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Total Synthesis and Molecular Target of Largazole, a Histone Deacetylase Inhibitor

Yongcheng Ying,[†] Kanchan Taori,[‡] Hyoungsu Kim,[†] Jiyong Hong,*[,†] and Hendrik Luesch*[,‡]

Department of Chemistry, Duke University, Durham, North Carolina 27708, and Department of Medicinal Chemistry, University of Florida, Gainesville, Florida 32610.

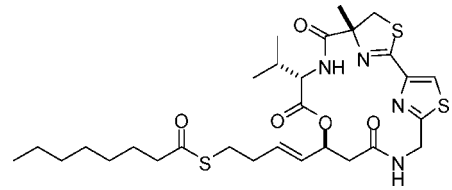
Received February 23, 2008; E-mail: luesch@cop.ufl.edu; jiyong.hong@duke.edu

Abstract: Full details of the concise and convergent synthesis (eight steps, 19% overall yield), its extension to the preparation of a series of key analogues, and the molecular target and pharmacophore of largazole are described. Central to the synthesis of largazole is a macrocyclization reaction for formation of the strained 16-membered depsipeptide core followed by an olefin cross-metathesis reaction for installation of the thioester. The biological evaluation of largazole and its key analogues, including an acetyl analogue, a thiol analogue, and a hydroxyl analogue, suggested that histone deacetylases (HDACs) are molecular targets of largazole and largazole is a class I HDAC inhibitor. In addition, structure−activity relationship (SAR) studies revealed that the thiol group is the pharmacophore of the natural product. Largazole's HDAC inhibitory activity correlates with its antiproliferative activity.

Introduction

The cyclic depsipeptide largazole (**1**, Figure 1), isolated from a cyanobacterium of the genus *Symploca*, is a marine natural product with novel chemical scaffold.[1] It consists of a potentially strained 16-membered macrocycle possessing a dense combination of unusual structural features, including a substituted 4-methylthiazoline linearly fused to a thiazole and a 3-hydroxy-7-mercaptohept-4-enoic acid unit. Another remarkable structural element in **1** is a thioester moiety, which is rarely encountered in natural products.

Macrocyclic natural products often exhibit unique biological properties and thus are attractive candidates for drug development in many diseases.[2] Largazole (**1**) represents such a class of compounds with highly differential growth-inhibitory activity, preferentially targeting transformed over nontransformed cells and favorably comparing to other natural products drugs such as paclitaxel in this respect.[1] Largazole (**1**) potently inhibited the growth of transformed mammary epithelial cells (MDA-MB-231, GI_{50} 7.7 nM) and induced cytotoxicity at higher concentrations (LC_{50} 117 nM). In contrast, nontransformed mammary epithelial cells (NMuMG) were less susceptible to **1** (GI_{50} 122 nM and LC_{50} 272 nM). Similarly, transformed fibroblastic osteosarcoma cells (U2OS, GI_{50} 55 nM and LC_{50} 94 nM) were more susceptible to **1** than nontransformed fibroblasts NIH3T3 (GI_{50} 480 nM and $LC_{50} > 8\ \mu M$). The potent biological activity and selectivity of **1** for cancer cells necessitates further investigation into the mode of action and potential as a cancer therapeutic. Herein we report the first total synthesis of **1** and its extension to preparation of key analogues, describe the identification of histone deacetylases (HDACs) as molecular targets of **1**, and define the pharmacophore through initial structure−activity relationship (SAR) studies.



Largazole (**1**)

Figure 1. Structure of largazole.

Results and Discussion

Total Synthesis of Largazole. As shown in Figure 2, we envisioned that the installation of the thioester moiety could be achieved by an olefin cross-metathesis of the 16-membered cyclic depsipeptide core **3** and the thioester **2**. Deliberate late-stage incorporation of the subunit bearing the thioester would allow convenient access to a series of analogues required to define the biological role of the thioester, the octanoyl group, and the side chain *E* double bond. A macrocyclization reaction of the linear precursor **4** would form the requisite 16-membered cyclic depsipeptide core **3**. Three key subunits (**5**, **6**, and **7**) could be assembled from readily available starting materials and sequentially coupled to provide the linear precursor **4**.

Condensation of **8**[3] with (*R*)-2-methyl cysteine methyl ester hydrochloride[4] (Et_3N, EtOH, 50 °C, 72 h) provided **7** in 51% (Scheme 1).[5] Removal of *N*-Boc group in **7** (TFA, CH_2Cl_2, 25

[†] Duke University.
[‡] University of Florida.

