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Largazole and Analogues with Modified Metal-Binding Motifs Targeting Histone Deacetylases: Synthesis and Biological Evaluation

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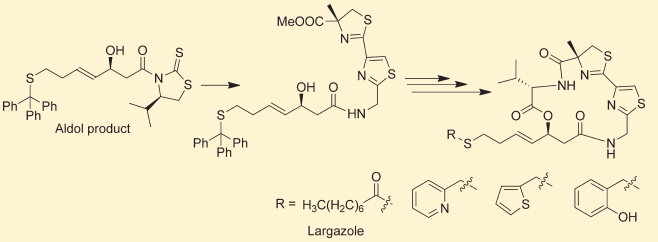
Supporting Information

ABSTRACT: The histone deacetylase inhibitor largazole **1** was synthesized by a convergent approach that involved several efficient and high yielding single pot multistep protocols. Initial attempts using *tert*-butyl as thiol protecting group proved problematic, and synthesis was accomplished by switching to the trityl protecting group. This synthetic protocol provides a convenient approach to many new largazole analogues. Three side chain analogues with multiple heteroatoms for chelation with Zn^{2+} were synthesized, and their biological activities were evaluated. They were less potent than largazole **1** in growth inhibition of HCT116 colon carcinoma cell line and in inducing increases in global H3 acetylation. Largazole **1** and the three side chain analogues had no effect on HDAC6, as indicated by the lack of increased acetylation of α-tubulin.



INTRODUCTION

A major challenge in cancer chemotherapy is the development of drugs that selectively kill cancer cells without affecting normal cells for increased potency and reduced toxic side effects. Changes in gene expression due to mutation, loss, or rearrangement are associated with cancer initiation and progression. With few exceptions, these changes have been difficult to target therapeutically. A new paradigm that is being extensively investigated as an anticancer drug development strategy is the targeting of epigenetic regulation of gene expression. Epigenetics refer to heritable alterations in gene expression that are not a result of changes in DNA sequences. In cancer, aberrant epigenetic silencing of several genes including tumor suppressor genes is a common occurrence.[1] Aberrant epigenetic silencing occurs as a result of aberrant promoter region CpG island DNA methylation in concert with changes in covalent modification of histone proteins.[1g] Enzymatic modifications such as acetylation, methylation, and phosphorylation of the lysine and arginine residues of the N-terminus of histones regulate the access to DNA by transcriptional factors, thereby regulating gene expression.[2] Histone acetylation modulated by two protein families, histone acetyltransferases (HATs) and histone deacetylases (HDACs), is the most extensively studied of these processes.[3] Most importantly, epigenetic changes, unlike DNA sequence changes, are reversible and thus there is interest in targeting epigenetic gene regulation as an anticancer strategy.

HDACs are a class of metalloenzymes responsible for removal of the acetyl group from lysine residues of histone and other non-histone substrate proteins. The deacetylated histone, positively charged at physiological pH, interacts with the negatively charged DNA phosphate backbone and can form transcriptionally inactive condensed forms of chromatin. These epigenetic changes can result in silencing of tumor suppressor genes promoting cancer initiation and/or progression. In some cases, inhibition of HDACs can be used to restore hyperacetylation to derepress or activate these undesirably silenced genes.[4] HDAC proteins are a family of at least 18 enzymes and are classified into four groups based on their size, cellular localization, active catalytic site numbers, and homology to yeast HDAC proteins:[5] class I, HDAC1, -2, -3, and -8; class IIa, HDAC4, -5, -7, and -9; class IIb, HDAC6 and -10; class III, sirtuin proteins; class IV, HDAC11. The HDAC proteins are associated with basic cellular functions and disease states such as cancer, but the importance of individual isoforms in cell function and cancer biology is not well understood. However, class I isoforms are regarded as promising cancer targets.[6] Two HDAC inhibitors (HDACis) suberoylanilide hydroxamic acid (SAHA, vorinostat) and FK228 (romidepsin) have recently been approved by the USFDA for the treatment of cutaneous T-cell lymphoma (CTCL). SAHA with a hydroxamic acid moiety as the metal binding domain is a pan-inhibitor of HDACs. FK228 has a thiol masked as a cyclic disulfide bond with a cysteine residue of the depsipeptide core. Many other HDACis are at different stages of clinical

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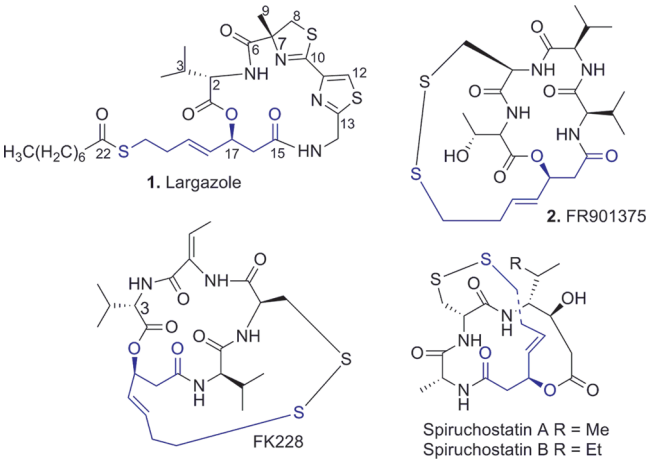


Figure 1. Natural HDAC inhibitors with anticancer properties.

investigation[7] with a preference for isoform-selective or class-selective inhibitors for higher therapeutic potential.[5] X-ray crystallography and SAR studies of HDAC inhibitors have shown that three key structural elements are involved in the binding of the inhibitor to the active site of the HDAC protein:[8] (a) a metal binding domain, which chelates with the Zn^{2+} cation present at the active site; (b) a surface recognition cap group which interacts with hydrophobic residues on the rim of the active site; (c) a linker, which mimics the *N*-acetyllysine side chain of histone and occupies a hydrophobic channel positioning the zinc binding moiety and the cap group in binding orientation.

Recently, a new natural product largazole (**1**, Figure 1), isolated from a marine cyanobacterium of the genus *Symploca* was reported to possess remarkable selective activity against highly invasive transformed human mammary epithelial cells (MDA-MB-231) and transformed fibroblastic osteosarcoma (U2OS) cells over the normal cell lines (NMuMG and NIH3T3, respectively).[9]

Mechanistic studies showed that largazole **1** is an inhibitor of HDACs.[10] It is a prodrug that undergoes hydrolysis of the thioester group by cellular esterases and/or lipases to release a free thiol function that constitutes the domain that chelates Zn^{2+}. The depsipeptide ring system represents the surface recognition cap group. The two domains are connected by a four-carbon olefinic linker. A striking similarity of largazole **1** to other natural HDAC inhibitors such as FK228,[10b] FR901375 **2**,[10b] and spiruchostatins (Figure 1) is that they all contain a masked 3-hydroxy-7-mercapto-4-heptenoic acid side chain. Upon activation, they release the free thiol as the active species.

Because of its highly selective activity on specific cancer cell lines and a strong and exceptional picomolar inhibitory bias for class I HDACs (HDAC1, -2, and -3) over class II HDAC6, largazole **1** has been subjected to extensive investigation since its discovery in 2008.[10b] A number of synthetic approaches to largazole **1** have appeared since then.[10a,b,11] Its high potency and selectivity, along with the presence of a metal-binding domain and a surface recognition group interacting with differently conserved regions of the receptor, make largazole **1** a fascinating lead molecule for further structural optimization in pursuit of molecules of higher potency and/or selectivity. An array of largazole analogues has been synthesized and their biological properties evaluated to uncover some of the SAR requirements of the molecule.[10,11,11c,11g−11i] These studies have

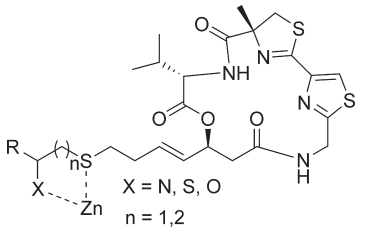


Figure 2. Largazole analogues targeted to Zn^{2+} binding motif.

