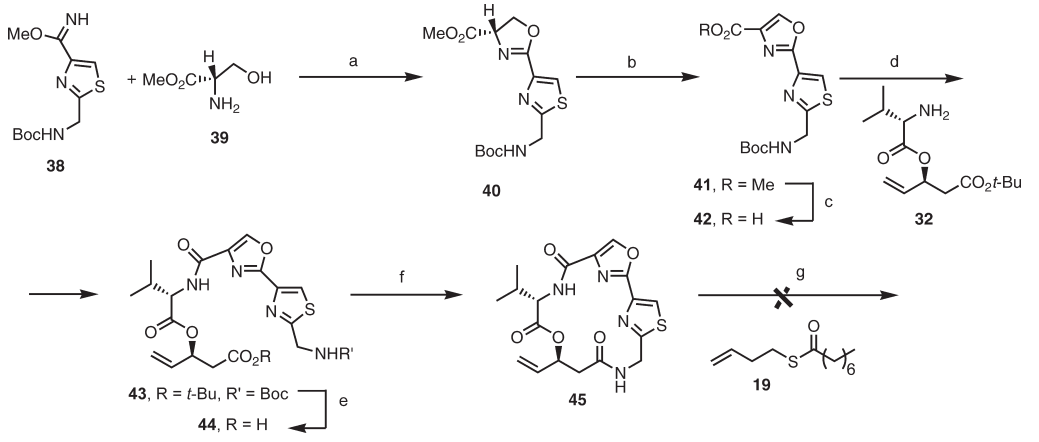
The preparation of the macrocycle with the thiazole-oxazole linear connection **5b** required a new synthetic route based on the formation of the imidate **38**, followed by condensation with serine methylester **39** to produce the thiazole-dihydroxazole unit **40** (Scheme 5). Application of the oxidative elimination

Scheme 4[a]



[a] Reagents and conditions: (a) piperidine, CH_3CN, 97%; (b) $NaHCO_3$, DMF, H_2O (phosphate buffer); (c) HATU, DIPEA, DMF (**34b**, 95%; **34c**, 89%; **34d**, 89%); (d) TFA, triethylsilane, CH_2Cl_2; (e) HATU, DIPEA, THF (**36b**, 87%; **36c**, 99%; **36d**, 88%); (f) alkene **19**, second generation Hoveyda−Grubbs catalyst, DCE, 90 °C (**4d**, 20%; **4c**, 23%; **4d**, 21%; **5a**, 35%); (g) DBU, $BrCCl_3$, CH_2Cl_2, 49%.

Scheme 5[a]



[a] Reagents and conditions: (a) SerOMe·HCl **39**, CH_2Cl_2, 60 °C, 45%; (b) DBU, pyridine, CCl_4, CH_3CN, 45%; (c) LiOH, THF; (d) **32**, HATU, DIPEA, DMF, 80% (two steps); (e) TFA, triethylsilane, CH_2Cl_2; (f) HATU, DIPEA, THF, 52%; (g) alkene **19**, second generation Hoveyda−Grubbs catalyst, DCE, 90 °C, 12 h.

reaction gave species **41** from which the desired cyclic product **45** was obtained as described above. Dissappointingly, the cross-metathesis reaction did not work in this case, in contrast to its successful use for the preparation of the α-methyldihydroxazole analogue reported by Williams and Schreiber,[41] and also for the synthesis of the parent system, previously documented by Phillips[37] and Cramer.[38]

Biological Evaluation. To evaluate the inhibitory activity of largazole (compound **4a**) and its derivatives (compounds **4b**, **4c**, **4d**, and **5a**) on human HDAC1 and HDAC4, two fluorimetric in vitro assays were performed. At 5 μM, these compounds showed inhibition on both isozymes (Figure 3), in particular of HDAC1, comparable to the pan-inhibitor SAHA[55] and class I-selective inhibitor

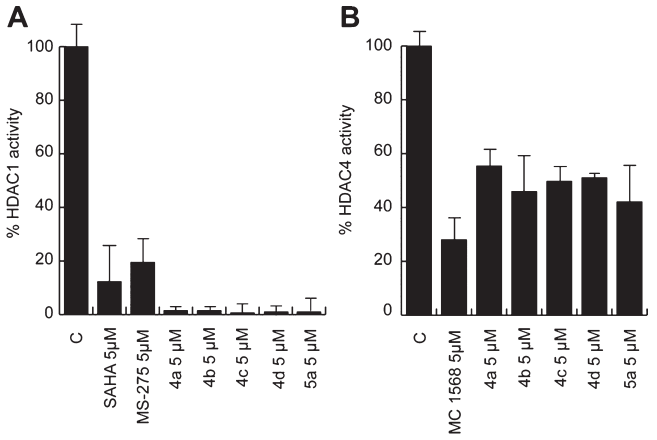


Figure 3. HDAC1 and HDAC4 in vitro assays in the presence of largazole and analogues. (A) HDAC1 inhibition by largazole and derivatives used at 5 μM; (B) HDAC4 inhibition on nonhistone substrate by largazole and derivatives used at 5 μM. SAHA, MS-275, and MC 1568 were used as reference compounds at 5 μM.

Table 1. Inhibitory Activity on Recombinant HDAC1 and HDAC4

compd	IC_{50} HDAC1 (nM)	IC_{50} HDAC4 (μM)
SAHA	120	6.2
4a (largazole)	11.4	3.0
4b	13.7	7.5
4c	161.3	4.9
4d	4.5	3.0
5a	4.7	3.0

N-(2-aminophenyl)-4-[*N*-(pyridin-3-ylmethoxycarbonyl)-aminomethyl]benzamide (MS-275)[56] used as positive controls (Figure 3a). The activity is more moderate in the HDAC4 assay and lower than that of the class II-specific inhibitor (*E*)-3-(4-((*E*)-3-(3-fluorophenyl)-3-oxoprop-1-enyl)-1-methyl-1*H*-pyrrol-2-yl)-*N*-hydroxyacrylamide (MC1568)[57] (Figure 3b) used at the same concentration. Nevertheless, the effect of the largazole derivatives **4b**−**d** seems to be comparable to that of the parent compound **4a**.

Values of IC_{50} for inhibition of these enzymes are given in Table 1. In addition to confirm the selectivity for the class I representative HDAC1 over the class II HDAC4, some other trends are observed in these measurements. The strongest inhibition of HDAC1 corresponds to the C7-benzyl and the bithiazole analogues (**4d** and **5a**, respectively). Addition of a methylene group at C7 (**4c**) results in a 10-fold decrease in inhibitory activity for HDAC1, whereas demethyl-largazole (**4b**) is roughly equipotent to the natural product.

NB4 cells stimulated with largazole **4a** and its derivatives **4b**, **4c**, and **4d** for 24 h at 1 μM showed a significant increase of pre-G_1 peak, similar to SAHA (Figure 4a). On the contrary, bithiazole **5a** (Figure 4a) did not alter the cell cycle even when used at 5 μM for 24 h (Figure 4b), and only a slight increase of pre-G_1 phase is noted after 48 h (Figure 4b). On the other hand, a massive cell death (Figure 4c) was induced when **4a**, **4b**, **4c**, and **4d** were used at 5 μM for 24 h. As reference compounds in these experiments, we selected not only the HDACis SAHA and MS-275, but also *all-trans*-retinoic acid (ATRA), because NB4 cells are characterized by the PML/RARα fusion protein and therefore they are sensitive to retinoic acid treatment.[58,59] Largazole and its analogues, with the exception of **5a**, have a comparable effect on the NB4 human leukemia cell line and are characterized as potent inducers of cell death even at low concentrations.

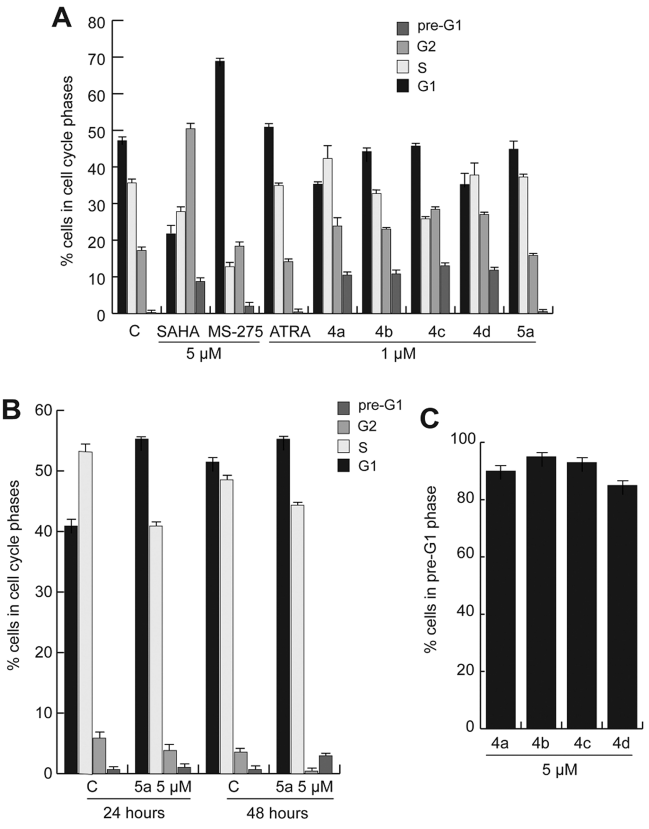


Figure 4. NB4 cell cycle analysis after treatment with largazole and analogues. (A) NB4 cell cycle analysis after 24 h treatment with largazole and derivatives at 1 μM; (B) NB4 cell cycle analysis after 24 or 48 h treatment with compound **5a** at 5 μM; (C) percentage of NB4 cells in pre-G_1 phase after treatment for 24 h with **4a**, **4b**, **4c**, and **4d** at 5 μM. ATRA (1 μM), SAHA, and MS-275 (both at 5 μM) were used as reference.

