Synthesis and HDAC Inhibitory Activity of Largazole Analogs: Alteration of the Zinc-Binding Domain and Macrocyclic Scaffold

Albert A. Bowers[1], **Nathan West**[3,4], **Tenaya L. Newkirk**[1], **Annie E. Troutman-Youngman**[1], **Stuart L. Schreiber**[4,5], **Olaf Wiest**[6], **James E. Bradner**[3,4,*], and **Robert M. Williams**[1,2,*]

1 *Department of Chemistry, Colorado State University, Fort Collins, Colorado 80523*

2 *University of Colorado Cancer Center, Aurora, Colorado 80045*

3 *Division of Hematologic Neoplasia, Dana-Farber Cancer Institute, Boston, MA 02115*

4 *Chemical Biology Program, Broad Institute of Harvard & MIT, Cambridge, MA 02142*

5 *Howard Hughes Medical Institute, Chemistry & Chemical Biology, Harvard University, Cambridge, MA 02138*

6 *Walther Cancer Research Center and Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556*

Abstract
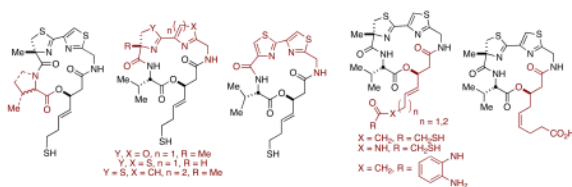
Fourteen analogs of the marine natural product largazole have been prepared and assayed against HDACs 1,2, 3, and 6. Olefin cross-metathesis was used to efficiently access six variants of the side-chain zinc-binding domain, while adaptation of our previously reported modular synthesis allowed probing of the macrocyclic cap group

Histone deacetylases (HDACs) control gene transcription *via* regulation of lysine acetylation and their selective inhibition is currently a major area of research in cancer chemotherapy.[1] The eighteen known HDACs are divided into four classes based on sequence homology to yeast counterparts.[2] The significance of individual classes and isoforms to cancer growth and inhibition is not well understood, yet the majority of data suggest that class I HDACs are the most relevant, tractible cancer targets.[3]

The pharmacophore model for HDAC inhibition consists of three elements: (1) a surface recognition unit which interacts with the rim of the binding pocket, (2) a metal-binding domain which coordinates to the active site zinc ion, and (3) a linker that connects the surface recognition site to the zinc-binding domain.[1b] Numerous HDAC inhibitors (HDACi), both

natural and synthetic, are known and variations in all three features have variably contributed to potency and selectivity in new HDACi.[4,5]

To date, the most potent and selective HDACi known is largazole (**1a**, Figure 1), a densely functionalized macrocyclic depsipeptide isolated from the cyanobacterium *Symploca sp.* by Luesch and co-workers.[6] We have recently disclosed a concise, modular, and scalable total synthesis of largazole and demonstrated its picomolar activity against HDACs 1, 2, and 3, as well as low nanomolar cytotoxicity against a number of chemoresistant cancer cell lines.[7,8] Additionally, we have disclosed a detailed conformation-activity relationship model for largazole, FK228, and their corresponding amide isosteres with insights into the key contacts and associated spatial determinants that provide this remarkable level of activity.[9] Herein we describe our continuing efforts to modify the structural scaffold of largazole with the goal of further defining and expanding structure-activity relationships within the family of macrocyclic HDACi's. Our over-arching aim in this context, is to perturb the class- and isoform-selectivity as initially observed in robust biochemical assays such that differences in specific enzymatic activity might later be correlated with phenotypic responses in cell-based assays.

Our reported route to largazole proved highly reproducible and we were able to rapidly adapt it to simple variants of the macrocyclic core.[7] Thus we were able to easily access milligram quantities of the C-2 epimer (**3**) and the enantiomer (**2**) of largazole. Additionally, we sought to perturb the conformation of the macrocycle by imparting greater rigidity; our initial foray in this capacity, replaced the valine residue with proline (**4**). Compound **4** could be obtained in only slightly diminished overall yield *via* the same synthetic route we deployed in the total synthesis of largazole (see Supporting Information).