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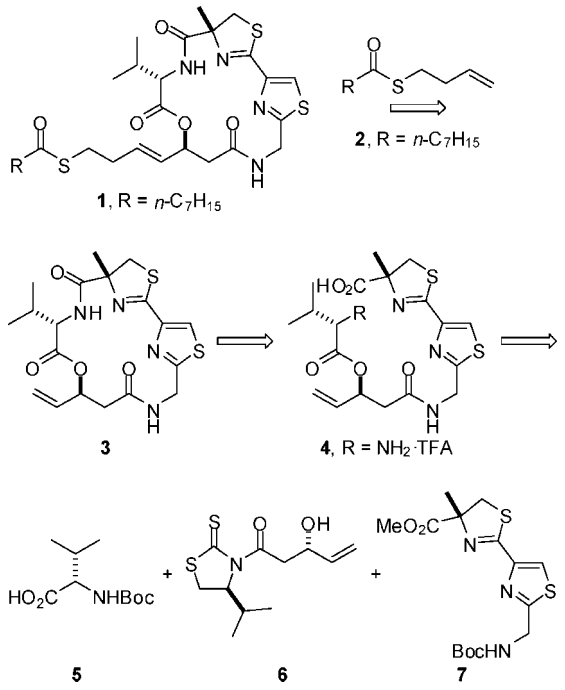
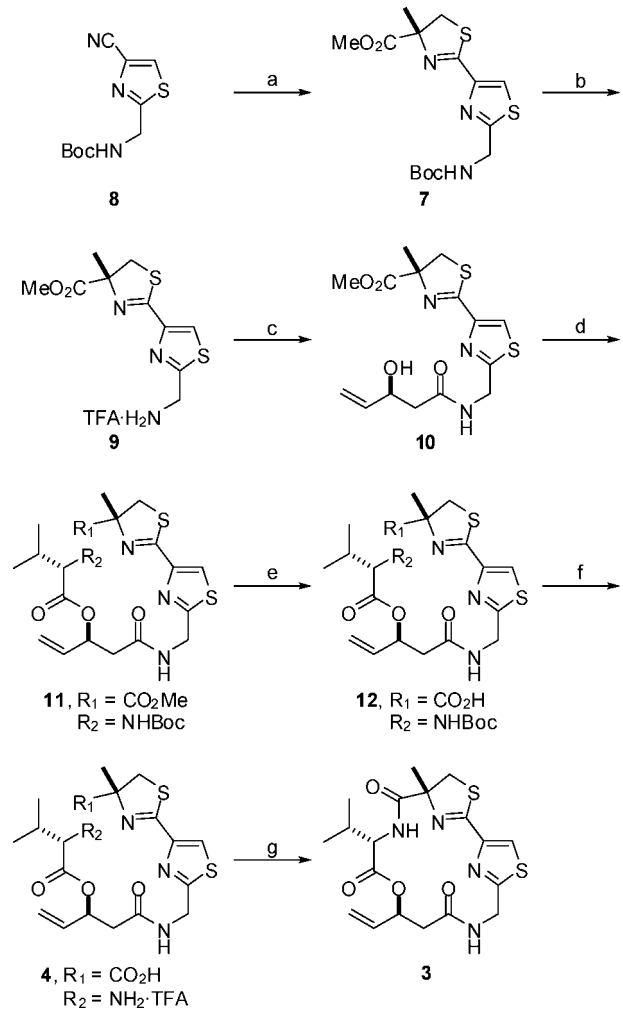


Figure 2. Retrosynthetic analysis.

