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Total Synthesis and Biological Mode of Action of Largazole: A Potent Class I Histone Deacetylase Inhibitor

Albert Bowers,[†] Nathan West,[§] Jack Taunton,[||] Stuart L. Schreiber,[⊥] James E. Bradner,[§] and Robert M. Williams*[,†,‡]

Department of Chemistry, Colorado State University, Fort Collins, Colorado 80523, University of Colorado Cancer Center, Aurora, Colorado 80045, Dana−Farber Cancer Institute, 44 Binney Street, Boston, Massachusetts 02115, Department of Cellular and Molecular Pharmacology, University of California−San Francisco, San Francisco, California 94158, Howard Hughes Medical Institute, Chemistry & Chemical Biology, Harvard University, Broad Institute of Harvard and MIT, Cambridge, Massachusetts 02142

Received May 6, 2008; E-mail: rmw@lamar.colostate.edu

Abstract: The efficient total synthesis of the recently described natural substance largazole (**1**) and its active metabolite largazole thiol (**2**) is described. The synthesis required eight linear steps and proceeded in 37% overall yield. It is demonstrated that largazole is a pro-drug that is activated by removal of the octanoyl residue from the 3-hydroxy-7-mercaptohept-4-enoic acid moiety to generate the active metabolite **2**, which is an extraordinarily potent Class I histone deacetylase inhibitor. Synthetic largazole and **2** have been evaluated side-by-side with FK228 and SAHA for inhibition of HDACs 1, 2, 3, and 6. Largazole and largazole thiol were further assayed for cytotoxic activity against a panel of chemoresistant melanoma cell lines, and it was found that largazole is substantially more cytotoxic than largazole thiol; this difference is attributed to differences in the cell permeability of the two substances.

Introduction

Largazole (**1**) is a densely functionalized macrocyclic depsipeptide, recently isolated from the cyanobacterium *Symploca* sp. by Luesch and co-workers.[1] This natural product exhibits exceptionally potent and selective biological activity, with 2- to 10-fold differential growth inhibition in a number of transformed and non-transformed human- and murine-derived cell lines. The remarkable selectivity of this agent against cancer cells prompts particular interest in its mode of action and its value as a potential cancer chemotherapeutic.

In their isolation paper, Luesch, et al. stated that "the 3-hydroxy-7-mercaptohept-4-enoic acid unit in **1** is unprecedented in natural products." [1] In contrast to this assertion, the (*S*)-3-hydroxy-7-mercaptohept-4-enoic acid is in fact an essential motif in several cytotoxic natural products, including FK228 (FR901228),[2] FR901375,[2] and spiruchostatin[3] (Figure 1), all of which are known histone deacetylase inhibitors (HDACi).[4,5]

The histone deacetylase enzymes are zinc metalloenzymes that catalyze the hydrolysis of acetylated lysine residues in chromatin, and thereby regulate transcription in eukaryotic cells.[6,7] Their selective inhibition has recently become a major area of research in cancer chemotherapy.[8] To date, 18 HDACs have been identified, which are generally divided into four classes on the basis of their sequence homology to yeast counterparts.[9] With respect to cancer therapy, there is an emerging consensus that Class I HDACs are clinically relevant and that the undesirable toxicity associated with the first generation of HDAC inhibitors may be related to class indiscriminancy. As a result, our laboratories have recently initiated programs aimed at the synthesis and modification of peptide- and depsipeptide-based HDACi with the objective of optimizing structures for class- and even isoform-specific inhibition.

[†] Colorado State University.
[§] Dana−Farber Cancer Institute.
[||] University of California−San Francisco.
[⊥] Howard Hughes Medical Institute.
[‡] University of Colorado Cancer Center.

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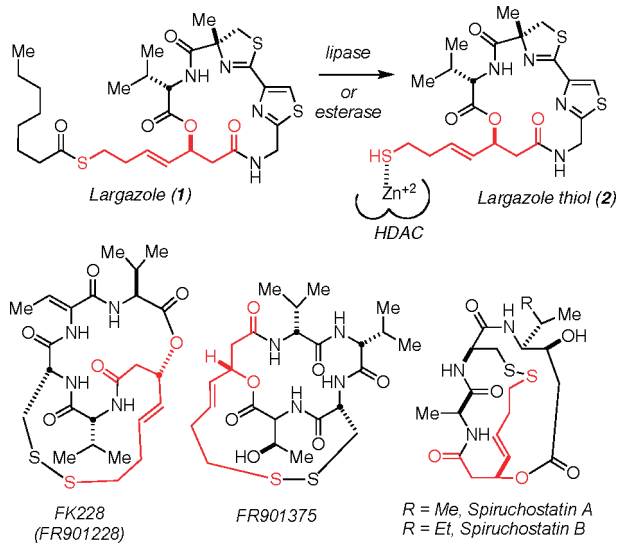


Figure 1. Structure of largazole (**1**), largazole thiol (**2**), and several known macrocyclic HDACi natural products containing the (*S*)-3-hydroxy-7-mercaptohept-4-enoic acid moiety.