Two methods were employed to alter side chain functionality and access a series of largazole chimeras. For the largazole-azumamide hybrid (**9**), the *cis*-geometry of the alkene residue necessitated its early introduction. Thus, aldol condensation of aldehyde **5**[10] with thiazolidine-2-thione **6** provided the necessary β-hydroxy acid building block (**7**, Scheme 1 & Supporting Information). For other variants investigated, late-stage introduction of the zinc-binding side arms *via* cross metathesis proved expedient. Cross metathesis had previously been used by Luesch, Phillips, and Cramer to attach the natural side-chain in their syntheses of largazole itself and was investigated independently in our laboratories.[8a–d]

Cramer et al. have demonstrated that, at least where largazole is concerned, the four-atom linker length relative to the thiol is optimal for maximum HDAC inhibition.[8d] However, literature precedent has shown that a four- to five- atom chain is optimal in small molecules bearing alternative zinc-binding functionality. Therefore, in the series of analogs prepared *via* metathesis both the four- and the five- atom tethers were synthesized.

Compounds **11** and **12** bear the well-known α-aminobenzamide group present in MS-275.[11] Meanwhile, compounds **13**, **14** and **15**, **16** contain α-thioamides and α-thioketones, respectively. These two motifs were identified as potential candidates in a computational study done by Vanommeslaeghe and have demonstrated promise in subsequent medicinal efforts.[12] Of note, our yields in the metathesis reaction conform to the results reported by Cramer.[8d] Our initial yields, employing Grubb's second-generation ruthenium catalyst were low with poor conversion. The Hoveyda-Grubbs second-generation catalyst proved much more efficient. All Boc- and Trityl- protecting groups were removed prior to biological assay (see Supporting Information).

We also sought to probe the significance of the methyl substituent on the thiazoline ring. Condensation of nitrile **17** with L-cysteine proved remarkably facile, proceeding in near quantitative yield (Scheme 3). Initial efforts at coupling to ester **19** provided poor yields of the

desired acyclic precursor **20**. The major product was thiazole-thiazole **21**, resulting from *in situ* oxidation. Optimization of conditions for this coupling eventually allowed for up to 62% yield of the desired product. Oxidation could be the cause of the somewhat diminished yields in the cyclization of **20**. Compound **23** could not be detected in NMR spectra of the crude reaction mixtures from cyclization of **20**. Moreover, **23** could not be prepared directly from its acyclic precursor **21**. Instead, oxidation of **22** under standard conditions provided **23**. This macrocycle clearly contains some added constraint as demonstrated by presence of rotamers in the ^{1}H NMR spectrum in CDCl$_3$. Both substrate **22a** and **23a** could be deprotected in good yield using our standard conditions.

Replacement of the thiazole moiety with a pyridine residue in the heterocyclic backbone was readily ammenable to our synthetic strategy (Scheme 4). Known chloro-nitrile **24** could be Boc-protected and then condensed with α-methyl cysteine to provide acid **27**. Subsequent deprotection, coupling, and cyclization provided analog **29**.

We also sought to perform additional single-atom replacements within the largazole macrocyclic scaffold to interrogate very small structural and attendant conformational changes manifest. Due to the inherent acid instability of oxazolines, additional protecting group manipulations were required for synthesis of oxazoline-oxazole substrate **39** (Scheme 5). Thus, oxazole **31** could be saponified and coupled to α-methyl serine.[13] Switching the nitrogen protecting group then allowed for cyclization and deprotection/acylation with thiazolidine-2-thione **35** to obtain alcohol **36**. Coupling to Fmoc-L-valine then provided the acyclic precursor **38**.

Finally, deprotection under basic conditions and cyclization gave macrocycle **39a**. In this case, removal of the trityl group was performed with iodine in methanol, yielding exclusively the disulfide homodimer (**39c**) resulting from spontaneous autoxidation of the incipient thiol (**39b**). The dimer is reduced to the active thiol (**39b**) under the reducing conditions (TCEP) of our biochemical assay. We next performed comparative profiling of largazole thiol and the thiol synthetic derivatives described herein, for inhibitory potency and selectivity against HDAC1, HDAC2, HDAC3/NCoR and HDAC6. A complete description of the assay has been provided previously[7] and is described in detail in Supporting Information.