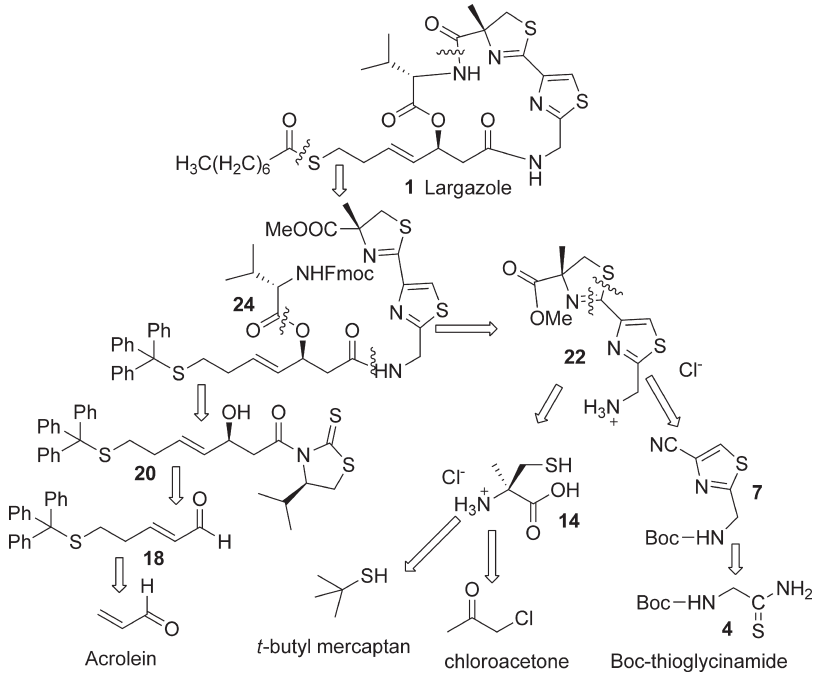
focused mostly on alterations in the cap group and to a lesser extent on the side chain. The four-atom linker between the hydrophobic cap group and the Zn^{2+} coordinating group in largazole **1** and other HDACis with a masked thiol as the Zn^{2+} binding domain such as FK228, spiruchostatins, and FR901375 **2** appears to be the optimal distance for highest potency in comparison to side chains with five to seven atoms which are common among most HDACis.[10d,11c] Replacement of the zinc-binding thiol of largazole **1** with a number of benzamide and thioamide headgroups failed to produce more potent analogues.[10c] The active sites of HDAC isoforms are highly conserved. However, the presence of an internal cavity within the active site has been exploited in designing isoform-selective HDACis with substituted benzamide derivatives as the zinc-binding group.[12] Therefore, it is conceivable that structural alterations in the zinc-binding domain of largazole **1** to modulate metal binding affinity may take advantage of such minor structural differences within the active site to develop isoform- or class-selective inhibitors. Isoform-selective inhibitors may help in understanding the role of different isoforms in cellular processes and disease states. Our approach to modifying the metal-binding domain consisted of introducing a second heteroatom in the side chain two to three atoms apart from sulfur and is thus capable of interacting with Zn^{2+} via a five- or six-membered cyclic transition state (Figure 2).

CHEMISTRY

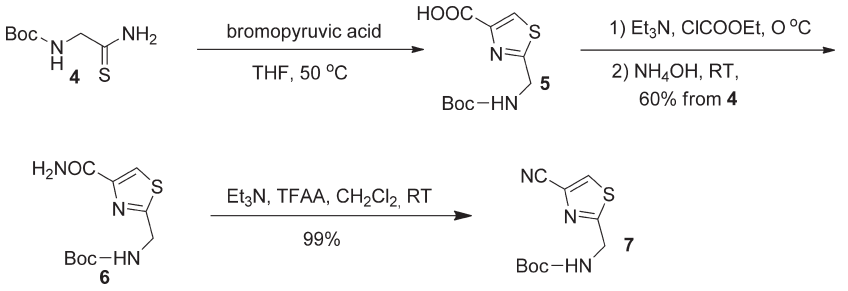
The convergent synthetic approach adopted as shown in retrosynthetic analysis (Scheme 1) involved macrolactamization and side chain thioesterification of the intermediate **24** which could be derived by acyl transfer from fragment **20** to **22** followed by coupling with Fmoc-valine. Intermediate **22** can be synthesized from the thiazole nitrile **7** and (*R*)-α-methylcysteine hydrochloride **14**. Synthesis of **14** would also generate its enantiomer useful for making the C-7 epimers of largazole **1** and analogues. Fragment **20** is the product of acetate aldol reaction of the aldehyde **18** using acetyl Nagao as the chiral auxiliary.[13] In our initial approach when *tert*-butyl was used as the thiol protecting group, deprotection and thioesterification to produce the final product became problematic (vide infra).

The synthesis of thiazole derivative **7** is shown in Scheme 2. Thiazole amide **6** was made in 60% overall yield from boc-thioglycinamide **4** and bromopyruvic acid in a one-pot reaction in which the initially formed carboxylic acid, after removal of water, was activated and treated with ammonia. This one-pot protocol was found to be more efficient and gave the product in higher yields and in a much shorter period of time than the previously reported methods.[11a] Amide **6** was converted to the nitrile **7** using standard conditions in 99% yield.[11a]

Scheme 1. Retrosynthetic Analysis of Largazole 1



Scheme 2. Synthesis of Fragment 7



The synthesis of (*R*)-α-methylcysteine HCl **14** and its (*S*)-enantiomer **15** was achieved via the enzymatic resolution of racemic precursor **11** (Scheme 3).[14] Reaction of chloroacetone **8** and *tert*-butyl mercaptan under basic conditions gave **9**, which was subjected to Bucherer−Berg conditions to produce hydantoin **10**. This one-pot protocol gave 90% yield of **10** after two steps. Hydantoin **10** was converted to **11** in one pot by hydrolysis with aqueous NaOH followed by KCNO treatment. Resolution of **11** with D-hydantoinase afforded **12** and **13** which were converted to **14** and **15**, respectively, in quantitative yields.[14]

Turning our attention to the synthesis of **20** (Scheme 4), conjugate addition of triphenylmethanethiol to acrolein **16** gave **17**, which was used in a Wittig reaction to yield **18**.[15] This one-pot protocol gave 65% overall yield for the two steps. Acetate aldol condensation of the aldehyde **18** with acetyl Nagao auxiliary was used to synthesize **20** in 84% diastereomeric excess.[13] The *S*-configuration of the newly created chiral center of molecule **20** was established by modified Mosher ester analysis.[16]

With the necessary building blocks in hand, the assembly of largazole **1** was undertaken as shown in Scheme 5. The nitrile **7** was condensed with (*R*)-α-methylcysteine HCl **14** to obtain the thiazole-thiazoline carboxylic acid **21**.[11a] After simultaneous removal of Boc group and esterification of the free carboxylic acid under acidic conditions in MeOH, the acyl group was transferred[17] from **20** to **22** to obtain **23**. The formation of **21** from nitrile **7** and the one pot conversion of **21** to **23** proved very efficient with 78% yield for three steps. Yamaguchi esterification[10a,18] was used to couple Fmoc-valine to **23** to afford the acyclic precursor **24**. After saponification and Fmoc group removal, macrocyclization with HOAt, HATU, and Hunig's base yielded the cyclized product **25** in 56% overall yield over the three steps.[10a] Deprotection of trityl group gave largazole thiol which was esterified with octanoyl chloride using Hunig's base to give largazole **1** in 79% yield (based on recovered largazole thiol in esterification step) over two steps.[10b]

Scheme 6 shows the general method used for the synthesis of largazole analogues with modification in the zinc-binding motif. These analogues are designed such that thiol and a second heteroatom in the side chain are two to three atoms apart and are thus capable of interacting with Zn^{2+} via five- or six-membered cyclic transition states (Figure 2).