°C, 1 h) followed by coupling (DMAP, CH_2Cl_2, 25 °C, 1 h) of the corresponding amine **9** to **6**,[6] prepared by an acetate *syn*-aldol reaction of *N*-acyl thiazolidinethione and acrolein, smoothly proceeded to provide **10** in 94% yield (for two steps). Yamaguchi esterification reaction of **10** and *N*-Boc-L-valine (**5**) (2,4,6-trichlorobenzoyl chloride, Et_3N, THF, 0 °C, 1 h; then **5**, DMAP, 25 °C, 10 h) afforded the linear depsipeptide **11** in 99%. Alternatively, DCC-coupling reaction of **10** and **5** (DCC, DMAP, toluene, 25 °C, 20 h) gave **11** in 85%. Hydrolysis of **11** under basic conditions (0.5 N LiOH, THF, H_2O, 0 °C, 3 h) to provide the carboxylic acid **12** and subsequent deprotection of *N*-Boc group in **12** (TFA, CH_2Cl_2, 25 °C, 2 h) provided **4**, a precursor to the 16-membered cyclic depsipeptide core **3**. Without further purification of **4**, we attempted to cyclize the crude **4** to provide the strained 16-membered cyclic depsipeptide core **3**. Initial attempts for macrocyclization reaction[7] under EDC- (EDC, *i*-Pr_2NEt, CH_2Cl_2, 25 °C, 12 h, 12% for three steps) or FDPP-coupling conditions (FDPP, *i*-Pr_2NEt, CH_3CN, 25 °C, 36 h, 37% for three steps) afforded the desired 16-membered cyclic depsipeptide core **3**, but in low yields. After exploration of various reaction conditions, macrocyclization reaction of the crude **4** utilizing HATU-HOAt (HATU, HOAt, *i*-Pr_2NEt, CH_2Cl_2, 25 °C, 24 h) proceeded smoothly to give **3** in 64% (for three steps).[8]

Scheme 1. Synthesis of 16-Membered Depsipeptide Core[a]



[a] (a) (*R*)-2-Methyl cysteine methyl ester hydrochloride, Et_3N, EtOH, 50 °C, 72 h, 51%; (b) TFA, CH_2Cl_2, 25 °C, 1 h; (c) **6**, DMAP, CH_2Cl_2, 25 °C, 1 h, 94% (two steps); (d) 2,4,6-trichlorobenzoyl chloride, Et_3N, THF, 0 °C, 1 h; then **5**, DMAP, 25 °C, 10 h, 99%; (e) 0.5 N LiOH, THF, H_2O, 0 °C, 3 h; (f) TFA, CH_2Cl_2, 25 °C, 2 h; (g) HATU, HOAt, *i*-Pr_2NEt, CH_2Cl_2, 25 °C, 24 h, 64% (three steps).

For synthesis of the thioester **2** (Scheme 2), coupling reaction of 4-(thiooctyl)-morpholine (**13**) and 4-bromo-1-butene (NaI, THF, H_2O, reflux, 16 h, 55%) was attempted following the modified procedure developed by Harrowven and co-workers,[9] but the procedure was not practical in preparation of the thioester **2** in large quantities. Instead, the thioacid **14**[10] was prepared and coupled to 4-bromo-1-butene (NaH, THF, 25 °C, 12 h) to provide the thioester **2** in 81%. With the 16-membered cyclic depsipeptide core **3** and the thioester **2** in hand, final olefin cross-metathesis reaction[11] of the macrocycle **3** and the thioester **2** was attempted under various conditions. After extensive optimization of reaction conditions, we were delighted to find that olefin cross-metathesis reaction of the macrocycle **3** and the

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(8) Attempts for macrolactonization under various conditions resulted in either no reaction or complex reaction mixtures. For details, see Supporting Information.
(9) Harrowven, D. C.; Lucas, M. C.; Howes, P. D. *Tetrahedron* **1999**, *55*, 1187–1196.
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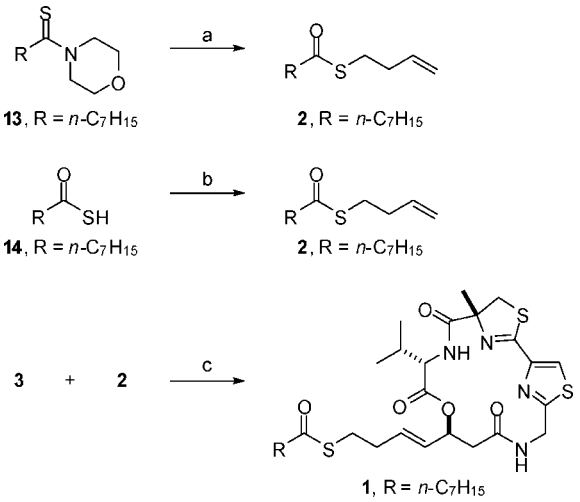
Scheme 2. Synthesis of Thioester and Completion of Synthesis[a]