In brief, small-molecule inhibitors are arrayed at twelve-point dose-response (3-fold increments) in 384-well library plates and transferred by robotic pin device to replicate assay plates containing assay buffer under reducing conditions (TCEP 200 μM). A liquid handling device then transfers a tripeptide substrate terminating in acetyl-lysine and amide conjugated to 4-methyl-7-aminocoumarin (AMC), recombinant human histone deacetylase (BPS Bioscience, San Diego, CA), and recombinant human trypsin (Sigma-Aldrich, St. Louis, MO). Following deacetylase hydrolysis of acetyl-lysine, trypsin cleavage liberates the AMC fluorophore. Kinetic (fluorescence per unit time) and end-point (total fluorescence) data are captured by a multilabel plate reader. Replicate data are analyzed by curve-fit using logistic regression (Spotfire Decision-Site). A summary of assay data are in Table 1.

Several striking observations emerge from this dataset. The largazole enantiomer (**2**) exhibits a decrease in potency by almost exactly three orders of magnitude for all isoforms tested, underscoring the obligate, stereochemical and conformation-activity relationship between the natural product and its protein targets. This is further substantiated by the intermediate potency of the C-2 epimer (**3**), the valine-to-proline substitution (**4**) and the oxdized and strained thiazole-thiazole derivative (**23b**). It is significant to note that the single-atom substitutions of the sulfur atoms for oxygen atoms in the oxazoline-oxazole derivative (**29b**) provided a compound equipotent to largazole itself. Our synthetic approach has allowed rapid diversification of the zinc-binding arm, modulating both potency and specificity (as for **11b**,

12b, **13b** and **14b**). While none of the zinc-binding replacements for the natural 3-hydroxy-7-mercaptohept-4-enoic acid moiety present in largazole, FK228 and spiruchostatin yielded more potent analogs, many additional zinc-binding replacements are evident and will be evaluated in due course. We were pleased to observe a significant increase in potency with pyridine substitution of the thiazole; this compound (**29b**) possesses sub-nanomolar activity against Class I HDACs, and will be studied further in translational models of cancer. *Compound 29b now constitutes the most biochemically potent Class I HDAC inhibitor known being between three~four times more potent than largazole itself against HDACs 1, 2 and 3.* Most notably, we have demonstrated that the methyl substituent of the thiazoline ring is non-essential for the dramatic potency of the natural product (cf. **22b**). The commercial availability of relatively inexpensive cysteine, compared with that of the α-methylcysteine residue of natural largazole, permitted for a reduction in the overall synthetic approach to **22b** by four steps, establishing a high-yielding, scalable, five-step synthesis of this agent. This highly efficient synthesis is compatible with further derivitization and potential for practical scale-up endeavors. The present study provides additional insight into the structural, functional, stereochemical and conformational aspects of the largazole molecular scaffold that constitutes the basis for the further design and synthesis of extraordinarily potent HDAC inhibitors with potential therapeutic significance. Studies along these lines are under intensive investigation in our laboratories.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