None of these compounds caused a significant increase of the CD11c basal expression levels on NB4 cells after treatment at 5 μM for 24 h (Figure 5a) or 48 h (Figure 5b) as indicated by the expression of this cell-surface marker using fluorescence-activated cell sorting (FACS). In fact, after stimulation, only a population of about 5% cells was CD11c positive.

Largazole derivatives **4b**, **4c**, and **4d** acetylate tubulin more efficiently than parent compound **4a**, but they are less potent than SAHA in this assay (Figure 6a). The series of largazole analogues also induced the expression of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ (Figure 6b). Compound **5a**, instead, failed to induce $p21^{WAF1/CIP1}$ protein expression and tubulin acetylation. We also studied the histone H3 acetylation levels in histone extracts from NB4 cells treated with these compounds at 5 μM for 24 h. The Western blot analysis showed an increase of histone H3 acetylation due to largazole action in comparison with the untreated control (Figure 6c).

Discussion

The epigenetic deregulation[4] has gained considerable attention recently as a rival of genetic aberrations to explain the onset and progression of cancer.[60,61] The epigenetic "marks" on chromatin, namely post-translational modifications of nucleosomal proteins and methylation of particular DNA sequences, are accomplished by enzymes that are part of multisubunit complexes.[62] These enzymes target the (un)packing of chromatin

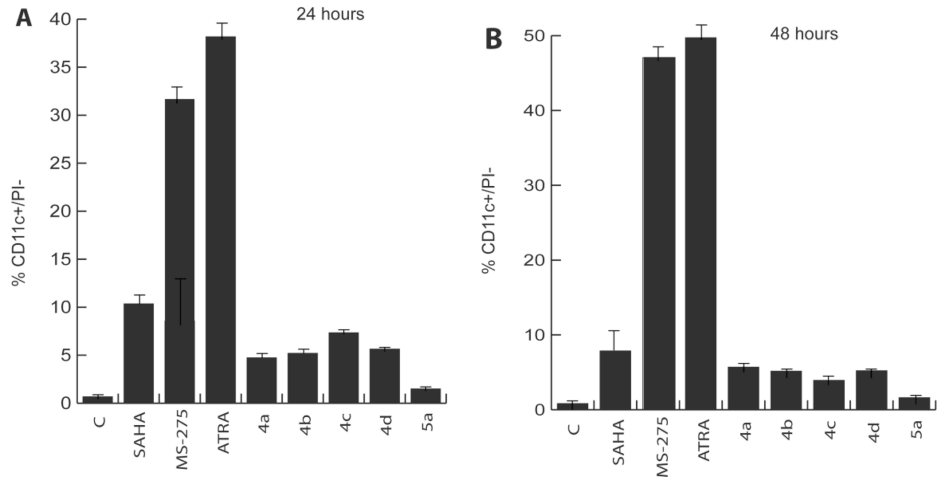


Figure 5. NB4 differentiation analysis. (A) CD11c expression after treatment with largazole and analogues at 5 μM for 24 h; (B) CD11c expression after treatment with largazole and analogues at 5 μM for 48 h. ATRA (1 μM), SAHA, and MS-275 (both at 5 μM) were used as reference.

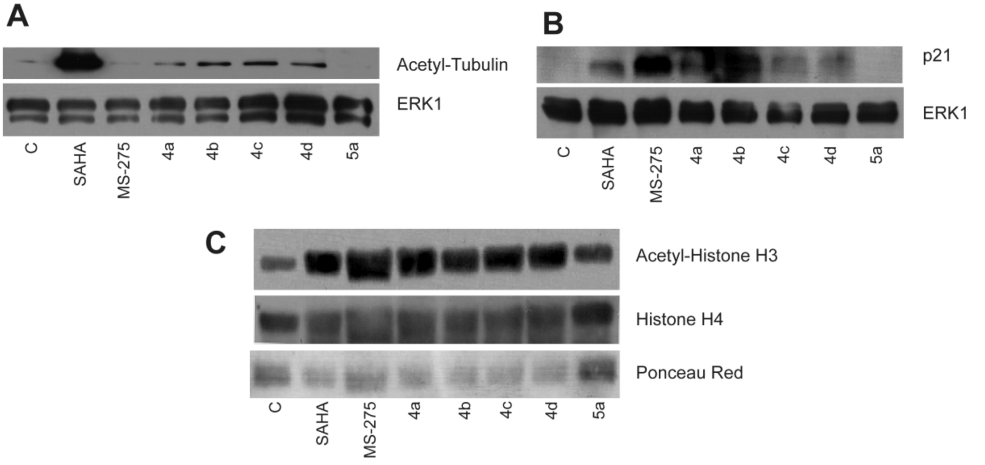


Figure 6. Western Blot analysis of NB4 cells after treatment with largazole derivatives or reference compounds at 5 μM for 24 h. (A,B) Acetyl-tubulin and p21 expression in total cell lysates from NB4 cells stimulated with largazole analogues; (C) acetylation of histone H3 in histone extracts stimulated with largazole analogues. ERK1 and histone H4 show loading control.

via reversible acetylation (HAT enzymes)/deacetylation (HDAC enzymes) of lysine residues,[7] methylation (HMTs)/demethylation (HDMs) of histone lysine and arginine residues,[63] and DNA methylation (DNMT enzymes)/demethylation, among other covalent modifications.[64] Because the accesibility of chromatin to the transcriptional machinery is an important step in the regulation of transcriptional events, chromating-remodeling enzymes, in particular HDACs and DNMTs, are new promising targets of the so-called "epigenetic drugs" for the treatment of cancer.[9−12,65] We and others have demonstrated that upon treatment with an HDAC inhibitor (HDACi), tumour necrosis factor-related apoptosis inducing ligand (TRAIL) is up-regulated and mediates acute apoptosis induced in both AML cells or patients blasts.[66,67]

Histone deacetylase inhibitors are currently entering therapy.[9−12] Following the approval of SAHA in 2006 for the treatment of cutaneous T-cell lymphoma, FK228 has recently been marketed for the same indication. Whereas the former features a hydroxamic acid as Zn^{2+} chelating group, a stronger Zn^{2+}-binding thiolate[33] is hidden in the structure of FK228 in the form of a cyclic disulfide bonded with a cysteine residue of the depsipeptide skeleton. In this regard, the novel depsipeptide natural product largazole[29] features a similar functional group of the same length but protected as an octanoyl thioester. Several synthesis and biological evaluation studies of largazole **4a** and analogues have confirmed the potent and selective (for transformed vs nontransformed cancer cells)[29] activity of the natural product, which is largely due to the inhibition of HDACs as primary target.[34] The structural and functional similarity of largazole with FK228 suggests also a common mechanism of action in the inhibition of the Zn^{2+}-dependent subfamily of HDAC enzymes (classes I, II, and IV). Therefore, both natural products are pro-drugs of the biologically active thiol derivatives, as shown by cell-based studies.[31,35] Whereas largazole exhibits a potency similar to that of SAHA, largazole thiol is a picomolar inhibitor of HDAC1/2.[35] The capping octanoyl group in largazole should make the compound more membrane permeable and more stable under physiological conditions relative to the free thiol, which would then be released by the action of a thioesterase within the cells.[35]

The flexibility of the modular approach to the synthesis of largazole **4a** has allowed access to a series of analogues and

provided preliminary SAR studies.[24] Among them, largazole homologues[36] and largazole alcohol,[34] largazole epimer,[34] largazole analogues with alanine scanning,[34] largazole bromide, side-chain hydrocarbon derivatives, homologues and ester at the side chain,[38] the peptide isostere,[42] thiols (instead of thioesters) of analogues with thiazole-thiazole, thiazoline-pyridine, and the oxazoline-oxazole[41] containing depsipeptides, *ent*-largazole, as well as chimeras with other Zn-chelating groups (*o*-anilinobenzamides, thiomethylketones, thioacetamides),[41] the chain length homologues,[41] and their combinations with the above head groups have all been synthesized.