The three natural substances FK228, FR901375, and spiruchostatin are all activated *in vitro* and *in vivo* by reductive cleavage of a disulfide bond to expose the free sulfhydryl residue of the pendant (*S*)-3-hydroxy-7-mercaptohept-4-enoic acid moiety that coordinates to the active-site Zn^{2+} residue of the HDACs, resulting in a potent inhibitory effect.[10] Given that largazole contains this well-known Zn^{2+}-binding arm, it seemed evident to us that largazole is simply a pro-drug that is activated by hydrolytic removal of the octanoyl residue by cellular lipases and/or esterases to produce the putative cytotoxic species **2** (the "largazole thiol"). Two laboratories have previously demonstrated that thioester analogues of FK228 retain their antiproliferative activity in cell-based assays.[11] As part of our program on the synthesis and chemical biology of FK228 and analogues,[12] we report herein an efficient total synthesis of largazole and the largazole thiol and demonstrate that **2** is an extraordinarily potent HDACi.

Results and Discussion

We envisioned disconnection of the macrocycle to the four key subunits illustrated in Figure 2; that is, α-methylcysteine (**3**), thiazole (**4**), (*S*)-valine (**5**), and (*S*)-3-hydroxy-7-mercaptohept-4-enoic acid (**6**). Given the ready availability of these building blocks from prior efforts,[13,14] the underlying synthetic

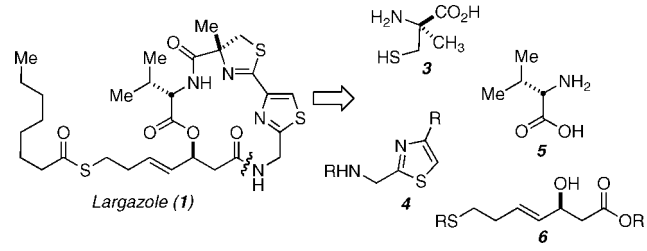
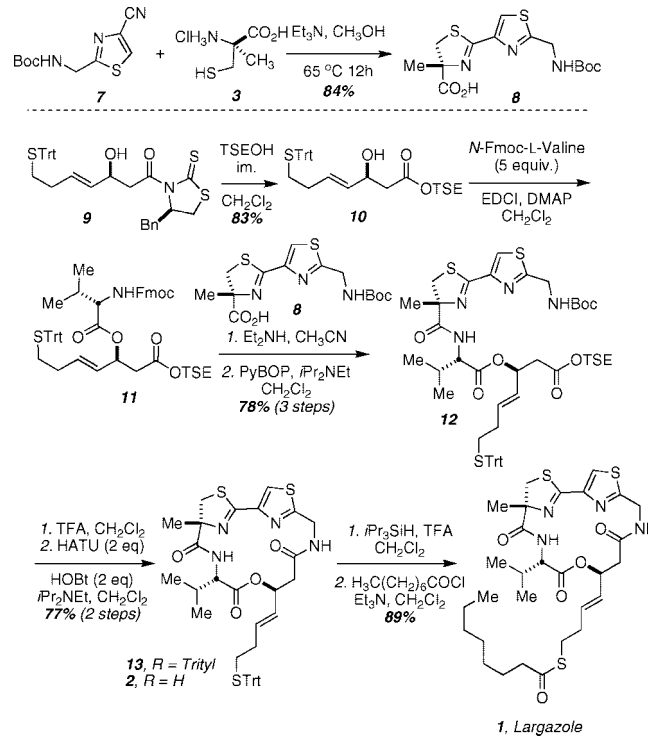


Figure 2. Synthetic strategy to assemble largazole.