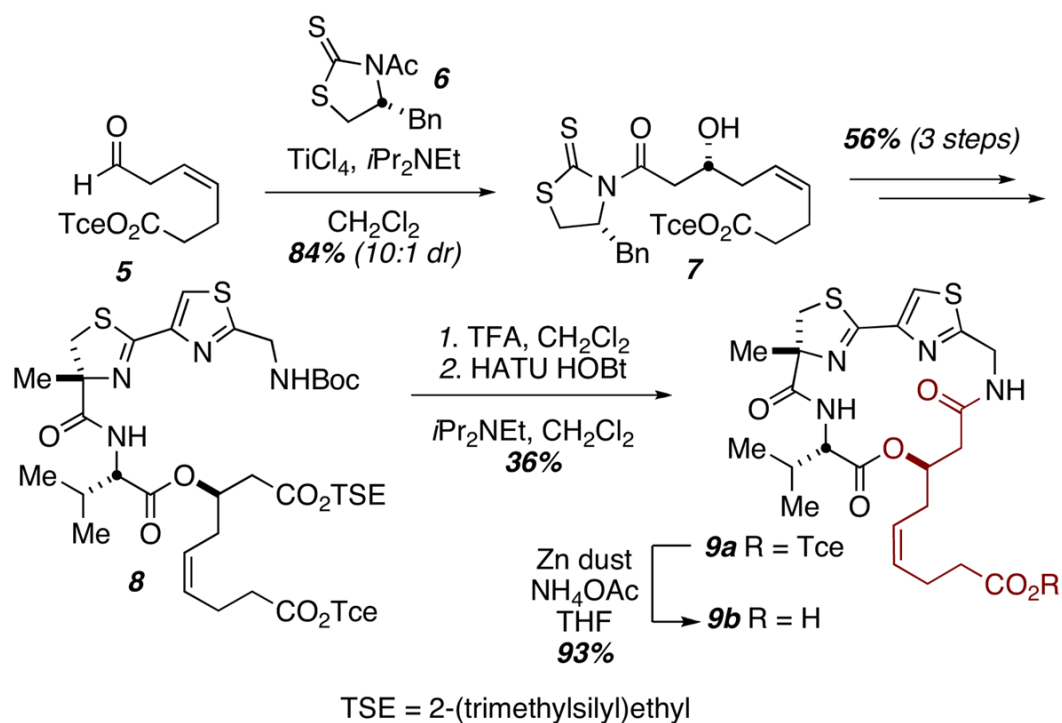## References

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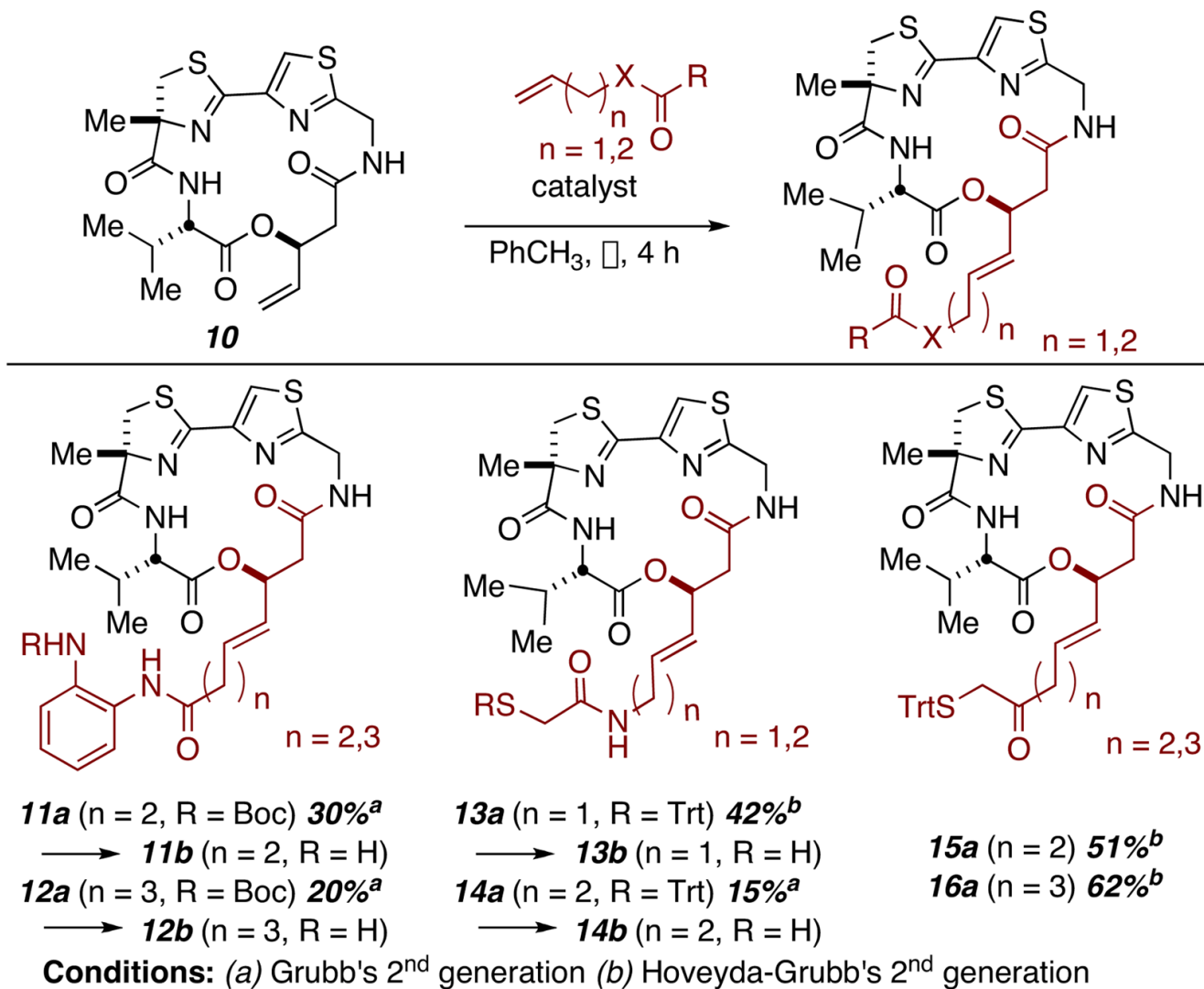