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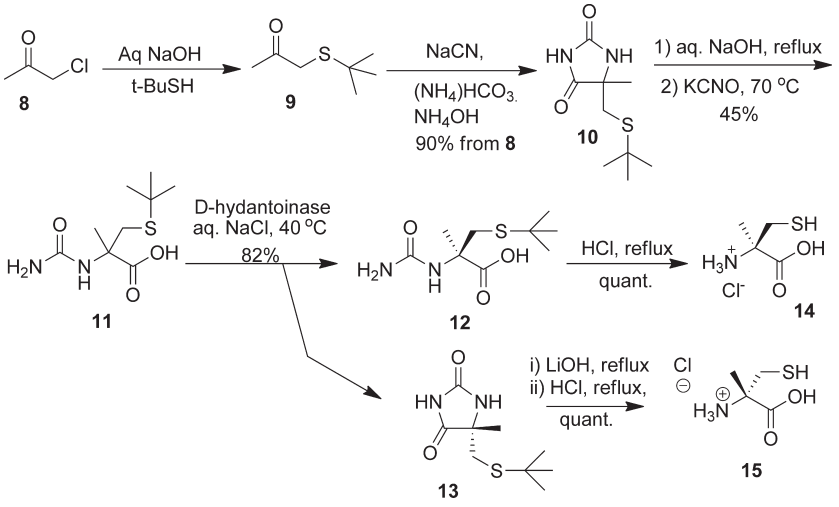
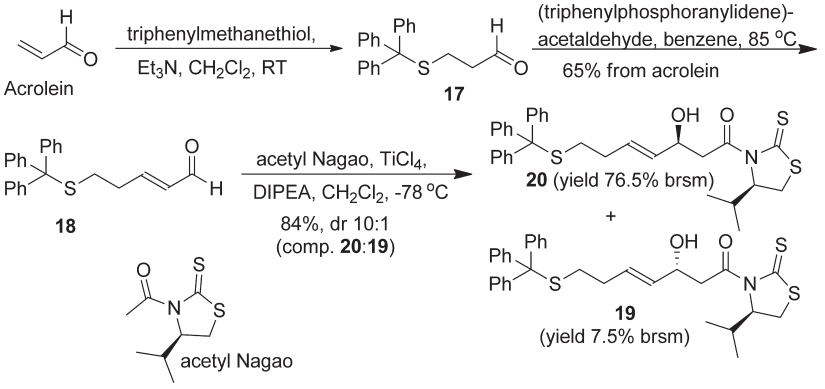
Scheme 3. Synthesis of (*R*)- and (*S*)-α-Methylcysteine



Scheme 4. Synthesis of the β-Hydroxycarboxamide 20



The analogues **27**, **28**, and **29** were synthesized following the removal of trityl group by nucleophilic substitution of the corresponding precursors by either method A (basic)[19] or one-pot method B (acidic)[20] as shown in Scheme 6.

In a previous attempt to synthesize largazole **1** by a similar approach, we used the *tert*-butyl group as the protecting group for thiol. The synthetic route to make the *tert*-butyl protected alcohol **35** is depicted in Scheme 7. Synthesis of alcohol **35** started from glycerol formal **30** which was converted to dioxene **31** in two steps. Alkylation of **31** with 2-[(2-bromoethyl)-sulfanyl]-2-methylpropane **32** via the lithium derivative gave **33**. Compound **33** underwent retro Diels–Alder reaction to give the aldehyde **34**[21] which was used in aldol reaction to furnish the alcohol **35**. It was used as described in Scheme 5 to make the cyclic core **38**. Attempts to remove the *tert*-butyl group as illustrated in Scheme 8 (1 M BBr_3,[22] trifluoroacetic acid, anisole, mercuric acetate[23]) and to esterify with octanoyl chloride to form largazole **1** failed (see Supporting Information for details). With BBr_3, starting material was recovered. Treatment with TFA/anisole/mercuric acetate indicated (TLC) the formation of a polar product which was subjected to thioesterification, but no largazole **1**, largazole thiol, or starting material was recovered.

BIOLOGICAL EVALUATION

The antiproliferative activity of analogues **27**, **28**, and **29** were evaluated in the HCT116 colon adenocarcinoma cell line by a standard 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) reduction assay (Promega) using largazole **1** as the control as previously reported.[24] Figure 3 shows the results of the MTS assay after 96 h of treatment with 10 nM, 100 nM, 1 μM, and 5 μM concentrations of the compounds. Largazole **1** inhibited the growth of HCT116 cells with a GI_{50} of 10 nM, whereas the three side chain analogues showed activity only at higher concentrations. The pyridine analogue **27** demonstrated the greatest effect on cell survival of the three with a GI_{50} of 1.0 μM.

In order to ascertain the inhibitory effects of these analogues, in vitro fluorimetric assays on recombinant HDACs were performed in the presence and absence of the analogues. Analogues **27** and **28** at 1 μM significantly inhibited HDAC1 activity by almost 80% (Figure 4). Analogue **29** had a modest inhibition of HDAC1, with only a ~30% decrease of activity. Largazole **1** demonstrated a decrease of HDAC1 activity similar to analogues **27** and **28**. Assays using HeLa nuclear extracts as a source for class 1 and class 2 HDAC activity were nearly identical to what

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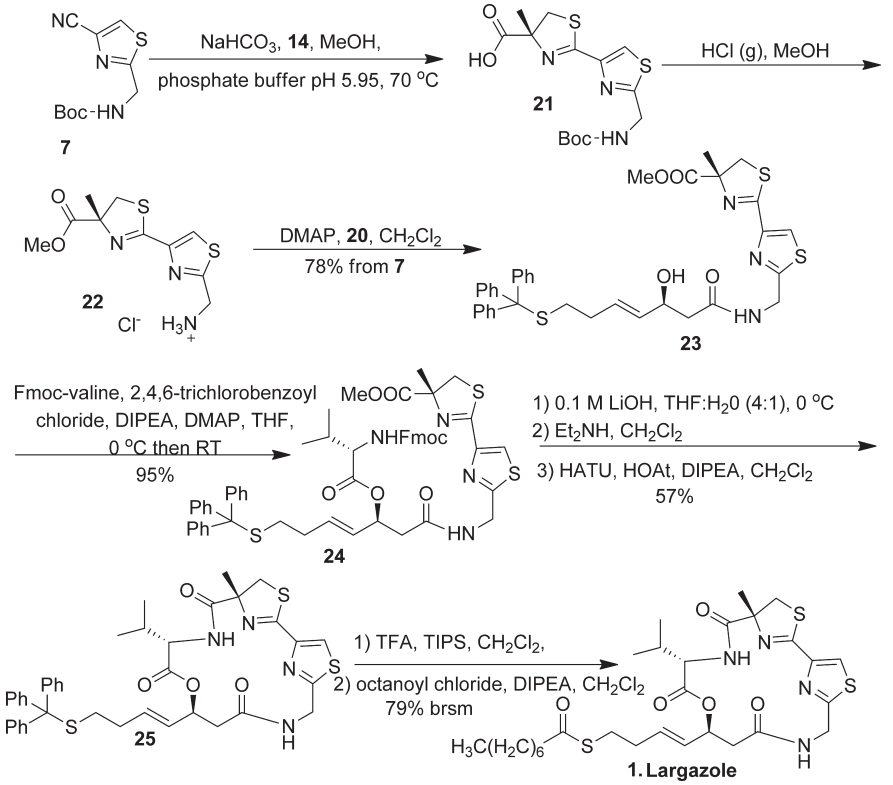
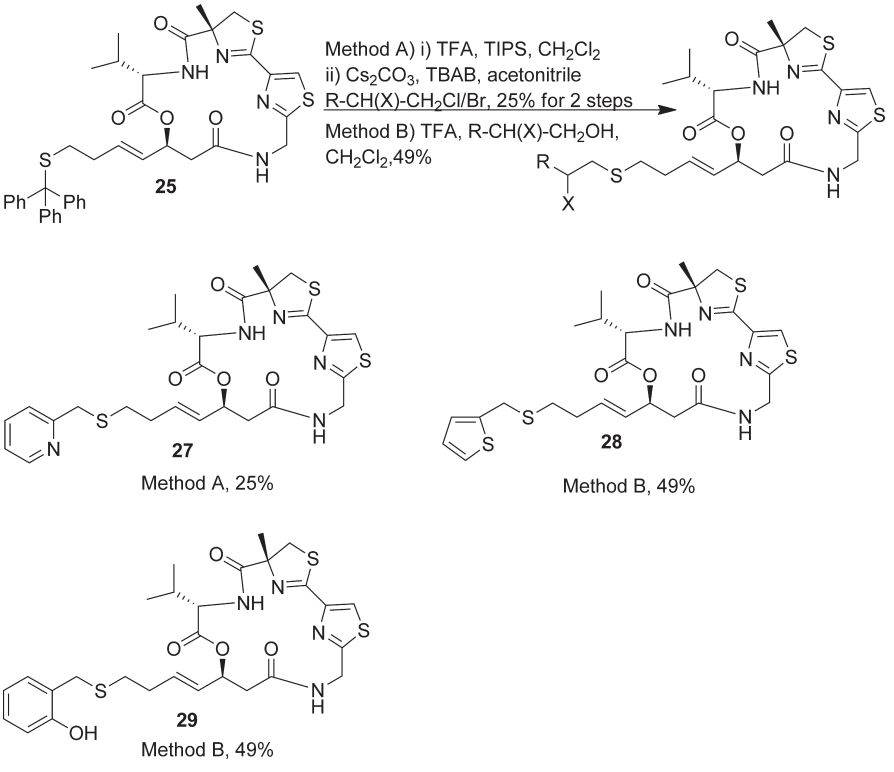
Scheme 5. Synthesis of Largazole 1