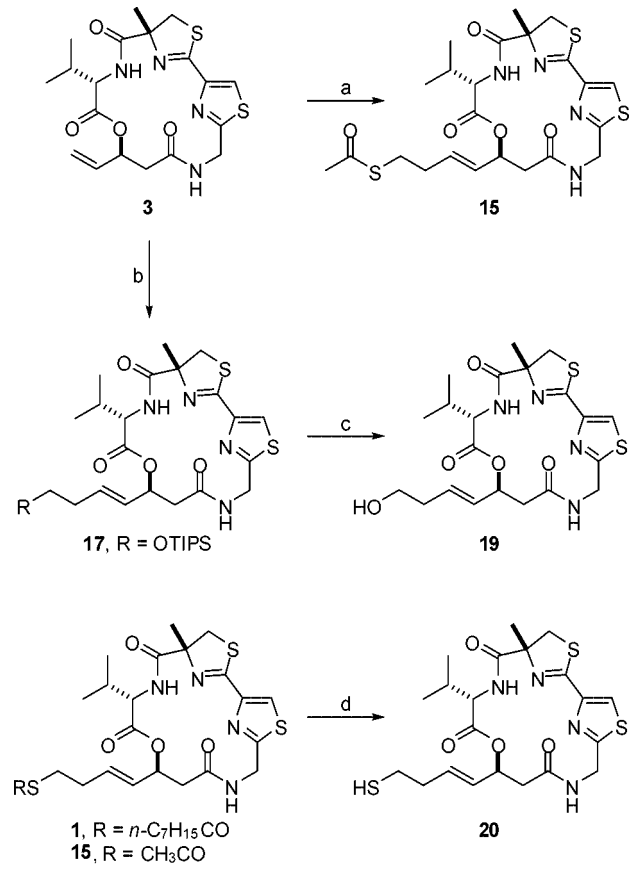
[a] (a) 4-Bromo-1-butene, NaI, THF, H_2O, reflux, 16 h, 55%; (b) NaH, 4-bromo-1-butene, THF, 25 °C, 12 h, 81%; (c) Grubbs' second-generation catalyst (50 mol %), toluene, reflux, 4 h, 41% (64% BRSM).

thioester **2** in the presence of Grubbs' second-generation catalyst (50 mol %, toluene, reflux, 4 h) provided **1** in 41% (64% BRSM, (*E*)-isomer only) yield identical in all respects with authentic largazole. The olefin cross-metathesis reaction of **3** and **2** in toluene occurred in a higher yield than in CH_2Cl_2 or benzene. Hoveyda−Grubbs' catalyst was not as effective as Grubbs' second-generation catalyst in the olefin cross-metathesis reaction.

Synthesis of Key Analogues. Following the completion of the total synthesis of **1**, our efforts turned to the preparation and evaluation of a series of key analogues (Scheme 3). To define the role of the octanoyl group in the thioester moiety, we prepared the acetyl analogue **15** of largazole by olefin cross-metathesis reaction of the macrocycle **3** with thioacetic acid *S*-but-3-enyl ester (**16**)[12] (50 mol % of Grubbs' second-generation catalyst, toluene, reflux, 4 h, 54% (71% BRSM), (*E*)-isomer only). Similarly, the hydroxyl analogue **19** (1-triisopropylsilyloxyl-3-butene (**18**),[13] 30 mol % of Grubbs' second-generation catalyst, toluene, reflux, 3 h, 50%, (*E*):(*Z*) = >9:1; TBAF, THF, 25 °C, 1 h, 76%) was synthesized as shown in Scheme 3. In addition, aminolysis of **1** or **15** (aq. NH_3, CH_3CN, 25 °C, 12−18 h) smoothly proceeded to afford the thiol analogue **20** in 70−80%.

HDACs as Molecular Targets. The presence of a thioester functionality in largazole (**1**) is intriguing since cellular metabolism is expected to rapidly generate the corresponding thiol **20**, suggesting that largazole may be a prodrug. The liberated thiol **20** would then correspond to the mercapto unit that is generated by reduction of the disulfide bond in FK228 and the related spiruchostatins, which are histone deacetylase (HDAC) inhibitors (Figure 3).[14] Histone deacetylation is an epigenetic mechanism that regulates gene transcription, including genes that control proliferation, which can lead to silencing of tumor

Scheme 3. Synthesis of Key Analogues[a]



[a] (a) Thioacetic acid *S*-but-3-enyl ester (**16**), Grubbs' second-generation catalyst (50 mol %), toluene, reflux, 4 h, 54% (71% BRSM); (b) 1-triisopropylsilyloxyl-3-butene (**18**), Grubbs' second-generation catalyst (30 mol %), toluene, reflux, 3 h, 50%; (c) TBAF, THF, 25 °C, 1 h, 76%; (d) aq. NH_3, CH_3CN, 25 °C, 12−18 h, 70−80%.