SAR studies combining the above information have already shed some light onto the structural determinants of the potency of largazole **4a** as HDAC inhibitor,[24] most importantly the requirement for a spacer length of 4 carbon atoms linking the thioester to the depsipeptide, the need of an intact depsipeptide structure (the acyclic precursors are not active), the critical configuration at C17, the similar potency of other thioesters, but the lack of activity of an ester or ketone, as well as the reduced activity of compounds with alternative Zn-chelating groups (hydroxamic acid, *o*-anilinobenzamide). Modifications at other positions are less important for activity: L-valine can be replaced by other amino acids, the thiazoline methyl group can be replaced by a H or oxidized to the thiazole, and the dihydroxazoline can substitute the dihydrothiazoline. The pyridine-by-thiazole replacement, remarkably, increased the activity 3−4-fold, and this derivative is the most potent HDACi reported.[24]

We also started our synthetic work aimed at developing a flexible synthetic approach to the natural depsipeptide on route to related analogues. Focusing on the regions in the natural product that could be modified, we reasoned that the thiazole-thiazoline unit was very important for the activity of largazole because it is one of the structural signatures of this depsipeptide when compared to the other members of the same family (see Figure 1) and directed our efforts toward the thiazoline heterocycle. Our efforts culminated in the preparation of the natural product **4a** by N−C6(O) late stage cycloamidation and acyclic olefin cross-metathesis. Although conceptually similar, with formation of the N−C=O bond at C6 as key step, the synthesis of the dihydrothiazole-thiazole dimer is different in the approach of Luesch et al.[34,36] Ghosh carried out the cross-metathesis at an early stage, and the thioester was made compatible with the reaction conditions employed along the synthetic route.[39] All other approaches carried out the N−C15(O) macrolactamization followed by cross-metathesis, which was the bond construction tactic followed in this work for the derivatives that differ from largazole by the nature of the group at C7 (**4b**−**d**). Although the number of analogues planned was much larger, the difficulties in some of the steps led us to discard other substituents, in particular halogenated benzyl derivatives. The 2,4′-bisthiazole **5a** was likewise constructed using as key step the N−C15(O) lactamization, but this strategy failed in the case of the thiazole-oxazole counterpart **5b**.

The biological evaluation of the largazole series **4a**−**d** and oxidized derivative **5a** included the characterization of the HDAC inhibitory profile using recombinant HDAC1 and HDAC4 as representative of class I and class II isozymes, respectively, as well as the effects on cell cycle and in the induction of differentiation of the NB4 leukemia cell line. In addition, functional assays included the expression levels of acetylated tubulin and $p21^{WAF1/CIP1}$, as well as global endogenous H3 acetylation by immunoblot analysis. Consistent with previous results, we show that largazole **4a** and C7-substituted analogues **4b**−**d** are potent inhibitors of HDAC, with selectivity for class I HDAC1 over class II HDAC4. Values of IC_{50} for inhibition of these enzymes are nano- and micromolar, respectively, with the strongest inhibition of HDAC1 due to the C7-benzyl and the bithiazole analogues (**4d** and **5a**, respectively). An ethyl group at C7 (**4c**) results in a 10-fold decrease in inhibitory activity for HDAC1 when compared to the natural product.

Largazole and synthetic derivatives have been tested in HCT-116 cells,[34,43] human malignant melanoma cell lines,[35] the human epithelial carcinoma cell line A432 and the pre-adipocyte cell line,[38] the transformed MDA-MB231 and non-transformed HME epithelial cell lines,[29,37] the transformed U2OS and nontransformed fibroblastic osteosarcoma,[29] and the HT29 colon and IMR-32 neuroblastoma cell lines.[29]

In the case of the maturation inducible NB4 cell line with a t(15;17) marker isolated from a human acute promyelocytic leukemia,[68] we have noticed massive apoptosis for all compounds (with the exception of **5a**) and only a moderate effect on cell differentiation, in contrast to the effect of other HDACis (SAHA, MS-275) or *all-trans*-retinoic acid.

In these cells, the HDACi action of the analogues **4a**−**d** modified at the dihydrothiazole ring is correlated with the expression of $p21^{WAF1/CIP1}$, tubulin acetylation, and global H3 acetylation levels as shown by immunoblotting. However, the compounds are less potent than SAHA. Similar observations in other cell lines[35] confirmed the "activities" of the corresponding thiols and the pro-drug hypothesis of these depsipeptides.[35] Clearly the potency of largazole thiol and analogues should be higher than those exhibited by the octanoyl thioesters. Thioester prodrugs of synthetic HDAC have been previously described.[69,70]

The moderate pro-apoptotic activity of the bithiazole **5a** is consistent with recent studies.[43] Evaluation in HCT-116 colon cancer cells revealed the significant decrease of the activity of **5a** (and its enantiomer at C17) relative to largazole both as HDACi and as antiproliferative agent. In NB4 cells, however, **5a** is 2-fold more potent than largazole. From the SAR standpoint, the results shown indicate the relative insensitivity of the C7 position in largazole to the size of the substituents with regard to their biological activities.

Summary and Conclusions

The depsipeptide largazole **4a** has been synthesized using a N−C6(O) late stage cycloamidation of a precursor depsipeptide followed by acyclic olefin cross-metathesis. Non-natural derivatives with different substituents at C7 (**4b**−**d**) as well as 2,4′-bithiazole **5a** were prepared by the alternatively N−C15-(O) macrolactamization. The series of largazole analogues **4a**−**d** showed a potent inhibition of recombinant HDAC1 and HDAC4 enzymes as representative members of class I and class II HDACs, with a marked selectivity for HDAC1, for which they exhibit nanomolar potency. They also exhibited strong pro-apoptotic effects on the NB4 leukemia cell line, although they lacked the ability to induce their differentiation to mature granulocytes. The in vitro activities correlated with functional assays because the expression levels of acetylated tubulin and $p21^{WAF1/CIP1}$, as well as global endogenous H3 acetylation, were increased as shown by immunoblot analysis, after treating NB4 cells with the compounds. Thus, substitution of the methyl group at C7 of the dihydrothiazole ring in largazole **4a** by groups of different size (H, Et, Ph) does not

greatly modify the profile of the parent natural product, although the C7-benzyl derivative and the bithiazole analogue showed increased (2-fold) potency for inhibition of HDAC1.

Experimental Section

General. Reagents and solvents were purchased as reagent grade and used without further purification unless otherwise stated. Solvents were dried according to standard methods and distilled before use or dispensed from a Puresolv solvent purification system of Innovative Technology, Inc. All reactions were performed in oven-dried or flame-dried glassware under an inert atmosphere of Ar unless otherwise stated. Chromatography refers to flash chromatography (FC) on SiO_2 60 (230−400 mesh) from Merck, head pressure of ca. 0.2 bar. TLC: UV_{254} SiO_2-coated plates from Merck, visualization by UV light (254 nm) or by spraying with a 15% ethanolic phosphomolybdic acid solution. NMR spectra were recorded in a Bruker AMX400 (400.13 and 100.61 MHz for proton and carbon, respectively) spectrometer at 298 K with residual solvent peaks as internal reference and the chemical shifts are reported in δ [ppm], coupling constants J are given in [Hz], and the multiplicities assigned with DEPT experiments and expressed as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. COSY, HMBC, and HSQC methods were used to establish atom connectivities. Electrospray ionization (ESI) mass spectra were recorded on a Bruker APEX3 instrument. Infrared spectra (IR) were obtained on a JASCO FT/IR-4200 infrared spectrometer. Peaks are quoted in wave numbers (cm^{-1}), and their relative intensities are reported as follows: s = strong, m = medium, w = weak. Melting points were measured in a Stuart Scientific apparatus. Elemental analyses were carried out in a Fisons EA-1108 elemental analyzer. Optical rotation values were determined in a Jasco P-1020 polarimeter. Irradiation with microwaves was performed using a CEM DISCOVER apparatus. Purity of the final products was assesed by HPLC to be greater than 95%. The HPLC traces are provided as Supporting Information.

2-(*tert*-Butoxycarbonylaminomethyl)-thiazole-4-carboxylic Acid (12). General procedure for the synthesis of thiazole derivatives. To a solution of *tert*-butyl 2-amino-2-thioxoethylcarbamate (**10**) (2.0 g, 10.6 mmol) in EtOH (80 mL) were added 3-bromo-2-oxopropanoic acid (2.6 g, 16 mmol) and $CaCO_3$ (1.6 g, 16 mmol), and the mixture was stirred overnight. The reaction was filtered and washed with EtOH, and the solvent was evaporated. The residue was dissolved with EtOAc and washed with a 5% aqueous $KHSO_4$ solution (1×) and brine (2×), dried (Na_2SO_4), and the solvent was evaporated. Further purification by crystallization (acetone/hexane) afforded 2.43 g (99%) of a brown solid identified as 2-(*tert*-butoxycarbonylaminomethyl)-thiazole-4-carboxylic acid (**12**); mp 178−179 °C (hexane/EtOAc). ^{1}H NMR (400.16 MHz, CD_3OD): δ 8.29 (s, 1H), 4.52 (s, 2H), 1.47 (s, 9H) ppm. ^{13}C NMR (100.62 MHz, CD_3OD): δ 172.5 (s), 162.6 (s), 156.8 (s), 146.8 (s), 127.8 (d), 79.6 (s), 41.6 (t), 27.3 (q, 3×) ppm. IR (NaCl): ν 3600−3000 (b, O−H), 3098 (w, C−H), 2978 (w, C−H), 1685 (s, C=O), 1507 (m, C=O), 1162 (m) cm^{-1}. MS (ESI^+): 297 ([M + K]$^+$, 29), 281 ([M + Na]$^+$, 100), 259 ([M$^+$ + H], 7), 224 (22), 203 (60). HRMS (ESI^+): calcd for $C_{10}H_{14}N_2O_4SNa$, 281.0567; found, 281.0573.