Scheme 6. Synthesis of Analogues with Modified Metal-Binding Motif



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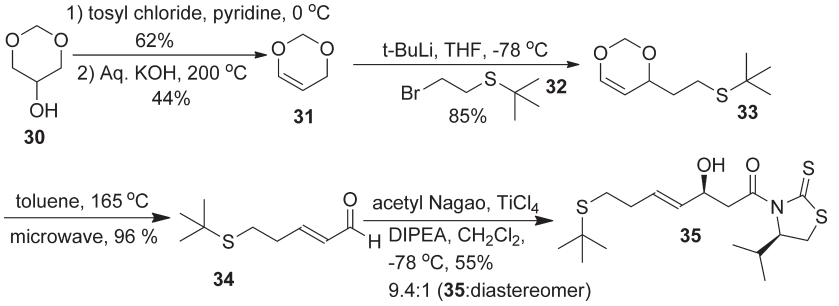
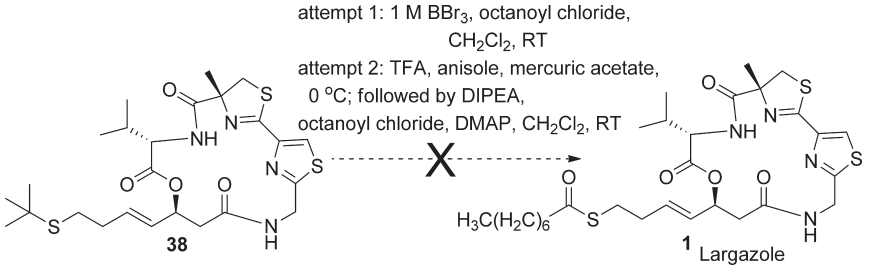
Scheme 7. Synthesis of *tert*-Butyl Protected β-Hydroxycarboxamide 35



Scheme 8. Attempted Synthesis of Largazole 1



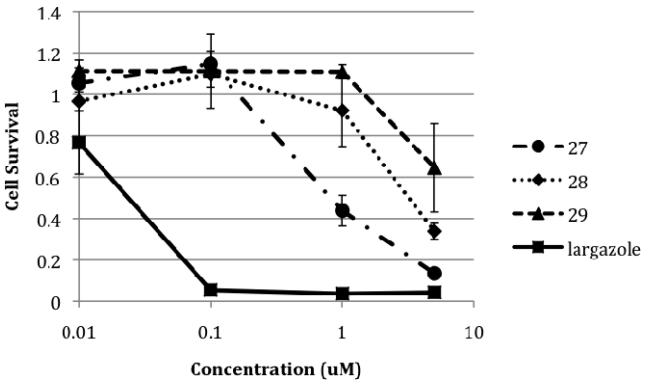


Figure 3. Growth inhibitory effects of largazole **1** and analogues on HCT116 colon carcinoma cells. Each point represents the mean of two experiments; each with 4 replicates with the error bars indicating the SEM.

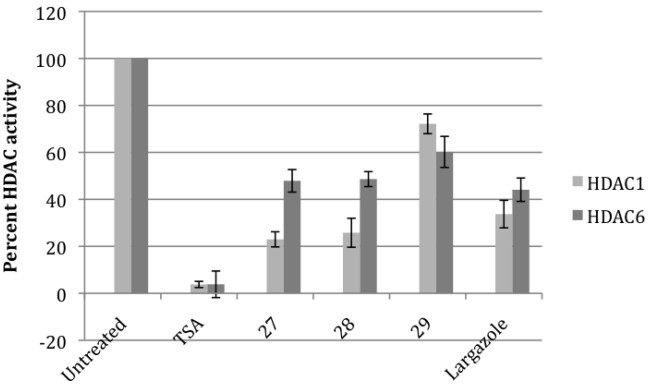


Figure 4. In vitro HDAC inhibitory activity – Recombinant HDAC1 and 6 activity was measured with and without the analogues. Reactions were carried out for 1 h at a concentration of 1 μM for all analogues. HDAC1 showed the greatest inhibition with analogues **27** and **28**, while HDAC6 inhibition was more modest and less specific to any of the largazole based analogues.

was observed with HDAC1 (data not shown). In vitro evaluation of the inhibition of recombinant HDAC6 enzyme showed ~50% decrease of activity with all of the analogues, including largazole **1**, at a concentration of 1 μM (Figure 4).

To evaluate the effects of these compounds on HDAC activity in a more physiologically relevant system, we treated a colorectal cancer cell line HCT116 with these analogues at increasing concentrations for 24 h. We examined the downstream effects of HDAC inhibition on global histone H3 acetylation by Western blot analysis after 24 h of cellular exposure to 10 nM, 100 nM, and 1 μM of each compound. No increase in global acetylation was observed in treated cells exposed to any of the compounds at 10 nM (Figure 5). Largazole **1** showed an increase of global acetylation at 100 nM. Analogues **27** and **28** showed a significant increase in global acetylation at 1 μM, though much lower than what was observed with largazole **1**.