suppressor genes.[15] HDAC inhibition is a validated approach for cancer therapy and an active area of pharmaceutical drug discovery research.[16] The recent FDA approval of suberoylanilide hydroxamic acid (SAHA) marketed by Merck as Zolinza for the treatment of cutaneous T-cell lymphoma is the first tangible result of these efforts.[17] Thus HDAC inhibition could indeed explain the largazole-induced phenotype, including selectivity for transformed cells.[1]

To test the hypothesis that largazole inhibits HDACs, we determined the cellular HDAC activity upon treatment with largazole in HCT-116 cells found to possess high intrinsic HDAC activity. We coincubated a cell-permeable fluorogenic artificial HDAC substrate (*fluor de Lys*, BIOMOL) and largazole (**1**) and determined that largazole treatment for 8 h resulted in a decrease of HDAC activity in a dose−response manner (Figure

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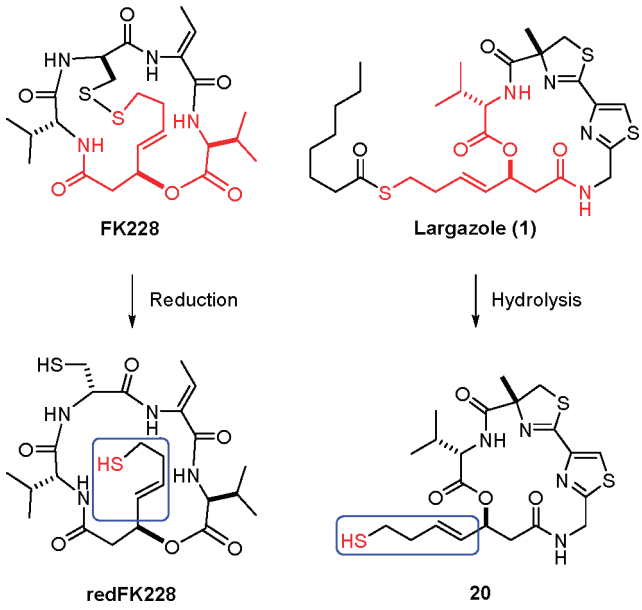

Figure 3. Structural similarity between FK228 and largazole (**1**) and modes of activation.

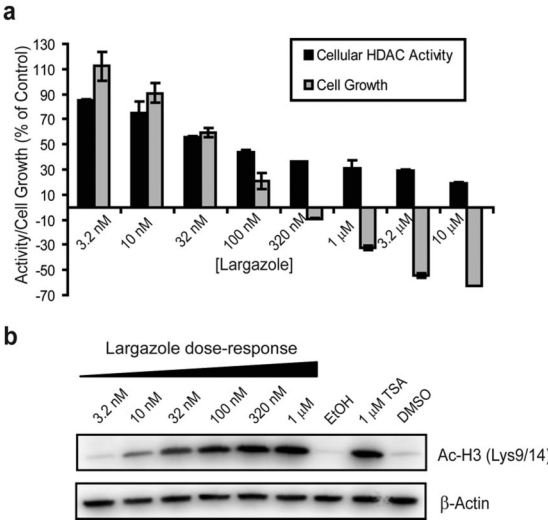

Figure 4. Target identification. (a) Largazole (**1**) inhibits cellular HDAC activity in HCT-116 cells (8 h) based on deacetylation of a fluorogenic substrate (BIOMOL). Growth-inhibitory activity (48 h) is similar. (b) Immunoblot analysis showing inhibition of endogenous histone H3 deacetylation in a dose−response fashion (8 h). Trichostatin A (TSA) was used as a positive control.

Table 1. IC_{50} and GI_{50} Values for HDACs and Growth Inhibition (nM)

	HCT-116 growth inhibition	HCT-116 HDAC cellular assay	HeLa nuclear extract HDACs
1	44 ± 10	51 ± 3	37 ± 11
20	38 ± 5	209 ± 15	42 ± 29
15	33 ± 2	50 ± 18	52 ± 27
3	>10000	>10000	>10000
19	>10000	>10000	>10000

4a) and, importantly, the IC_{50} for HDAC inhibition closely corresponded with the GI_{50} of largazole in this cell line (Figure 4a, Table 1). This correlation suggested that HDAC is the relevant target responsible for largazole's antiproliferative effect. Confirmatory, immunoblot analysis of an endogenous HDAC substrate, acetylated histone H3, revealed the same dose−response relationship (Figure 4b).