2-(*tert*-Butoxycarbonylaminomethyl)-thiazole-4-carboxamide (13). To a solution of 2-(*tert*-butoxycarbonylaminomethyl)-thiazole-4-carboxylic acid (**12**) (0.5 g, 1.90 mmol) in DMF (20 mL) were added HOBt (0.3 g, 1.90 mmol) and NH_3 (0.94 mL, 1.90 mmol, 2 M in MeOH). After cooling down to 0 °C, DCC (0.6 g, 2.9 mmol) was added and the mixture was stirred at room temperature for 12 h. The reaction mixture was filtered, and the solids were washed with EtOAc. A saturated aqueous $NaHCO_3$ solution was added, and after separating the layers, the aqueous layer was extracted with EtOAc (3×). The combined organic layers were washed with brine (2×), dried (Na_2SO_4), and the solvent was evaporated. The residue was purified by column chromatography (silica gel, 95:5 CH_2Cl_2/MeOH) to afford 256 mg (52%) of a white solid identified as 2-(*tert*-butoxycarbonylaminomethyl)-thiazole-4-carboxamide (**13**); mp 160−161 °C (hexane/EtOAc). Elemental analysis: calcd for $C_{10}H_{15}N_3O_3S$, C, 46.68; H, 5.88; found, C, 46.66; H, 5.62. ^{1}H NMR (400.16 MHz, $(CD_3)_2CO$): δ 8.09 (s, 1H), 7.36 (br, 1H), 6.95 (br, 1H), 6.82 (br, 1H), 4.55 (d, J = 6.2 Hz, 2H), 1.44 (s, 9H) ppm. ^{13}C NMR (100.62 MHz, $(CD_3)_2CO$): δ 173.2 (s), 164.1 (s), 157.8 (s), 152.2 (s), 125.4 (d), 80.8 (s), 44.1 (t), 29.5 (q, 3×) ppm. IR (NaCl): 3600−3000 (br, N−H), 2976 (m, C−H), 2930 (w, C−H), 1670 (s, C=O), 1519 (s, C=O), 1368 (m), 1283 (m), 1166 (s) cm^{-1}. MS (ESI^+): 296 ([M + K]$^+$, 20), 280 ([M + Na]$^+$, 100), 258 ([M + H]$^+$, 5), 224 (23), 202 (50). HRMS (ESI^+): calcd for $C_{10}H_{15}N_3O_3SNa$, 280.0726; found, 280.0720.

2-(*tert*-Butoxycarbonylaminomethyl)-thiazole-4-carbonitrile (14). To a cooled (0 °C) solution of 2-(*tert*-butoxycarbonylaminomethyl)-thiazole-4-carbothioamide (**13**) (0.067 g, 0.26 mmol) in CH_2Cl_2 (0.5 mL) were sequentially added Et_3N (0.097 mL, 0.702 mmol) and freshly distilled $(CF_3CO)_2O$ (0.05 mL, 0.338 mmol). After stirring for 1 h at 0 °C and 1 h at room temperature, H_2O was added and the mixture was extracted with CH_2Cl_2 (3×), the combined organic layers were dried (Na_2SO_4), and the solvent was evaporated. The residue was purified by column chromatography (silica gel, 80:20 hexane/EtOAc) to afford 32 mg (57%) of a white solid identified as 2-(*tert*-butoxycarbonylaminomethyl)-thiazole-4-carbonitrile (**14**); mp 147−148 °C (hexane/acetone). Elemental analysis: calcd for $C_{10}H_{13}N_3O_2S$, C, 50.19; H, 5.48; found, C, 50.20; H, 4.91. ^{1}H NMR (400.16 MHz, $CDCl_3$): δ 7.96 (s, 1H), 5.31 (br, 1H), 4.63 (d, J = 6.1 Hz, 2H), 1.48 (s, 9H) ppm. ^{13}C NMR (100.62 MHz, $CDCl_3$): δ 171.8 (s), 155.7 (s), 131.0 (d), 126.3 (s), 113.9 (s), 80.8 (s), 42.3 (t), 28.3 (q, 3×) ppm. IR (NaCl): ν 3500−3200 (br, N−H), 3110 (w, C−H), 2978 (m, C−H), 2933 (w, C−H), 2236 (m, C≡N), 1699 (s, C=O), 1519 (s), 1281 (s), 1251 (s), 1165 (s) cm^{-1}. MS (ESI^+): 262 ([M + Na]$^+$, 46), 240 ([M + H]$^+$, 61), 184 (100). HRMS (ESI^+): calcd for $C_{10}H_{14}N_3O_2S$, 240.08012; found, 240.08037.

(*S*)-*tert*-Butyl 3-Hydroxypent-4-enoate (*S*)-8.[37] To a cooled (0 °C) solution of diisopropylamine (13.22 mL, 94.7 mmol) in THF (100 mL) was added *n*-BuLi (59 mL, 94.7 mmol, 1.6 M in hexane), and the reaction mixture was stirred for 15 min. The reaction was cooled down at −78 °C, and a solution of *tert*-butyl acetate (11.6 mL, 86.09 mmol) in THF (50 mL) was added. After stirring for 1 h, acrolein **6** (5.75 mL, 86.09 mmol) was added and the mixture was stirred for 1 h at −78 °C and for 30 min at room temperature. A saturated aqueous NH_4Cl solution was added and the mixture was extracted with Et_2O (3×), the combined organic layers were dried (Na_2SO_4), and the solvent was evaporated to afford 14.34 g (97%) of a colorless oil identified as *tert*-butyl 3-hydroxypent-4-enoate (**8**). To a solution of the residue obtained above (9.0 g, 52.25 mmol) in pentane (242 mL) was added Amano lipase (from *Bulkholderia cepacia*, 4.45 g), 4 Å molecular sieves (8.2 g), and vinylacetate (4.8 mL, 52.25 mmol) and the reaction was stirred for 18 h at 30 °C. The reaction was filtered through a celite pad, the solids were washed with hexane, and the solvents were evaporated. The residue was purified by column chromatography (silica gel, 90:10 hexane/EtOAc) to afford 4.14 g (37%) of a colorless oil identified as (*S*)-*tert*-butyl 3-acetoxypent-4-enoate (***S*)-9**, which was dissolved in MeOH (40 mL). The solution was cooled down to 0 °C, K_2CO_3 (5.33 g, 38.6 mmol) was added, and the resulting mixture was stirred 30 min. The solids were filtered off and washed with EtOAc, the organic solution was washed with H_2O (1×) and brine (1×), dried (Na_2SO_4), and the solvent was evaporated to afford 3.22 g (97%) of a colorless oil identified as (*S*)-*tert*-butyl 3-hydroxypent-4-enoate (***S*)-8**. ^{1}H NMR (400.16 MHz, $CDCl_3$): δ 5.89 (ddd, J = 17.2, 10.5, 5.5 Hz, 1H), 5.32 (td, J = 17.2, 1.5 Hz, 1H), 5.16 (td, J = 10.5, 1.4 Hz, 1H), 4.6−4.5 (m, 1H), 2.52 (dd, J = 16.2, 4.0 Hz, 1H) 2.44 (dd, J = 16.2, 8.3 Hz, 1H), 1.48 (s, 9H) ppm. ^{13}C NMR (100.62 MHz, $CDCl_3$): δ 171.8 (s), 138.9 (d), 115.2 (t), 81.7 (s), 69.0 (d), 42.0 (t), 28.1 (q, 3×) ppm. IR (NaCl):

ν 3600−3100 (br, O−H), 2978 (m, C−H), 2922 (s, C−H), 2852 (m, C−H), 1727 (s, C=O), 1367 (m), 1157 (m) cm^{-1}. MS (ESI^+): 195 ($[M + Na]^+$, 100), 173 ($[M + H]^+$, 28), 155 (8), 117 (98). HRMS (ESI^+): calcd for $C_9H_{16}O_3Na$, 195.0992; found, 195.0997.