In order to ascertain the effect of the analogues on HDAC6 activity, we studied the levels of α-tubulin acetylation after exposure to 10 nM, 100 nM, and 1 μM. Again, we measured the α-tubulin acetylation using Western blot analysis of whole cell lysates from cells treated for 24 h. No changes in α-tubulin acetylation were observed by exposure to any of the analogues, including largazole **1** (Figure 5). This concurs with earlier findings that HDAC6 is not a target for largazole **1**.[10b]

DISCUSSION

Histone deacetylase inhibitors that modulate post-transcriptional modification of proteins and target reversible epigenetic

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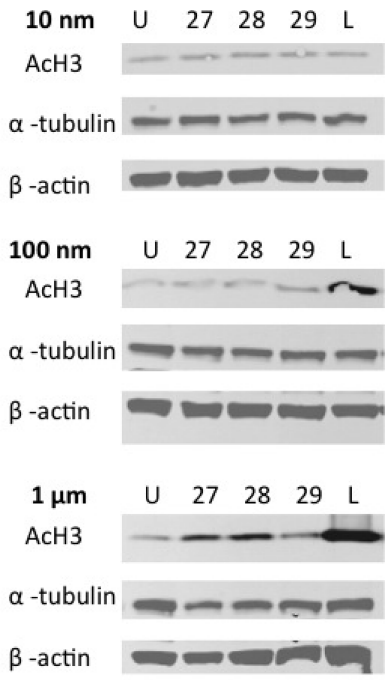

Figure 5. Western blot analysis of global acetylated H3 and acetylated α-tubulin proteins following the treatment of HCT116 cells with largazole **1** (L) and analogues at 10 nM, 100 nM and 1 μM concentrations after 24 h of treatment. Increases of global acetylation were observed with largazole **1** at 100 nM and 1 μM and modest increases with analogues **27** and **28**. α-tubulin levels remained unchanged.

repression of gene expression are a promising source of selective anticancer agents. Although the basic cellular functions of individual HDAC isoforms are not fully characterized, the aberrant expression of specific HDAC isoforms in some types of cancer cells suggests that specific isoforms may be targeted to develop selective anticancer agents. With two inhibitors SAHA (vorinostat) and FK228 (romidepsin) already approved by the USFDA for treatment of cutaneous T-cell lymphoma (CTCL) and a number of other candidates in the drug pipelines, HDACis are being extensively investigated as potential selective anticancer agents. SAHA with a promiscuous hydroxamic acid group as the metal-binding domain is a pan-inhibitor of HDACs, whereas FK228 with a 3-hydroxy-7-mercapto-4-heptenoic acid side chain masked as a disulfide bridge as the metal-binding domain is a class I inhibitor with no effect on HDAC6. Although the thiol group is not as strong a Zn^{2+} binding functionality as hydroxamic acid,[25] FK228 has a higher potency than SAHA due to additional compensatory binding interaction of the cap group with the rim region of the active site. The recently isolated depsipeptide largazole **1** has a high degree of structural and functional similarity to FK228 and, like FK228, is a prodrug of the active thiol. A range of largazole analogues synthesized by a number of groups has unraveled some SAR information related to potency and selectivity. The cyclic cap of the molecule which interacts with the less conserved rim region of the receptor has been the major target of structural modifications. Notable among them are the variable substitution of thiazoline methyl at C-7 or oxidation of thiazoline to thiazole[11i] and replacement of L-valine with other amino acids[10d,11h] without adverse effect on potency, reduction in potency accompanying change of configuration at C2[10c,26] and C17[10d] and conversion of lactone to lactam,[10e] equipotent replacement of dihydrothiazoline with dihydrooxazoline,[10c] and the substitution of pyridine for thiazole[10c] to generate the most potent HDACi known to date. Major SAR determinants of the side chain are the preference for a *trans*-alkene vs a *cis*-alkene,[11h] preference for thiol vs esters,[11a] ketones,[11a] benzamides, and α-thioamides[10c] as the metal-binding moiety, and most importantly the requirement of an optimal four-atom linker between the hydrophobic cap group and the Zn^{2+} coordination thiol group[10d,11c] in comparison to five to seven atom side chains common among most HDACis. We modified the metal-binding domain by introducing a second heteroatom in the side chain two to three atoms apart from sulfur to facilitate chelation with Zn^{2+} via a five- or six-membered cyclic transition state.

We adopted a convergent approach to the synthesis of largazole **1** and used it as a general method to synthesize side chain analogues by nucleophilic substitution of the desired precursors with largazole thiol. In our initial approach to largazole **1** we used *tert*-butyl for thiol protection. The *tert*-butyl protected 5-mercapto-2-pentenal **34** was conveniently obtained by the retro Diels−Alder reaction of the 6-substituted-1,3-dioxene **33**. After conversion to the *tert*-butyl protected 3-hydroxy-7-mercapto-4-heptenoic acid derivative **35**, it was used in our convergent approach to synthesize *tert*-butyl protected largazole thio ether **38**. Attempts to convert **38** to largazole **1** by removal of the *tert*-butyl group by a number of standard protocols and esterification with octanoyl chloride were unsuccessful. This was overcome by switching to the trityl protecting group. Largazole **1** and the side chain analogues were synthesized via largazole thiol following removal of trityl under acid conditions.

Largazole **1** inhibited the growth of HCT116 cells with a GI_{50} of ~10 nM. The three side chain analogues were less active, the pyridine analogue **27** being the most active of the three with a GI_{50} of ~1.0 μM. The other two analogues were active at higher concentrations. In vitro analysis of HDAC activity demonstrated that all of these analogues have some inhibitory effects on recombinant HDAC1 and HDAC6. The inhibition of HDAC6 observed with analogues **27** and **28** was modest in comparison to their effects on HDAC1 or global class 1 and 2 HDACs. The modest inhibition of HDAC1 by largazole **1** observed is somewhat in contrast to previous reports.[10a,b] However, the increased global acetylation of H3 in treated cells indicates efficient in situ inhibition of HDAC1. The effects on cellular proliferation are consistent with significant increase of global H3 acetylation observed with largazole **1** at 1 μM and modest induction of global H3 acetylation observed for analogues **27** and **28**. There was no change in acetylated α-tubulin levels with any of the compounds, indicating they have no effect on HDAC6. The marked difference in the activity of largazole **1** in the cellular assays compared to the in vitro HDAC activity assays can be attributed to the hydrolysis of the prodrug largazole **1** to largazole thiol by cellular esterases. Thioethers bind weakly to hard or borderline metal ions like Mg^{2+}, Mn^{2+}, Cu^{2+}, and Zn^{2+}.[27] These largazole analogues were designed to see whether the introduction of a second heteroatom would lead to stronger binding to the metal ion and also to isoform/class selectivity. However, it is not possible to suggest if the diminished biological activity observed is due to poor affinity of thioether group for Zn^{2+} ion or incompatibility of the modified metal binding moiety with the HDAC active site. Depsipeptides such as largazole **1** and FK228 constitute a distinct class of HDACis characterized by a masked thiol function as the metal-binding domain and a side chain with a characteristic four-atom spacer between the macrocycle and the

metal binding domain. The side chain occupies the hydrophobic channel between the metal binding site and the hydrophobic rim of the active site and positions the two end groups of the inhibitor for binding interaction with the receptor. Despite the high sequence similarity within the active site, the presence of discrete binding cavities in the vicinity of the metal ion may be taken advantage of to achieve isoform selectivity as exemplified by HDACis with substituted benzamide metal-binding domains and class I selectivity.[12] The exploration of alternative metal-binding domains is the way forward to the development of such isoform and class-selective HDACis.

CONCLUSION

Largazole **1** and its analogues were synthesized efficiently and in high yields using several one-pot two-reaction protocols. The stereochemistry of **20** was installed by acetate aldol reaction. Macrolactamization with HATU, HOAt, and Hunig's base gave cyclic product **25**. An initial attempt to synthesize largazole **1** using the *tert*-butyl group to protect thiol proved problematic because of difficulty in removing the protecting group for thioesterification. By use of the trityl group instead, deprotection followed by thioesterification with octanoyl chloride provided largazole **1**. This method provides an efficient synthetic approach to largazole analogues. Three side chain analogues with multiple heteroatoms for metal binding synthesized using this approach were found to be less potent than largazole **1** as measured by growth inhibition in HCT116 colorectal carcinoma cell line and induction of global H3 acetylation. However, the moderate selectivity for HDAC1 over HDAC6 observed with analogues **27** and **28** in the in vitro HDAC inhibitory activity assay may guide the structural modification of the metal-binding motif to design selective HDAC inhibitors. Further studies in this direction are continuing.