Largazole (**1**) inhibited HDAC activity from a HeLa cell nuclear protein extract rich in class I HDACs 1, 2, and 3 (BIOMOL); however, it is possible that the thioester is cleaved under assay conditions. To substantiate the hypothesis that the thiol analogue **20** is the reactive species, we liberated **20** from largazole (**1**) or the acetyl analogue **15** (Scheme 3) and measured enzymatic activity directly; compound **20** inhibited the HDACs in the nuclear extract of HeLa cells with a similar IC_{50} value (Table 1). Largazole (**1**) and the thiol analogue **20** exhibited similar cellular activity against HDACs derived from nuclear HeLa extracts as well as antiproliferative activity. We presume that Nature engineered the biosynthesis of the protected form rather than the target-reactive metabolite itself to increase stability and avoid potential oxidation.

Structure−Activity Relationship (SAR). Initial SAR studies further defined the pharmacophore of largazole. The acetyl analogue **15** showed the same activity as largazole (**1**) in the cellular HDAC assay and the same antiproliferative activity, underscoring that the carboxylic acid portion of the thioester merely plays a transient protective role and can be varied as long as thioester hydrolysis is achieved. To further define the pharmacophore we tested key analogues of **1** with modified side-chain functionality. Since hydroxyl groups do not chelate Zn^{2+}, the hydroxyl analogue **19** was expectedly not an HDAC inhibitor, paralleling its lack of growth-inhibitory activity (Table 1). Additionally, the macrocyle **3**, which lacks the sulfur-containing aliphatic chain, also lacks any inhibitory activity in the HDAC as well as cell viability assay (Table 1), highlighting that the thiol group is indispensable for both activities. The correlation of HDAC inhibitory activity and antiproliferative effect for all key compounds synthesized indicates that both activities are closely related and that the growth inhibition is a functional consequence of HDAC inhibition.

HDAC Isoform Selectivity. HDACs are grouped into four major classes. While class III enzymes require NAD^+ as a cofactor, enzymes of class I, II and of the more recently discovered class IV are Zn^{2+}-dependent enzymes.[16] The activity of thiols related to largazole derivative **20** against Zn^{2+}-dependent HDACs has been attributed to their ability to chelate Zn^{2+}.[16] Particularly, class I HDACs are critical in controlling proliferation in mammalian cells.[18] HDAC1 and HDAC3 (both class I) overexpression has been observed in prostate and colon tumors, respectively,[19] while inhibition of certain class II HDACs may cause cardiac hypertrophy.[20] Consequently, the selective inhibition of class I enzymes is desirable in anticancer drug discovery. To establish a preliminary selectivity profile, we tested largazole (**1**) and **20** against recombinant HDAC1 (class I) and HDAC6 (class II) (Table 2). Compound **20** inhibited HDAC1 activity at low nanomolar concentrations and was 150-fold less active against HDAC6. This strong class I

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Table 2. IC_{50} Values for HDAC1 and HDAC6 Inhibition (nM)

	HDAC1 (class I)	HDAC6 (class II)
largazole (**1**)	25 ± 11	5700 ± 3600
thiol **20**	2.5 ± 1.4	380 ± 76
trichostatin A	4.9 ± 0.8	18 ± 12

selectivity had also been observed for FK228; conversely, the hydroxamic acids trichostatin A (Table 2) and SAHA are less discriminatory.[21] Furthermore, largazole (**1**) was 10- to 15-fold less active against both enzymes than **20**, indicating that hydrolysis did not as readily occur under assay conditions with the purified enzyme compared with HeLa nuclear extract, further supporting the hypothesis that largazole (**1**) is a prodrug and **20** is the reactive metabolite.

Conclusion

In summary, we have devised a concise and convergent synthesis of largazole (**1**) (eight steps, 19% overall yield) that is amenable to the synthesis of key analogues. We provided evidence that HDACs are the molecular targets of largazole responsible for its antiproliferative activity. HDACs are validated targets for anticancer therapy; first-in-class Zolinza (Merck) was recently approved for the treatment of cutaneous T-cell lymphoma, and various other HDAC inhibitors are in advanced clinical trials.[16,17] Largazole (**1**) is class I HDAC selective, suggesting that it may be a valuable addition to the weaponry against certain types of cancers. Further biological studies with largazole-based inhibitors are currently underway to assess the chemotherapeutic potential of this group of antiproliferative agents.

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Supporting Information Available: General experimental procedures including spectroscopic and analytical data for **1**−**4**, **7**, **9**, **10**−**12**, **15**, **17**, **19**, and **20** along with copies of ^{1}H and ^{13}C NMR spectra; attempts for macrolactonization route; detailed assay procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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