tert*-Butyl (3*S*,2′*S*)-3-(-2-(9*H*-fluoren-9-ylmethoxycarbonylamino)-3-methylbutanoyloxy)-pent-4-enoate (26).**[37] To a solution of (*S*)-*tert*-butyl 3-hydroxypent-4-enoate (S*-8**) (1.26 g, 7.31 mmol) in CH_2Cl_2 (37 mL) was added Fmoc-(L)-valine (2.98 g, 8.78 mmol). The reaction was cooled down to 0 °C, and a solution of DMAP (0.89 g, 7.31 mmol) and DIC (1.37 mL, 8.78 mmol) in CH_2Cl_2 (7 mL) was added. After stirring the mixture for 10 min at 0 °C, and for 2 h at room temperature, a phosphate buffer solution (pH = 7, 7 mL) was added, the layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (3×), dried (Na_2SO_4), and the solvent was evaporated. The residue was purified by column chromatography (silica gel, 90:10 hexane/EtOAc) to afford 3.50 g (97%) of a colorless foam identified as (3*S*,2′*S*)-*tert*-butyl-3-(-2-(9*H*-fluoren-9-ylmethoxycarbonylamino)-3-methylbutanoyloxy)-pent-4-enoate (**26**). ^1H NMR (400.16 MHz, $CDCl_3$): δ 7.79 (d, J = 7.6 Hz, 2H) 7.63 (d, J = 7.5 Hz, 2H), 7.43 (t, J = 7.4 Hz, 2H), 7.34 (t, J = 7.4 Hz, 2H), 5.84 (ddd, J = 17.2, 10.5, 6.7 Hz, 1H), 5.70 (dd, J = 13.8, 6.7 Hz, 1H), 5.37 (d, J = 17.1 Hz, 1H), 5.32 (d, J = 9.5 Hz, 1H), 5.26 (d, J = 10.5 Hz, 1H), 4.41 (d, J = 7.2 Hz, 2H), 4.34 (dd, J = 9.2, 4.6 Hz, 1H), 4.25 (t, J = 7.1 Hz, 1H), 2.70 (dd, J = 15.6, 7.9 Hz, 1H), 2.57 (dd, J = 15.7, 6.1 Hz, 1H), 2.3−2.2 (m, 1H), 1.44 (s, 9H), 1.00 (d, J = 6.9 Hz, 3H), 0.91 (d, J = 6.9 Hz, 3H) ppm. ^{13}C NMR (100.62 MHz, $CDCl_3$): δ 170.8 (s), 168.6 (s), 156.1 (s), 143.9 (s), 143.7 (s), 141.2 (s, 2×), 134.5 (d), 127.6 (d, 2×), 127.0 (d, 2×), 125.0 (d, 2×), 119.9 (d, 2×), 118.4 (t), 81.3 (s), 72.2 (d), 66.9 (t), 58.8 (d), 47.1 (d), 40.4 (t), 31.3 (d), 27.9 (q, 3×), 19.0 (q), 17.2 (q) ppm. IR (NaCl): ν 3500−3200 (br, N−H), 2961 (m, C−H), 2920 (s, C−H), 2849 (s, C−H), 1731 (s, C=O), 1509 (m, C=C), 1450 (m, C=C), 1156 (m) cm^{-1}. MS (ESI^+): 516 ($[M + Na]^+$, 22), 494 ($[M + H]^+$, 48), 438 (100). HRMS (ESI^+): calcd for $C_{29}H_{36}NO_6$ 494.2537; found: 494.2537.

(2*S*,3′*S*)-(5-(4-Cyanothiazol-2-ylmethylamino)-5-oxopent-1-en-3-yl) 2-(9*H*-fluoren-9-ylmethoxycarbonylamino)-3-methylbutanoate (28). To a solution of (3*S*,2′*S*)-*tert*-butyl 3-(-2-(9*H*-fluoren-9-ylmethoxycarbonylamino)-3-methylbutanoyloxy)-pent-4-enoate (**26**) (0.44 g, 0.99 mmol) in CH_2Cl_2 (4.3 mL) at 0 °C was added TFA (4.3 mL, 45.7 mmol), and the reaction was stirred for 2 h at room temperature. The solvent was evaporated to afford (3*S*,2′*S*)-(9*H*-fluoren-9-ylmethoxycarbonylamino)-3-methylbutanoyloxy)-pent-4-enoic acid (**27**), which was used in the next step without further purification. To a solution of the residue obtained above (**14**) (0.357 g, 1.49 mmol) in CH_3CN (14 mL) at 0 °C was added TMSI (0.42 mL, 2.98 mmol), and the mixture was stirred for 30 min. The reaction was warmed up to room temperature, and a solution of polymer-bound diisopropylamine (0.427 g) in MeOH (10 mL) was added and the resulting mixture was stirred for 10 min. The solids were filtered off washed with CH_3CN and MeOH, and the solvents were removed to afford 2-(aminomethyl)-thiazole-4-carbonitrile (**15**), which was used in the next step without further purification. To a cooled (0 °C) solution of the product **27** (0.99 mmol) and 2-(aminomethyl)-thiazole-4-carbonitrile (**15**) (1.49 mmol) in CH_3CN (14 mL) was added via cannula a solution of PyBOP (1.03 g, 1.98 mmol) and DMAP (0.48 g, 3.96 mmol) in CH_3CN (1 mL). After stirring at room temperature for 1 h, the solvent was removed in vacuo and the residue was dissolved in EtOAc. The solution was washed with a saturated aqueous NH_4Cl solution (3×), water (1×), and brine (1×), dried (Na_2SO_4), and evaporated to dryness The residue was purified by column chromatography (silica gel, 60:40 hexane/EtOAc) to afford 0.51 g (93%) of a colorless foam identified as (2*S*,3′*S*)-(5-(4-cyanothiazol-2-ylmethylamino)-5-oxopent-1-en-3-yl) 2-(9*H*-fluoren-9-ylmethoxycarbonylamino)-3-methylbutanoate (**28**). $[\alpha]^{25}_D$ −15.2° (*c* 0.55, MeOH); mp 154−155 °C (hexane/acetone). ^1H NMR (400.16 MHz, $CDCl_3$): δ 7.82 (s, 1H), 7.76 (dd, J = 7.5, 3.4 Hz, 2H). 7.56 (d, J = 7.5 Hz, 2H), 7.40 (dd, J = 7.4, 4.7 Hz, 2H), 7.32 (d, J = 7.4 Hz, 2H), 5.87 (ddd, J = 17.1, 10.5, 6.5 Hz, 1H), 5.66 (dd, J = 11.3, 6.3 Hz, 1H), 5.35 (d, J = 17.2 Hz, 1H), 5.25 (d, J = 10.3 Hz, 2H), 4.73 (dd, J = 14.8, 6.4 Hz, 1H), 4.65 (dd, J = 16.8, 6.0 Hz, 1H), 4.39 (dd, J = 10.8, 7.2 Hz, 1H), 4.32 (dd, J = 10.8, 6.8 Hz, 1H), 4.19 (t, J = 6.8 Hz, 1H), 4.02 (t, J = 7.0 Hz, 1H), 2.65 (dd, J = 14.0, 5.6 Hz, 1H), 2.60 (dd, J = 14.8, 7.2 Hz, 1H), 2.09 (td, J = 13.0, 6.7 Hz, 1H), 0.97 (d, J = 6.9 Hz, 3H), 0.95 (d, J = 6.9 Hz, 3H) ppm. ^{13}C NMR (100.62 MHz, $CDCl_3$): δ 171.3 (s), 170.3 (s), 169.6 (s), 156.6 (s), 143.6 (s), 143.5 (s), 141.3 (s), 141.2 (s), 134.0 (d), 131.3 (d), 127.8 (d), 127.7 (d), 127.2 (d), 127.1 (d), 125.9 (s), 125.0 (d), 124.9 (d), 120.0 (d), 119.9 (d), 118.5 (t), 113.8 (s), 72.3 (d), 67.2 (t), 59.7 (d), 47.0 (d), 41.1 (t), 41.0 (t), 30.6 (d), 19.0 (q), 18.0 (q) ppm. IR (NaCl): ν 3319 (br, N−H), 2964 (m, C−H), 2850 (m, C−H), 1715 (s, C=O), 1529 (s, C=C), 1241 (m), 1195 (m) cm^{-1}. MS (ESI^+): 581 ($[M + Na]^+$, 100), 559 ($[M + H]^+$, 50), 321 (22), 201 (28). HRMS (ESI^+): calcd for $C_{30}H_{31}N_4O_5S$, 559.2010; found, 559.1993.