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13. α-Methylserine provided as a generous gift by Ajinomoto Co., Japan.
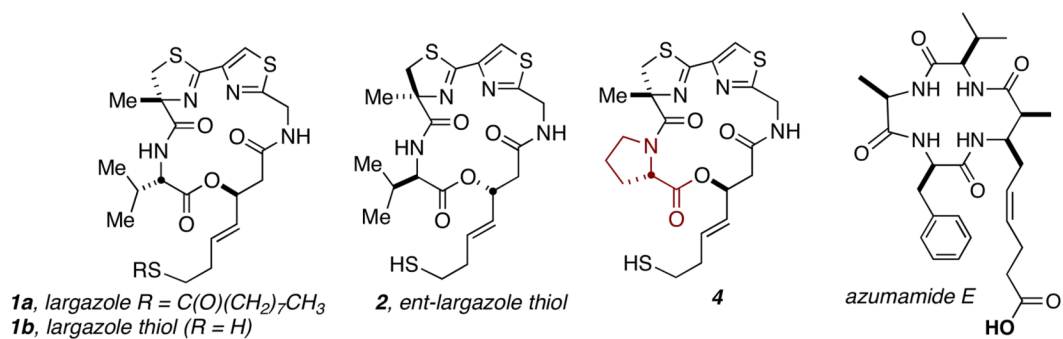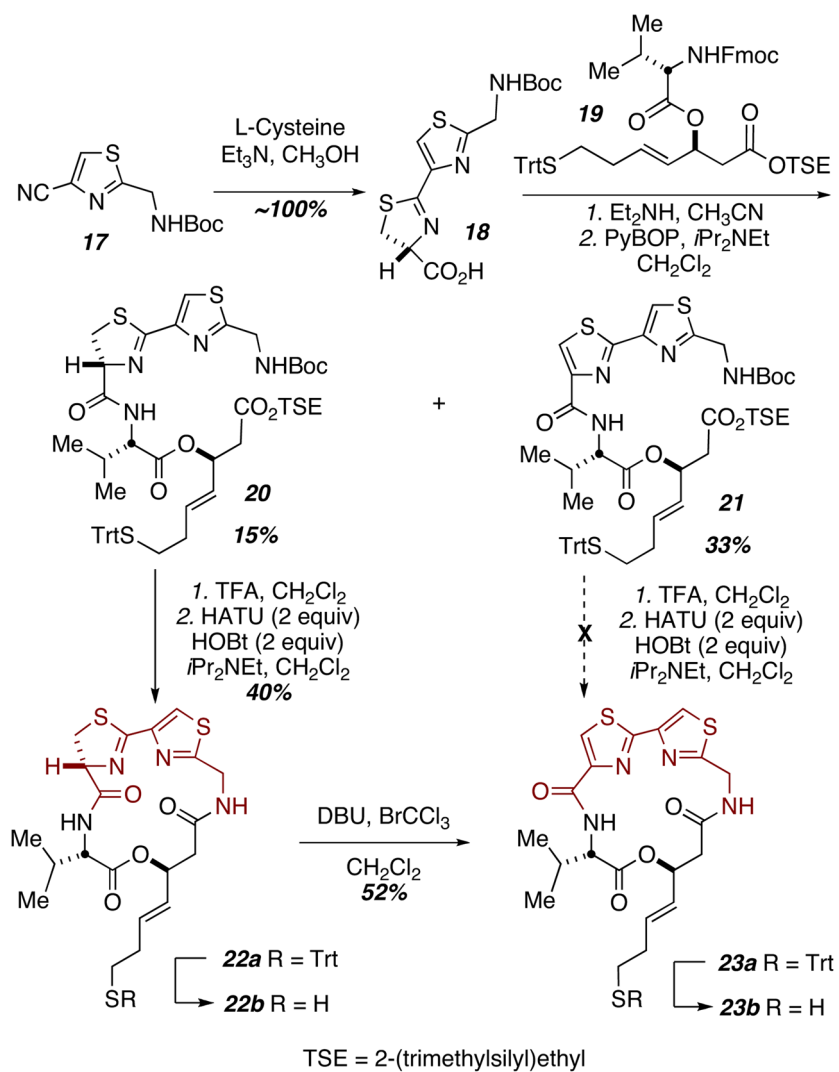
Figure 1.
Selected largazole analogs and azumamide E.