EXPERIMENTAL SECTION

THF was refluxed with Na and benzophenone and freshly distilled prior to use. NMR spectra were recorded on Varian INOVA 600 MHz and Varian VXRS 400 MHz instruments and calibrated using residual undeuterated solvent as internal reference ($CDCl_3$, ^1H NMR at δ 7.26, ^{13}C NMR at δ 77.23; D_2O, ^1H NMR at δ 4.8; DMSO-d_6, ^1H NMR at δ 2.5, ^{13}C NMR at δ 39.51). Optical rotations were recorded on an AUTOPOL III 589/546 polarimeter. High-resolution mass spectra (HRMS) were recorded on a Micromass LCT electrospray mass spectrometer at the Central Instrument Facility, Wayne State University, Detroit, MI, and on a Micromass Q-Tof II electrospray mass spectrometer at the Mass Spectrometry and Proteomics Facility, The Ohio State University, Columbus, OH. Crude products were purified by flash column chromatography on silica gel (32−63 μm) purchased from Dynamic Adsorbents Inc. and by preparative thin layer chromatography on 1000 μm uniplates purchased from Analtech Inc. using commercial solvents as specified. HPLC analyses were performed on a Waters 1525 binary pump HPLC system with Waters 2487 dual wavelength absorbance detector on a Symmetry C18 column (reverse phase, 5 μm, 4.6 mm × 150 mm) using a linear gradient of 10−100% H_2O/MeOH over 15−20 min; flow rate of 1 mL/min and UV detection at 254 nm. Structural integrity and purity of the test compounds were determined by the composite of ^1H and ^{13}C NMR, HRMS, and HPLC and were found to be >95% pure. D-Hydantoinase, recombinant, immobilized from *E. coli* was purchased from Fluka Chemie AG (catalog no. 53765, CAS no. 9030-74-4).

***tert*-Butyl (4-Carbamoylthiazol-2-yl)methylcarbamate (6).** A mixture of Boc-thioglycinamide **4** (0.95 g, 5 mmol, 1 equiv) and bromopyruvic acid (0.835 g, 5 mmol, 1 equiv) in dry THF (20 mL) was stirred at 50 °C under nitrogen for 2 h. The reaction mixture was concentrated to dryness, and the residue was dried by repeated azeotropic removal of moisture with toluene. The crude carboxylic acid thus obtained was dissolved in dry THF (50 mL), and triethylamine (1.3 g, 12.94 mmol, 2.6 equiv) and ethyl chloroformate (0.681 g, 6.24 mmol, 1.25 equiv) were added at 0 °C and stirred for 30 min at the same temperature. Ammonium hydroxide (∼ 1.1 g, 31.76 mmol, 5 equiv) was added, and the reaction mixture was stirred at room temperature for 2 h. It was concentrated in vacuo and purified by flash column chromatography on silica gel in ethyl acetate/hexanes, 33−100%, to yield **6** (0.768 g, 60% over two steps from boc-thioglycinamide): mp 153−154 °C (lit.[11c] mp 153 °C). ^1H NMR (400 MHz, $CDCl_3$): δ 8.08 (s, 1H), 7.13 (br s, 1H), 5.94 (brs, 1H), 5.34 (br s, 1H), 4.59 (d, J = 5.6 Hz, 2H), 1.47 (s, 9H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.7, 163.1, 155.8, 149.3, 124.8, 80.8, 42.5, 28.5 ppm.

***tert*-Butyl (4-Cyanothiazol-2-yl)methylcarbamate (7).** To a solution of amide **6** (0.204 g, 0.797 mmol, 1 equiv) in dichoromethane (20 mL) at 0 °C was added triethylamine (0.174 g, 1.725 mmol, 2.16 equiv) followed by dropwise addition of trifluoroacetic anhydride (0.181 g, 0.863 mmol, 1.08 equiv). The reaction mixture was stirred at room temperature for 1 h, concentrated in vacuo, and purified by flash chromatography on silica gel in ethyl acetate/hexanes, 20−50%, to obtain the nitrile **7** (0.189 g, 99%): mp 84−85 °C (lit.[11c] mp 84 °C). ^1H NMR (600 MHz, $CDCl_3$): δ 7.95 (s, 1H), 5.3 (s, 1H), 4.62 (d, J = 6 Hz, 2H), 1.47 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.7, 155.8, 131.1, 126.6, 114, 81, 42.5, 28.5.

(2*E*)-5-[(Triphenylmethyl)thio]-2-pentenal (18). To a solution of triphenylmethanethiol (6.91 g, 25 mmol, 2.09 equiv) in dichloromethane (100 mL) was added acrolein **16** (1.965 g, 35 mmol, 2.9 equiv) and triethylamine (3.56 g, 35 mmol, 2.9 equiv). The resulting mixture was stirred for 1 h at room temperature and was concentrated to give the aldehyde **17** as a white solid, which was used in the next step without purification. A solution of the aldehyde **17** obtained above and (triphenylphosphoranylidene)acetaldehyde (3.64 g, 11.96 mmol, 1 equiv) in dry benzene (150 mL) was refluxed for 8 h. The reaction mixture was concentrated and purified by flash chromatography on silica gel in dichloromethane/hexanes, 20−25%, to afford aldehyde **18** (5.83 g, 65% over the two steps): mp 140−141 °C. ^1H NMR (400 MHz, $CDCl_3$): δ 9.43 (d, J = 8.0 Hz, 1H), 7.42 (dd, J = 2.4, 7.6 Hz, 6H), 7.29 (dt, J = 2.0, 6.8 Hz, 6H), 7.22 (dt, J = 2.4, 7.2 Hz, 3H), 6.60−6.67 (m, 1H), 5.95−6.01 (dd, J = 8.0, 15.6 Hz, 1H), 2.29−2.37 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 194, 156, 144.7, 133.8, 129.7, 128.2, 127, 67.2, 31.9, 30.2.

3*S*-Hydroxy-1-(4*R*-isopropyl-2-thioxothiazolidin-3-yl)-7-tritylsulfanylhept-4*E*-en-1-one (20). To a solution of acetyl Nagao chiral auxiliary (1.493 g, 7.355 mmol, 1 equiv) in dichloromethane (60 mL) at 0 °C was added $TiCl_4$ (1.72 g, 9.05 mmol, 1.23equiv). After the reaction mixture was stirred for 5 min and cooled to −78 °C, Hunig's base (1.872 g, 9.2 mmol, 1.25 equiv) was added. The mixture was stirred for 2 h at the same temperature, and to it was added the aldehyde **18** (2.6 g, 7.263 mmol, 0.987 equiv) in dichloromethane (8 mL) dropwise. The reaction mixture was stirred for 1 h at −78 °C. It was removed from cooling bath, treated with water (15 mL), and diluted with dichloromethane (50 mL). The aqueous portion was extracted with dichloromethane; the organic layer was washed with saturated NaCl (40 mL) and dried over anhydrous Na_2SO_4. It was concentrated in vacuo and the residue was purified by flash chromatography on silica gel in dichloromethane/hexanes, 25−90%, to obtain the major isomer **20** as a thick yellow oil (1.963 g, 76.5% brsm), and the diastereomer **19** (0.193 g, 7.5%) with recovery of acetyl Nagao chiral auxiliary (0.513 g). Major isomer: $[\alpha]_D^{20}$ −149 (c 3.7, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$): δ 7.41 (d, J = 7.2 Hz, 6H), 7.28 (t, J = 7.2 Hz, 6H), 7.21 (t, J = 7.2 Hz, 3H), 5.61−5.55 (m, 1H), 5.46 (dd, J = 6.0, 15.2 Hz, 1H), 5.12 (t, J = 6.8 Hz,

1H), 4.57 (t, J = 5.8 Hz, 1H), 3.56 (dd, J = 2.8, 17.6 Hz, 1H), 3.47 (dd, J = 7.6, 11.6 Hz, 1H), 3.28 (dd, J = 8.8, 17.6 Hz, 1H), 2.99 (d, J = 11.6 Hz, 1H), 2.82 (s, 1H), 2.37−2.32 (m, 1H), 2.21 (t, J = 7.2 Hz, 2H), 2.09 (q, J = 7.2 Hz, 2H), 1.05 (d, J = 6.8 Hz, 3H), 0.97 (d, J = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 203.1, 172.7, 145, 132, 130.2, 129.7, 128, 126.8, 71.6, 68.6, 66.7, 45.4, 31.6, 31.6, 31, 30.8, 19.3, 18.0.