Macrocycle 31.[34] General procedure for the synthesis of macrocycle (method A). To a solution of (2*S*,3′*S*)-(5-(4-cyanothiazol-2-ylmethylamino)-5-oxopent-1-en-3-yl) 2-(9*H*-fluoren-9-ylmethoxycarbonylamino)-3-methylbutanoate (**28**) (0.07 g, 0.125 mmol) in DMF (2.3 mL) was added α-methylcysteine (**24a**)[48] (0.043 g, 0.313 mmol), and the mixture was stirred at room temperature. A solution of $NaHCO_3$ (0.042 g, 0.5 mmol) in water (0.7 mL) was added, and the reaction was stirred at room temperature for 12 h. A second portion of $NaHCO_3$ (0.042 g, 0.5 mmol) and α-methylcysteine (0.043 g, 0.313 mmol) in water (0.7 mL) were added until total conversion of the starting material. The solids were filtered off, and the solution was acidified to pH = 1 by addition of formic acid and the solvents were removed under reduced pressure. The residue was dissolved in EtOAc and washed with H_2O (5×), dried (Na_2SO_4), and the solvent was evaporated to afford 74 mg of a yellow foam identified as the carboxylic acid derivative (**29**), which was used in the next step without further purification. To a solution of this residue (0.047 g, 0.069 mmol) in CH_3CN (0.9 mL) was added piperidine (0.027 mL, 0.28 mmol), and the resulting mixture was stirred at room temperature for 30 min. The solvents were removed, and the residue was used in the next step without further purification. To a cooled (0 °C) solution of the residue in CH_2Cl_2 (69 mL) were sequentially added HATU (0.052 g, 0.138 mmol), HOBt (0.021 g, 0.138 mmol), and DIPEA (0.047 mL, 0.276 mmol) and the reaction was allowed to reach room temperature and stirred for 12 h. The solvents were removed, and the residue was redissolved using a H_2O/EtOAc mixture. The aqueous layer was extracted with EtOAc (3×), and the combined organic layers were washed with brine, dried (Na_2SO_4), and the solvent was evaporated. The residue was purified by column chromatography (silica gel, 10:10:1 hexane/EtOAc/MeOH) to afford 16 mg (53% for the three steps) of a yellow foam, which was identified as the macrocycle **31**. ^1H NMR (400.16 MHz, $CDCl_3$): δ 7.83 (s, 1H), 7.14 (d, J = 9.2 Hz, 1H), 6.54 (br, 1H), 5.85 (ddd, J = 16.9, 10.6, 6.2 Hz, 1H), 5.7−5.6 (m, 1H), 5.36 (d, J = 17.3 Hz, 1H), 5.29 (d, J = 9.8 Hz, 1H), 5.28 (d, J = 10.1 Hz, 1H), 4.62 (dd, J = 9.3, 3.4 Hz, 1H), 4.26 (dd, J = 17.7, 3.1 Hz, 1H), 4.08 (d, J = 11.4 Hz, 1H), 3.32 (d, J = 11.4 Hz, 1H), 2.91 (dd, J = 14.8, 10.8 Hz, 1H), 2.70 (dd, J = 16.4, 2.6 Hz, 1H), 2.2−2.1 (m, 1H), 1.87 (s, 3H), 0.68 (d, J = 6.9 Hz, 3H), 0.51 (d, J = 6.9 Hz, 3H) ppm. ^{13}C NMR (100.62 MHz, $CDCl_3$): δ 173.0 (s), 169.4 (s), 168.7 (s), 168.4 (s), 167.9 (s), 146.8 (s), 134.7 (d), 125.3 (d), 118.1 (t), 83.7 (s), 72.5 (d), 57.8 (d), 43.3 (t), 41.1 (t), 40.1 (t), 34.1 (d), 23.8 (q), 18.9 (q), 16.6 (q) ppm. MS (ESI^+): 459 ($[M + Na]^+$, 100), 437 ($[M + H]^+$, 33), 321 (8). HRMS (ESI^+): calcd for $C_{19}H_{24}N_4O_4S_2$, 437.1312; found, 437.1317

Largazole (4a).[34] General procedure for the metathesis reaction. A solution of the macrocycle (**31**) (0.016 g, 0.037 mmol) and (*S*)-but-3-enyl octanethionate (**6.72**) (0.032 g, 0.148 mmol) in 1,2-dichloroethane (0.3 mL) was set in a sealed tube, and a solution of second generation Hoveyda−Grubbs catalyst (3.5 mg,

0.005 mmol) in 1,2-dichloroethane (0.1 mL) was added. The mixture was briefly degassed and then stirred at 90 °C overnight. The solvent was removed, and the residue was purified by column chromatography (silica gel, 10:10:1 hexane/EtOAc/MeOH) to afford 6 mg (27%) of a dark-yellow oil identified as largazole (**4a**). ^{1}H NMR (400.16 MHz, $CDCl_3$): δ 7.85 (s, 1H), 7.19 (d, J = 8.8 Hz, 1H), 6.64 (br, 1H), 5.86 (td, J = 15.0, 6.8 Hz, 1H), 5.68 (t, J = 8.8 Hz, 1H), 5.56 (dd, J = 15.8, 6.8 Hz, 1H), 5.34 (dd, J = 17.7, 9.1 Hz, 1H), 4.63 (dd, J = 9.0, 3.1 Hz, 1H), 4.30 (d, J = 17.1 Hz, 1H), 4.12 (d, J = 10.9 Hz, 1H), 3.34 (d, J = 11.4 Hz, 1H), 3.1−3.0 (m, 1H), 2.93 (t, J = 7.3 Hz, 2H), 2.69 (dd, J = 16.3, 1.5 Hz, 1H), 2.55 (t, J = 7.5 Hz, 2H), 2.34 (q, J = 7.2 Hz, 2H), 2.2−2.1 (m, 1H), 1.78 (s, 3H), 1.7−1.6 (m, 2H), 1.4−1.2 (m, 8H), 0.90 (t, J = 6.5 Hz, 3H), 0.70 (d, J = 6.9 Hz, 3H), 0.54 (d, J = 6.8 Hz, 3H) ppm. ^{13}C NMR (100.62 MHz, $CDCl_3$): δ 199.3 (s), 173.0 (s), 169.6 (s), 168.7 (s), 168.2 (s), 165.5 (s), 147.1 (s), 132.8 (d), 128.4 (d), 124.7 (d), 83.6 (s), 71.9 (d), 57.9 (d), 44.2 (t), 43.3 (t), 41.1 (t), 40.5 (t), 34.1 (d), 32.3 (t), 31.6 (t), 28.9 (t, 2×), 27.9 (t), 25.7 (t), 23.8 (q), 22.6 (t), 18.8 (q), 16.7 (q), 14.1 (q) ppm. MS (ESI$^+$): 645 ([M + Na]$^+$, 57), 623 ([M + H]$^+$, 100), 413 (15), 321 (51). HRMS (ESI$^+$): calcd for $C_{29}H_{43}N_4O_5S_3$, 623.2390; found, 623.2379.

(3*S*,2′*S*)-*tert*-Butyl 3-(2-Amino-3-methylbutanoyloxy)pent-4-enoate (32).[37] Following the general procedure described above for the cleavage of Fmoc-carbamates, the reaction of (3*S*,2′*S*)-*tert*-butyl-3-(-2-(9*H*-fluoren-9-ylmethoxycarbonylamino)-3-methylbutanoyloxy)-pent-4-enoate (**26**) (0.6 g, 1.22 mmol) and piperidine (0.48 mL, 4.86 mmol) in CH_3CN (23 mL) afforded, after purification by column chromatography (silica gel, 50:50 hexane/EtOAc) 320 mg (97%) of a colorless oil identified as (3*S*,2′*S*)-*tert*-butyl 3-(2-amino-3-methylbutanoyloxy)pent-4-enoate (**32**). ^{1}H NMR (400.16 MHz, $CDCl_3$): δ 5.85 (ddd, J = 17.1, 10.5, 6.6 Hz, 1H), 5.68 (dd, J = 13.6, 6.7 Hz, 1H), 5.36 (td, J = 17.2, 1.1 Hz, 1H), 5.24 (td, J = 10.5, 1.0 Hz, 1H), 3.30 (d, J = 4.8 Hz, 1H), 2.65 (dd, J = 15.4, 8.0 Hz, 1H), 2.55 (dd, J = 15.4, 5.7 Hz, 1H), 2.2−2.0 (m, 1H), 1.65 (br, 2H), 1.46 (s, 9H), 1.00 (d, J = 6.9 Hz, 3H), 0.91 (d, J = 6.9 Hz, 3H) ppm. ^{13}C NMR (100.62 MHz, $CDCl_3$): δ 174.2 (s), 168.5 (s), 134.7 (d), 117.5 (t), 80.8 (s), 71.1 (d), 59.5 (d), 40.2 (t), 31.6 (d), 27.7 (q, 3×), 18.4 (q), 16.6 (q) ppm. IR (NaCl): ν 3500−3300 (br, N−H), 2963 (s, C−H), 2923 (s, C−H), 2850 (s, C−H), 1735 (s, C=O), 1458 (m, C=C), 1368 (m), 1157 (s) cm^{-1}. MS (ESI$^+$): 294 ([M + Na]$^+$, 5), 272 ([M + H]$^+$, 100). HRMS (ESI$^+$): calcd for $C_{14}H_{26}NO_4$, 272.1856; found, 272.1869.