Scheme 1.
Synthesis of largazole-azumamide Hybrid.

10

catalyst

PhCH$_3$, □, 4 h

n = 1,2

11a (n = 2, R = Boc) **30%**[a]
⟶ **11b** (n = 2, R = H)
12a (n = 3, R = Boc) **20%**[a]
⟶ **12b** (n = 3, R = H)

13a (n = 1, R = Trt) **42%**[b]
⟶ **13b** (n = 1, R = H)
14a (n = 2, R = Trt) **15%**[a]
⟶ **14b** (n = 2, R = H)

15a (n = 2) **51%**[b]
16a (n = 3) **62%**[b]

Conditions: *(a)* Grubb's 2nd generation *(b)* Hoveyda-Grubb's 2nd generation
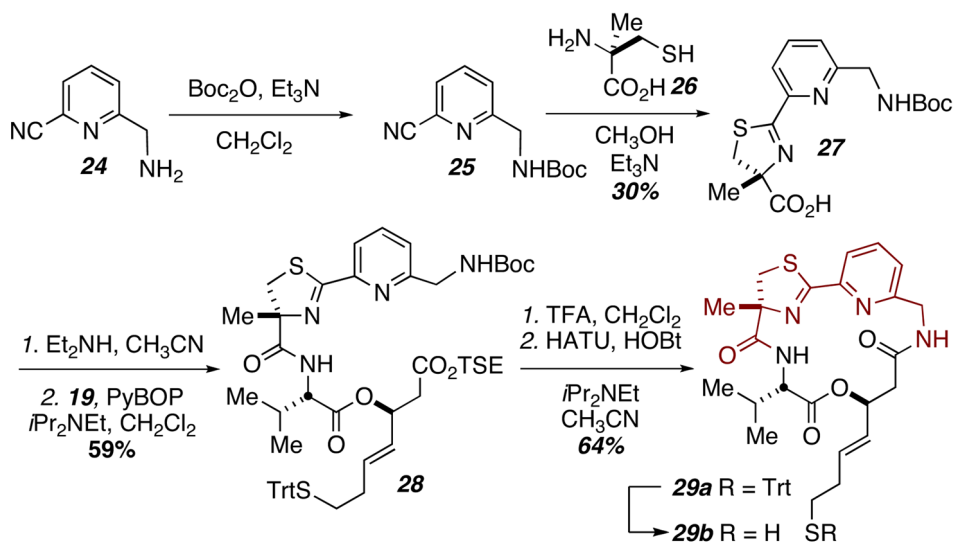
Scheme 2.
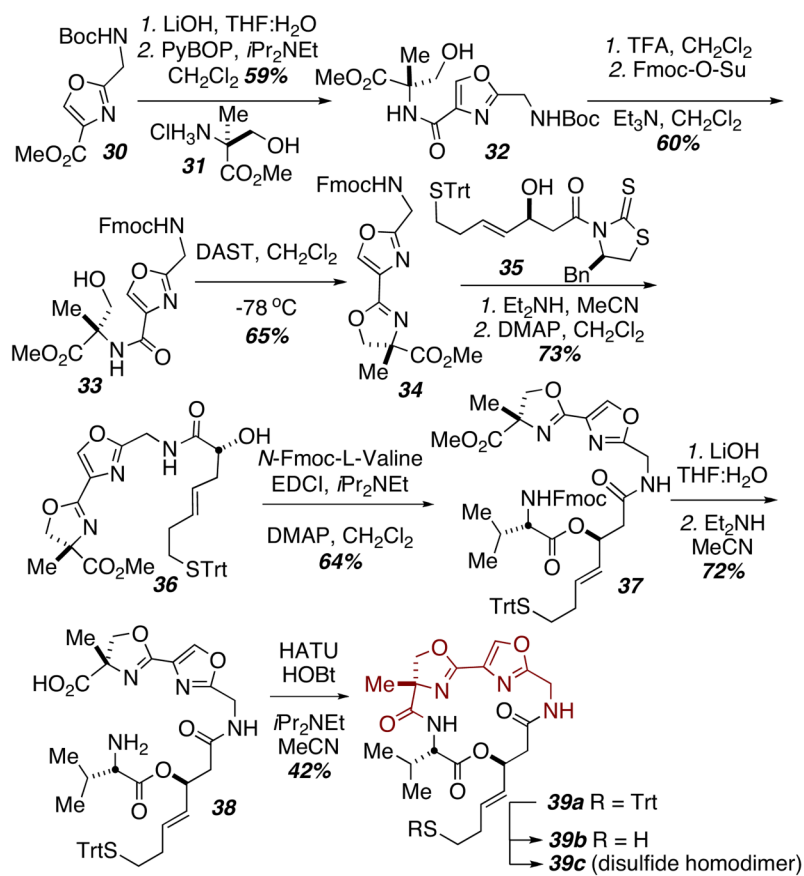Metathesis route to largazole hybrids.

Scheme 3.
Synthesis of cysteine & thiazole-thiazole analogs.

Scheme 4.
Synthesis of thiazole to pyridine substitution.

Scheme 5.
Synthesis of the oxazoline-oxazole analog.

Table 1

Biochemical inhibiton of human HDACs (IC$_{50}$, μM).

compound	HDAC1	HDAC2	HDAC3	HDAC6
largazole thiol (**1b**)	0.0012	0.0035	0.0034	0.049
enantiomer (**2**)	1.2	3.1	1.9	2.2
C-2 epimer (**3**)	0.030	0.082	0.084	0.68
proline substitution (**4**)	0.11	0.80	0.58	13
largazole-azumamide hybrid (**9b**)	> 30	> 30	> 30	> 30
benzamide (**11b**)	0.27	4.1	4.1	> 30
benzamide (**12b**)	23	29	14	> 30
thioamide (**13b**)	0.67	1.6	0.96	0.7
thioamide (**14b**)	1	1.9	1.5	0.24
cysteine substitution **22b**	0.0019	0.0048	0.0038	0.13
thiazole-thiazole (**23b**)	0.077	0.12	0.085	> 30
thiazole-pyridine substitution (**29b**)	0.00032	0.00086	0.0011	0.029
oxazoline-oxazole (**39c**)	0.00069	0.0017	0.0015	0.045
SAHA	0.010	0.026	0.017	0.013
MS-275	0.045	0.13	0.17	> 30