(*R*)-2-(2-((*tert*-Butoxycarbonylamino)methyl)thiazol-4-yl)-4,5-dihydrothiazole-4-carboxylic Acid (21). To a well stirred mixture of the nitrile **7** (0.096 g, 0.4 mmol, 1 equiv) and $NaHCO_3$ (0.232 g, 2.76 mmol, 5.6 equiv) in methanol (5 mL) was added (*R*)-α-methylcysteine hydrochloride **14** (0.084 g, 0.491, 1.23 equiv) followed by phosphate buffer, pH 5.95 (2.5 mL). The reaction mixture was degassed with nitrogen before stirring it under nitrogen at 70 °C for 1 h. It was acidified with 1 M HCl and extracted with ethyl acetate (15 mL) three times. The combined organic extract was washed with saturated NaCl solution, dried over anhydrous sodium sulfate, and concentrated to obtain the carboxylic acid **21** (0.137 g) which was used in the next step without further purification.

(*R*)-Methyl 2-(2-((3*S*-Hydroxy-7-(tritylthio)hept-4*E*-enamido)methyl)thiazol-4-yl)-4-methyl-4,5-dihydrothiazole-4-carboxylate (23). A solution of carboxylic acid **21** (0.136 g, 0.38 mmol, 1 equiv) in anhydrous methanol (5 mL) was bubbled with HCl gas for 5 min. The reaction mixture was stirred overnight and concentrated in vacuo to give compound **22** which was azeotroped using toluene before taking it to the next step. A mixture of above obtained compound **22** and DMAP (0.121 g, 0.992 mmol, 2.61 equiv) in dichloromethane (2 mL) was stirred for 5 min, and a solution of aldol product **20** (0.214, 0.38 mmol, 1 equiv) in dichloromethane (1 mL) was added. The reaction mixture was stirred for 1 h, concentrated in vacuo, and purified by flash chromatography on silica gel in ethyl acetate/hexanes, 20−100%, to afford the alcohol **23** (0.191 g, 78% over three steps a−c). $[\alpha]_D^{20}$ −11 (c 3.55, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.90 (s, 1H), 7.38 (d, J = 8.4 Hz, 6H), 7.26 (t, J = 7.6 Hz, 6H), 7.19 (t, J = 7.6 Hz, 3H), 7.07 (t, J = 6.0 Hz, 1H), 5.50−5.58 (m, 1H), 5.37−5.43 (dd, J = 6.0, 15.2 Hz, 1H), 4.63−4.74 (m, 2H), 4.43 (m, 1H), 3.86 (d, J = 11.6 Hz, 1H), 3.78 (s, 3H), 3.48 (s, 1H), 3.25 (d, J = 11.6 Hz, 1H), 2.34−2.46 (m, 2H), 2.18 (t, J = 7.2 Hz, 2H), 2.05 (q, J = 7.2, Hz, 2H), 1.63 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.8, 172.0, 168.1, 162.9, 148.4, 144.9, 132.4, 130.3, 129.7, 128, 126.8, 122.4, 84.6, 69.2, 66.7, 53.1, 42.9, 41.6, 40.9, 31.6, 31.4, 24.1. HRMS-ESI (m/z): $[M + H]^+$ calcd for $C_{36}H_{38}N_3O_4S_3$, 672.2019; found, 672.2024

(4*R*)-Methyl 2-(2-((8*S*)-1-(9*H*-Fluoren-9-yl)-5-isopropyl-3,6,10-trioxo-8-((*E*)-4-(tritylthio)but-1-enyl)-2,7-dioxa-4,11-diazadodecan-12-yl)thiazol-4-yl)-4-methyl-4,5-dihydrothiazole-4-carboxylate (24). To a solution of Fmoc-L-valine (0.09 g, 0.0266 mmol, 1 equiv) in THF (1 mL) at 0 °C was added Hunig's base (0.045 g, 0.345 mmol, 1.29 equiv) and 2,4,6-trichlorobenzoyl chloride (0.078 g, 0.32 mmol, 1.2 equiv). The reaction mixture was stirred at 0 °C for 1 h. When TLC indicated formation of the anhydride, alcohol **23** in THF (1 mL) was added to the reaction mixture at 0 °C. It was stirred overnight at room temperature. The reaction mixture was concentrated in vacuo, and flash chromatography purification on silica gel in ethyl acetate/hexanes, 20−100%, yielded the acyclic precursor **24** (0.125 g, 94%). $[\alpha]_D^{20}$ −12 (c 6.83, $CHCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.89 (s, 1H), 7.76 (d, J = 7.2 Hz, 2H), 7.57 (d, J = 7.2 Hz, 2H), 7.41−7.37 (m, 8 H), 7.30 (t, J = 7.2 Hz, 2H), 7.26−7.29 (m, 6 H), 7.20 (t, J = 7.2 Hz, 3H), 6.74 (t, J = 8.4 Hz, 1H), 5.69−5.65 (m, 1H), 5.61 (dd, J = 6.0, 13.2 Hz, 1H), 5.42 (dd, J = 7.8, 15.0 Hz, 1H), 5.21 (d, J = 7.8 Hz, 1H), 4.7 (d, J = 6.0 Hz, 2H), 4.38 (dd, J = 7.2, 10.8 Hz, 1H), 4.33 (dd, J = 6.6, 10.8 Hz, 1H), 4.19 (t, J = 6.6 Hz, 1H), 4.05 (dd, J = 6.0, 8.4, 1H), 3.85 (d, J = 10.8 Hz, 1H), 3.78 (s, 3H), 3.24 (d, J = 10.4 Hz, 1H), 2.58 (d, J = 6. Hz, 2H), 2.2−2.12 (m, 2H), 2.07−2.01 (m, 2H), 1.62 (s, 3H), 0.9 (d, J = 7.2 Hz, 3H), 0.85 (d, J = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.8, 169.2, 168.6, 163, 156.6, 148.5, 144.9, 143.6, 141.5, 134.2, 129.7, 128.03, 127.9, 127.7, 127.3, 126.8, 125.2, 122.3, 120.15, 84.7, 72.42, 67.2, 66.8, 59.6, 53.1, 47.3, 41.7, 41.6, 41.3, 31.5, 31.3, 31, 29.9, 24.1, 19.2, 18.1. HRMS-ESI (m/z): $[M + H]^+$ calcd for $C_{56}H_{57}N_4O_7S_3$, 993.3397; found, 993.3389.