(*R*)-2-(2-*tert*-Butoxycarbonylaminomethyl-thiazol-4-yl)-4-ethyl-4,5-dihydrothiazole-4-carboxylic acid (33c). General procedure for the condensation of nitriles and alkylcysteines. To a solution of 2-(*tert*-butoxycarbonylaminomethyl)-thiazole-4-carbonitrile (**14**) (0.043 g, 0.18 mmol) and ethylcysteine hydrochloride (**24c**) (0.053 g, 0.29 mmol) in MeOH (2.3 mL) and phosphate buffer (pH = 7, 1.4 mL) was added $NaHCO_3$ (0.076 g, 0.9 mmol) and the reaction was stirred for 24 h at 70 °C. H_2O (1 mL) and a saturated aqueous $NaHCO_3$ solution (1 mL) was added and the mixture was extracted with EtOAc (3×). The aqueous layer was acidified until pH = 1 by addition of $KHSO_4$ and then extracted with EtOAc (4×). The combined organic layers were washed with brine, dried (Na_2SO_4) and the solvent was evaporated. The residue was purified by column chromatography (C18 silica gel, CH_3CN) to afford 30 mg (49%) of a colorless foam identified as (*R*)-2-(2-*tert*-butoxycarbonylaminomethyl-thiazol-4-yl)-4-ethyl-4,5-dihydrothiazole-4-carboxylic acid (**33c**). $[\alpha]^{27}_D$ −2.97° (*c* 0.31, MeOH). ^{1}H NMR (400.16 MHz, $CDCl_3$): δ 8.11 (s, 1H), 5.39 (br, 1H), 4.65 (d, J = 5.8 Hz, 2H), 3.91 (d, J = 11.8 Hz, 1H), 3.42 (d, J = 11.8 Hz, 1H), 2.10 (q, J = 7.4 Hz, 2H), 1.49 (s, 9H), 1.07 (t, J = 7.4 Hz, 3H) ppm. ^{13}C NMR (100.62 MHz, $CDCl_3$): δ 174.3 (s), 170.3 (s), 166.0 (s), 155.7 (s), 147.4 (s), 123.5 (d), 88.3 (s), 80.6 (s), 42.3 (t), 38.6 (t), 31.8 (t), 28.3 (q, 3×), 8.6 (q) ppm. IR (NaCl): ν 3500−3200 (br, N−H), 2972 (m, C−H), 2927 (m, C−H), 1705 (s, C=O), 1654 (m, C=C), 1515 (s, C=C), 1249 (m), 1169 (s) cm^{-1}. MS (ESI$^+$): 391 ([M + Na]$^+$, 16), 372 ([M + H]$^+$, 100). HRMS (ESI$^+$): calcd for $C_{15}H_{22}N_3O_4S_2$ 372.1046; found, 372.1039.

(3*S*,2′*S*,4″*R*)-*tert*-Butyl 3-(2-(2-(2-(*tert*-Butoxycarbonylaminomethyl-thiazol-4-yl)-4-ethyl-4,5-dihydrothiazole-4-carboxamido)-3-methylbutanoyloxy)pent-4-enoate (34c). General procedure for the formation of peptide bonds. To a solution of (3*S*,2′*S*)-*tert*-butyl 3-(2-amino-3-methylbutanoyloxy)pent-4-enoate (**32**) (0.02 g, 0.075 mmol) and (*R*)-2-(2-*tert*-butoxycarbonylaminomethyl-thiazol-4-yl)-4-ethyl-4,5-dihydrothiazole-4-carboxylic acid (**33**) (0.028 g, 0.075 mmol) in DMF (0.5 mL) were added HATU (0.034 g, 0.09 mmol) and DIPEA (0.039 mL, 0.225 mmol) and the reaction was stirred at room temperature for 2 h. A phosphate buffer (pH = 7, 0.5 mL) was added, and the mixture was extracted with EtOAc (3×). The combined organic layers were washed with H_2O (2×), brine (2×), and dried (Na_2SO_4) and the solvent was removed under vacuum. The residue was purified by column chromatography (silica gel, 80:20 hexane/EtOAc) to afford 14 mg (89%) of a colorless oil identified as a mixture of conformers of (3*S*,2′*S*,4″*R*)-*tert*-butyl 3-(2-(2-(2-(*tert*-butoxycarbonylaminomethyl-thiazol-4-yl)-4-ethyl-4,5-dihydrothiazole-4-carboxamido)-3-methylbutanoyloxy)pent-4-enoate (**34c**). $[\alpha]^{25}_D$ −29.3° (*c* 1.067, MeOH). ^{1}H NMR (400.16 MHz, $CDCl_3$) (major conformer): δ 7.99 (s, 1H), 7.14 (d, J = 9.0 Hz, 1H), 5.81 (ddd, J = 17.2, 10.4, 6.8 Hz, 1H), 5.66 (dd, J = 13.7, 6.8 Hz, 1H), 5.35 (d, J = 17.2 Hz, 1H), 5.23 (d, J = 10.4 Hz, 1H), 4.63 (d, J = 6.1 Hz, 2H), 4.55 (dd, J = 9.2, 4.6 Hz, 1H), 3.81 (d, J = 11.7 Hz, 1H), 3.34 (d, J = 11.7 Hz, 1H), 2.69 (dd, J = 15.5, 7.9 Hz, 1H), 2.57 (dd, J = 15.5, 9.5 Hz, 1H), 2.19 (dq, J = 11.4, 6.8 Hz, 1H), 2.04 (dq, J = 15.0, 7.5 Hz, 1H), 1.93 (qd, J = 14.6, 7.4 Hz, 1H), 1.47 (s, 9H), 1.43 (s, 9H), 0.99 (t, J = 5.9 Hz, 3H), 0.86 (d, J = 6.8 Hz, 3H), 0.79 (d, J = 6.9 Hz, 3H) ppm. ^{13}C NMR (100.62 MHz, $CDCl_3$) (major conformer): δ 174.0 (s), 170.5 (s), 168.7 (s), 160.8 (s), 155.7 (s), 134.6 (d), 123.9 (d), 121.2 (s), 118.5 (t), 89.2 (s), 81.3 (s), 80.5 (s), 77.3 (d), 72.3 (d), 56.8 (d), 42.3 (t), 40.6 (t), 39.7 (t), 32.4 (t), 31.1 (d), 28.3 (q, 3×), 28.0 (q, 3×), 19.1 (q), 17.4 (q), 8.8 (q) ppm. IR (NaCl): ν 3500−3200 (br, N−H), 2920 (s, C−H), 2850 (s, C−H), 1733 (m, C=O), 1716 (m, C=O), 1683 (m, C=O) cm^{-1}. MS (ESI$^+$): 647 ([M + Na]$^+$, 37), 625 ([M + H]$^+$, 100), 534 (22), 512 (18). HRMS (ESI$^+$): calcd for $C_{29}H_{45}N_4O_7S_2$, 625.2724; found, 625.2728.

Ethyl-thiazoline Macrocycle 36c. General procedure for the synthesis of macrocycles (method B). (*S*)-*tert*-butyl 3-((*S*)-2-((*R*)-2-(2-*tert*-butoxycarbonylaminomethylthiazol-4-yl)-4-ethyl-4,5-dihydrothiazole-4-carboxamido)-3-methylbutanoyloxy)pent-4-enoate (**34c**) (0.02 g, 0.034 mmol) and triethylsilane (6.8 μL, 0.043 mmol) in CH_2Cl_2 (0.2 mL) and TFA (0.08 mL, 0.85 mmol) was stirred at 0 °C. After 4 h stirring, the solvents were removed and the residue was used in the next step without further purification. To a cooled (0 °C) solution of HATU (0.045 g, 0.119 mmol) and DIPEA (0.058 mL, 0.34 mmol) in THF (8.8 mL) was added a solution of the previously synthesized product (0.034 mmol) in THF (1.6 mL) and the reaction was stirred for 15 h, allowing it to reach room temperature. H_2O was added, and the layers were separated. The aqueous layer was extracted with EtOAc (3×), the combined organic layers were washed with brine (2×), dried (Na_2SO_4), and the solvent was evaporated under vacuum. The residue was purified by column chromatography (silica gel, 10:10:1 hexane:EtOAc/MeOH) to afford 18 mg (99%) of a colorless foam identified as the ethyl-thiazoline macrocycle **36c**. $[\alpha]^{25}_D$ +39.0° (*c* 0.24, MeOH). ^{1}H NMR (400.16 MHz, CD_3OD): δ 8.14 (s, 1H), 7.41 (d, J = 9.2 Hz, 1H), 5.96 (ddd, J = 16.6, 10.6, 5.8 Hz, 1H), 5.8−5.6 (m, 1H), 5.30 (dt, J = 17.3, 1.1 Hz, 1H), 5.25 (dt, J = 10.6, 1.0 Hz, 1H), 5.16 (d, J = 17.6 Hz, 1H), 4.6−4.5 (m, 1H), 4.39 (d, J = 17.6 Hz, 1H), 3.87 (d, J = 11.6 Hz, 1H), 3.45 (d, J = 11.6 Hz, 1H), 2.98 (dd, J = 16.5, 11.2 Hz, 1H), 2.79 (dd, J = 16.5, 2.7 Hz, 1H), 2.24 (qd, J = 14.7, 7.4 Hz, 1H), 2.12 (dtd, J = 13.7, 6.9, 3.4 Hz, 1H), 2.1−1.9 (m, 1H), 1.13 (t, J = 7.4 Hz, 3H), 0.74 (d, J = 6.9 Hz, 3H), 0.51 (d, J = 6.9 Hz, 3H) ppm. ^{13}C NMR (100.62 MHz, $CDCl_3$): δ 174.2 (s), 170.9 (s), 168.8 (s), 168.4 (s), 166.2 (s), 147.0 (s), 135.1 (d), 125.0 (d), 116.0 (t), 88.1 (s), 72.5 (d),

57.4 (d), 40.4 (t), 40.2 (t), 39.0 (t), 34.0 (d), 29.8 (t), 18.3 (q), 15.6 (q), 8.6 (q) ppm. IR (NaCl): ν 3500−3200 (br, N−H), 2919 (s, C−H), 2850 (s, C−H), 1712 (m, C═O), 1649 (m, C═O), 1513 (m, C═C) cm^{-1}. MS (ESI^+): 473 ($[M + Na]^+$, 100), 451 ($[M + H]^+$, 11). HRMS (ESI^+): calcd for $C_{20}H_{26}N_4O_4S_2Na$, 473.1288; found, 473.1288.