Cyclic Core 25. To a stirred solution of **24** (133 mg, 0.134 mmol, 1 equiv) in THF/H_2O (4:1, 4 mL) at 0 °C was added 0.1 M LiOH (1.4 mL, 0.14 mmol, 1.045 equiv) dropwise over a period of 15 min. After the mixture was stirred at 0 °C for 1 h, it was acidified with 1 M HCl solution and was extracted with EtOAc three times. The combined organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated. The reaction mixture was purified by preparative TLC in ethyl acetate to get the carboxylic acid. It was dissolved in dichloromethane (13 mL) and treated with diethylamine (0.462 g, 6.32 mmol, 47.16 equiv). After the mixture was stirred at room temperature for 3 h, it was concentrated to dryness to afford the free amino derivative. After the sample was dried azeotropically with toluene, it was treated with HATU (0.105 g, 0.276 mmol, 2.06 equiv), HOAt (0.038 g, 0.279 mmol, 2.08 equiv), dichloromethane (130 mL, ∼ 1 mmol), and Hunig's base (0.074 g, 0.575 mmol, 4.29 equiv) and the mixture was stirred for 30 h at room temperature. The reaction mixture was concentrated to dryness and was purified by flash chromatography on silica gel in ethyl acetate/hexanes, 10−60%, to yield the cyclic core **25** (0.056 g, 57% over three steps from **24**). $[\alpha]_D^{20}$ +2.5 (c 0.95, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.73 (s, 1H), 7.37 (d, J = 8.4 Hz, 6H), 7.26 (t, J = 8 Hz, 6H), 7.20−7.15 (m, 4H), 6.49 (dd, J = 2.8, 9.2 Hz, 1H), 5.68−5.71 (m, 1H), 5.60 (m, 1H), 5.38 (dd, J = 6.8, 15.6 Hz, 1H), 5.19 (dd, J = 8.8, 17.6 Hz, 1H), 4.55 (dd, J = 3.2, 9.6 Hz, 1H), 4.11 (dd, J = 3.2, 17.6 Hz, 1H), 4.01 (d, J = 11.2 Hz, 1H), 3.26 (d, J = 11.6 Hz, 1H), 2.77 (dd, J = 9.6, 16.4 Hz, 1H), 2.64 (dd, J = 3.2, 16 Hz, 1H), 2.15−2.19 (m, 2H), 1.98−2.06 (m, 2H), 1.82 (s, 3H), 0.67 (d, J = 6.8 Hz, 3H), 0.50 (d, J = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.7, 169.4, 168.9, 168.1, 164.6, 147.6, 144.9, 133.3, 129.7, 128.1, 126.8, 124.3, 84.6, 72.0, 66.8, 58.0, 43.5, 41.2, 40.8, 34.2, 31.5, 31.4, 29.9, 24.4, 19.1, 16.9.

Largazole (1). To a solution of **25** (0.033 g, 0.045 mmol, 1 equiv) in dichloromethane (5 mL) at 0 °C was added triisopropylsilane (0.013 g, 0.083 mmol, 1.85 equiv) followed by trifluoroacetic acid (0.307 g, 2.69 mmol, 59.85 equiv). After being stirred for 3 h at room temperature, the reaction mixture was concentrated in vacuo. The crude product was purified by flash chromatography on silica gel in 20% ethyl acetate/hexanes to first remove impurity followed by ethyl acetate to obtain the largazole thiol (0.022 g). To a stirred solution of largazole thiol in dichloromethane (7 mL) at 0 °C were added Hunig's base (0.045 g, 0.35 mmol, 7.7 equiv) and octanoyl chloride (0.044 g, 0.27 mmol, 6 equiv). Catalytic DMAP (1 mg) in dichloromethane (0.1 mL) was added to the reaction mixture. After the mixture was stirred for 4 h at room temperature, the reaction was quenched with methanol and the mixture was concentrated in vacuo. Purification of the crude product by preparative thin layer chromatography on silica gel with ethyl acetate as solvent gave largazole **1** (0.010 g, 79%, based on recovered largazole thiol 0.012 g). $[\alpha]_D^{20}$ +19.5 (c 0.2, $CHCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.77 (s, 1H), 7.15 (d, J = 9.6 Hz, 1H), 6.41 (dd, J = 2.4, 9.0 Hz, 1H), 5.79−5.84 (m, 1H), 5.66 (m, 1H), 5.50 (dd, J = 6.6, 15.6 Hz, 1H), 5.29 (dd, J = 9.6, 18.0 Hz, 1H), 4.61 (dd, J = 3.0, 9.0 Hz, 1H), 4.27 (dd, J = 3.0, 17.4 Hz, 1H), 4.04 (d, J = 11.4 Hz, 1H), 3.28 (d, 11.4 Hz, 1H), 2.90 (t, J = 7.2 Hz, 2H), 2.85 (dd, J = 10.8, 16.2 Hz, 1H), 2.53 (t, J = 7.8 Hz, 2H), 2.31 (q, J = 7.2, Hz, 2H), 2.10−2.12 (m, 1H), 1.87 (s, 3H), 1.62−1.67 (m, 2H), 1.26−1.31 (m, 8H), 0.87 (t, J = 7.2 Hz, 3H), 0.68 (d, J = 7.2 Hz, 3H), 0.5 (d, 6.6 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 199.6, 174.7, 169.6, 169.1, 168.1, 164.7, 147.7, 133.0, 128.6, 124.4, 84.7, 72.3, 57.9, 44.4, 43.6, 41.3, 40.7, 34.4, 32.5, 31.8, 29.1, 28.1, 25.9, 24.4, 22.8, 19.1, 16.8, 14.3. HRMS-ESI (m/z): $[M + Na]^+$ calcd for $C_{29}H_{42}N_4O_5S_3Na$, 645.2211; found, 645.2215

Cytoproliferation Assay. Cell proliferation was measured using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) reduction assay with the CellTiter

96 One solution MTS assay as described by the manufacturer (Promega, Madison, WI). Briefly, cells (3×10^4 cells/well) were seeded in quadruplicate in 96-well plates and allowed to attach overnight. The medium was replaced with 100 μL of fresh medium containing the appropriate concentration of **27**, **28**, **29**, or largazole **1**. Following incubation at 37 °C, 5% CO_2, 20 μL of CellTiter 96 One solution was added per well and incubated 1.5 h at 37 °C and absorbance measured at 490 nm.

HDAC Activity Assays. HDAC activity was assayed using the Enzo Life Sciences (Plymouth Meeting, PA) fluorimetric drug discovery kits with specificity for HDAC1 and -6 or using cellular extracts kits. Inhibition of global class 1 and 2 HDAC activity was ascertained using the included HeLa nuclear extracts. Experiments were performed as described by the protocol supplied by the manufacturer. Briefly, the indicated inhibitor, enzyme, and Fluor de Lys substrate were incubated for 1 h when the developing solution was added and incubated for the designated time prior to reading. Experiments were repeated 3 times and done in triplicate each time. Analogues incubated with the substrate and developer was performed as a control and showed no influence on the fluorescent signal. Data show a representative experiment with error bars indicating standard deviation of the triplicates.

Evaluation of Global Histone Acetylation Levels. HCT116 cells were treated for 24 h with 10 nM, 100 nM, or 1 μM or DMSO. Cell lysates were extracted using RIPA buffer, and equal amounts (30 μg/lane) were loaded onto an SDS−PAGE gel. Standard Western blotting protocols were used using the Invitrogen NuPAGE Western blotting system. Primary antibodies used were AcH3 (Millipore), α-tubulin (Sigma), and β-actin (Sigma). Dye-conjugated secondary antibodies from Li-Cor Biosciences were used for detection and scanned using the Odyssey infrared detection system (LI-COR Biosciences, Lincoln, NE).

■ ASSOCIATED CONTENT

Supporting Information. Experimental procedures, analytical and spectral characterization data, and NMR spectra for compounds **10**−**14**, **31**−**35** and analogues **27**−**29**; NMR spectra for compounds **6**, **7**, **18**, **20**, **23**−**25**, and largazole **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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■ ABBREVIATIONS USED

HDAC, histone deacetylase; HAT, histone acetyltransferase; SAHA, suberoylanilide hydroxamic acid; MDA-MB-231, invasive transformed human mammary epithelial cells; U2OS, transformed fibroblastic osteosarcoma; NMuMG, nontransformed mouse mammary gland epithelial cell line; HCT116, human colorectal carcinoma cell line; HATU, 2-(1*H*-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HDACis, histone deacetylase inhibitors; NMR, nuclear magnetic resonance; USFDA, United States Food and Drug Administration; SAR, structure−activity relationship; TIPS, triisopropylsilane; TBAB, tetrabutylammonium bromide; TFAA, trifluoroacetic anhydride; HOAt, 1-hydroxy-7-azabenzotriazole; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; TLC, thin layer chromatography; CTCL, cutaneous T-cell lymphoma

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