Ethyl-thiazoline Largazole Analogue 4c. Following the general procedure described above for the metathesis reaction, ethylthiazoline macrocycle **36c** (0.01 g, 0.022 mmol), (*S*)-but-3-enyl octanethioate (**19**) (0.019 g, 0.088 mmol), and second generation Hoveyda−Grubbs catalyst (2.8 mg, 0.004 mmol) in 1,2-dichloroethane (0.23 mL) afforded, after purification by column chromatography (silica gel, 10:10:1 hexane/EtOAc/MeOH), 3 mg (21%) of a yellow foam identified as the ethylthiazoline thioester **4c**. $[\alpha]^{25}_D$ +15.5° (*c* 0.228, MeOH). ^1H NMR (400.16 MHz, $CDCl_3$): δ 7.74 (s, 1H), 6.49 (d, J = 8.7 Hz, 1H), 5.9−5.8 (m, 1H), 5.68 (t, J = 7.2 Hz, 1H), 5.53 (dd, J = 15.4, 5.9 Hz, 1H), 5.26 (dd, J = 17.4, 8.9 Hz, 1H), 4.61 (dd, J = 9.4, 3.1 Hz, 1H), 4.30 (d, J = 15.9 Hz, 1H), 3.95 (d, J = 11.2 Hz, 1H), 3.32 (d, J = 11.3 Hz, 1H), 2.88 (dd, J = 14.4, 7.3 Hz, 1H), 2.80 (dd, J = 16.6, 9.3 Hz, 1H), 2.70 (dd, J = 16.0, 2.4 Hz, 1H), 2.51 (t, J = 7.3 Hz, 2H), 2.28 (dt, J = 14.2, 6.7 Hz, 2H), 2.1−2.0 (m, 2H), 1.7−1.5 (m, 2H), 1.4−1.2 (m, 8H), 1.13 (t, J = 7.0 Hz, 3H), 0.87 (t, J = 6.0 Hz, 5H), 0.70 (d, J = 6.8 Hz, 3H), 0.53 (d, J = 6.7 Hz, 3H) ppm. ^{13}C NMR (100.62 MHz, $CDCl_3$): δ 199.4 (s), 173.0 (s), 169.4 (s), 168.7 (s), 167.6 (s), 164.6 (s), 147.6 (s), 132.3 (d), 128.4 (d), 123.9 (d), 88.7 (s), 71.5 (d), 57.8 (d), 44.2 (t), 41.2 (t), 41.1 (t), 40.7 (t), 34.0 (d), 32.3 (t), 31.6 (t), 30.3 (t), 28.9 (t, 2×), 28.0 (t), 25.6 (t), 22.6 (t), 18.9 (q), 16.8 (q), 14.1 (q), 10.0 (q) ppm. IR (NaCl): ν 2920 (s, C−H), 2850 (s, C−H), 1739 (w, C═O), 1712 (w), 1649 (w), 1538 (w), 1513 (w), 1460 (m) cm^{-1}. MS (ESI^+): 659 ($[M + Na]^+$, 53), 637 ($[M + H]^+$, 48), 245 (42). HRMS (ESI^+): calcd for $C_{30}H_{45}N_4O_5S_3$, 637.2547; found, 637.2564.

Chemicals. SAHA (Merck) and MS 275 (Bayer-Schering AG) were dissolved in DMSO (Sigma-Aldrich) and used at 5 μM concentration. The largazole derivatives were dissolved in DMSO and used at 5 or 50 μM as indicated.

Cell Lines. NB4 human acute promyelocytic cell line was grown in RPMI 1640 medium (Euroclone) supplemented with heat-inactivated FBS, 1% glutamine, 1% penicillin/streptomycin, and 0.1% gentamycin at 37 °C in air and 5% CO_2.

HDAC1 and 4 Fluorimetric Assay. HDACs assays were performed in the presence of 3 mM HDAC buffer (16.7 mM Tris-HCl pH 8, 45.7 mM NaCl, 0.9 mM KCl, 0.3 mM $MgCl_2$) and 5 μg of BSA. For these experiments 0.25 μg of human recombinant HDAC1 or 2.5 ng of human recombinant HDAC4 were added to the reaction mixture and after a preincubation of 15 min at 37 °C was also added a 50 μM solution of the specific substrate. The reaction was carried out for 1 h at 37 °C with gentle shaking, and fluorescence was quantified with a TECAN Infinite M200 station.

Cell Cycle Analysis by FACS. NB4 cells (2.5×10^5) were collected by centrifugation after 24 h stimulation with reference compounds or largazole analogues. NB4 cells were resuspended in 500 μL of ahypotonic buffer (0.1% NP-40, 0.1% sodium citrate, 50 μg/mL PI, RNase A) and incubated in the dark for 30 min. The analysis was performed using a FACS-Calibur (Becton Dickinson) with the Cell Quest Pro software (Becton Dickinson) and ModFit LT version 3 software (Verity). The experiments were performed in triplicate.

Granulocytic Differentiation Analysis. NB4 cells (2.5×10^5) were collected by centrifugation after 24 h of stimulation with reference compounds or largazole analogues. NB4 cells were washed with PBS and incubated in the dark at 4 °C for 30 min with 10 μL of PE-conjugated anti-CD11c surface antigen antibody or with 10 μL of PE-conjugated IgG, as isotopic control in order to remove the background. At the end of incubation, the samples were washed and resuspended in 500 μL of PBS containing 0.25 μg/mL PI. The analysis was performed by FACS-Calibur (Becton Dickinson) using the Cell Quest Pro software (Becton Dickinson). The experiments were performed in triplicate, and PI positive cells were excluded from the analysis.

Protein Extraction. NB4 cells were treated with reference compounds or largazole derivatives and harvested by centrifugation. After PBS wash, the samples were resuspended in a lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1%NP40, 10 mM sodium fluoride, 1 mM PMSF and protease inhibitor cocktail), and the lysis reaction was carried out for 15 min at 4 °C. The samples were centrifuged at 13000 rpm for 30 min at 4 °C, and the proteins were quantified by the Bradford assay (Bio-Rad).

Histone Extraction. After stimulation with the compounds, the cells were collected by centrifugation and washed two times with PBS. Then the samples were resuspended in Triton extraction buffer (PBS containing 0.5% Triton X 100 (v/v), 2 mM PMSF, 0.02% (w/v) NaN_3), and the lysis was performed for 10 min at 4 °C. Next, the samples were centrifugated at 2000 rpm for 10 min at 4 °C, and the pellets were washed in TEB (half the volume). After a new centrifugation under the same conditions, the samples were resuspended in 0.2 N HCl and the acid histone extraction was carried out overnight at 4 °C. The next day, the supernatant was recovered by centrifugation and the protein content was ensured by Bradford assay (Bio-Rad).

Western Blot. First, 50 μg of total protein for each sample were loaded on 10% polyacrylamide gel in order to evaluate acetyl-tubulin expression (Sigma Aldrich, dilution 1:500), while the same amount of proteins but on 15% polyacrylamide gel was used for $p21^{CIP1/WAF1}$ analysis (Transduction Laboratories, dilution 1:500). ERK1 antibody (Santa Cruz, dilution 1:1000) was used as loading control.

In addition, 10 μg of histonic extracts were loaded on 15% polyacrylamide gel, and the histone H3 acetylation was assessed with antiacetyl-histone H3 antibody (Upstate, dilution 1:500). To check for equal loading, Ponceau Red (Sigma) staining and the antihistone H4 antibody (Abcam, dilution 1:500) were used.

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Supporting Information Available: Experimental procedures, analytical and spectral characterization data for compounds **33b**, **34b**, **36b**, **4b**, **33d**, **34d**, **36d**, **4d**, **37**, **5a**, **40**, **41**, **43**, **45**, **55**, **57**, and **58**. This material is available free of charge via the Internet at http://pubs.acs.org.

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