Scheme 1. Total Synthesis of Largazole (**1**) and Largazole Thiol (**2**)



challenge turned out to be the macrocyclization strategy. Due to the anticipated susceptibility of the β-carboxylate linkage to undergo elimination, our initial efforts focused on installing this linkage last. However, all methods, both direct (macrolactonization *via* Yamaguchi, Mukaiyama, Keck, and Shiina procedures) and indirect (inversion *via* Mitsunobu reaction), failed to provide the desired macrocycle. An additional attempt at closure of the depsipeptide ring *via* a late-stage thiazoline-forming reaction also failed to provide the desired macrocyclic product. Thus, we turned to a strategy involving early installation of the ester and subsequent closure about the least-hindered amide bond.

We were able to readily access the necessary α-methylcysteine subunit with the requisite (*R*)-stereochemistry *via* the Pattenden modification of the Seebach protocol on L-cysteine methyl ester (Scheme 1).[14] Alternatively, we obtained α-methylserine as a generous gift from Ajinomoto Co., Japan, and converted this substance into α-methylcysteine by a published procedure.[15] Gram quantities of this amino acid were obtained in high enantiomeric purity and condensed with the known nitrile **7**[16] to provide the thiazoline−thiazole subunit **8** in high yield.

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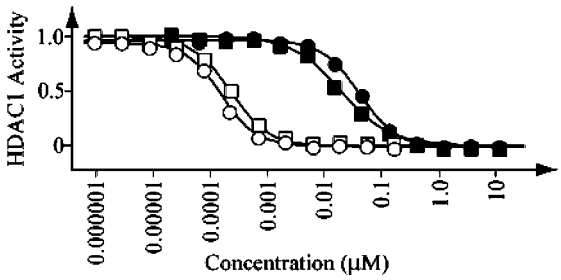

Figure 3. Inhibition of HDAC1 by largazole (●), largazole thiol (○), SAHA (■), and FK228 (□).

We recently disclosed a new synthetic route to the β-hydroxy acid **10** based on a Noyori asymmetric transfer hydrogenation.[12a] In the present work, we found a recently published synthesis of this subunit to also be expedient and high yielding.[17] Thiazolidinethione (**9**) was treated with 2-(trimethylsilyl)ethanol to provide the TSE-protected acid **10**, which was subsequently coupled to *N*-Fmoc-L-valine to afford **11**. Due to the sluggish reactivity of allylic alcohol **10**, we found it necessary to use an excess (5 equiv) of the commercially available amino acid. Removal of the Fmoc group and PyBOP-mediated coupling to the thiazoline−thiazole carboxylic acid (**8**) furnished the acyclic precursor **12**.

Cyclization was effected under high dilution in the presence of 2 equiv each of HOBt and HATU, furnishing the desired macrocycle **13** in 77% isolated yield from **12**. Removal of the *S*-trityl protecting group was accomplished with iPr$_3$SiH and TFA to provide an authentic sample of **2** in excellent yield.

Acylation of **2** with octanoyl chloride under standard conditions afforded synthetic largazole in 89% yield from **13**. The spectroscopic data (^{1}H NMR, ^{13}C NMR, and HRMS) for the synthetic substance were in excellent agreement with those published for the natural product (see Supporting Information).[1]

We next interrogated the biochemical activity of synthetic largazole (**1**) and **2** against HDACs 1, 2, 3, and 6, for which we have developed robust, kinetic biochemical assays. To measure the inhibitory effect on deacetylase function *in vitro*, we optimized a continuous kinetic biochemical assay miniaturized to a 384-well plate format. In this assay, purified, full-length HDAC protein (HDAC1 1.67 ng/μL, HDAC2 0.067 ng/μL, HDAC3/NCor2 0.033 ng/μL, HDAC6 0.67 ng/μL; BPS Biosciences) was incubated with a commercially available fluorophore-conjugated substrate at a concentration equivalent to the substrate K_m (Upstate 17−372; 6 μM for HDAC1, 3 μM for HDAC2, 6 μM for HDAC3/NCoR2, and 20 μM for HDAC6). As presented in Figures 3, 4 and Table 1, **2** is an extraordinarily potent inhibitor of HDAC1 and HDAC2 (K_i = 70 pM).

The parent natural product largazole itself, on the other hand, is a comparatively weak HDAC inhibitor with potency approximating that of the nonselective pharmaceutical product SAHA (Vorinostat, Merck Research Laboratories). In fact, the measurement of potency obtained in these studies of largazole defines the maximal possible HDAC inhibitory effect. That is, even a trace contamination of **2** or free thiol liberated under aqueous assay conditions or by trypsin (present in this enzyme-coupled reaction) could account for the substantial decrease in enzyme potency observed.

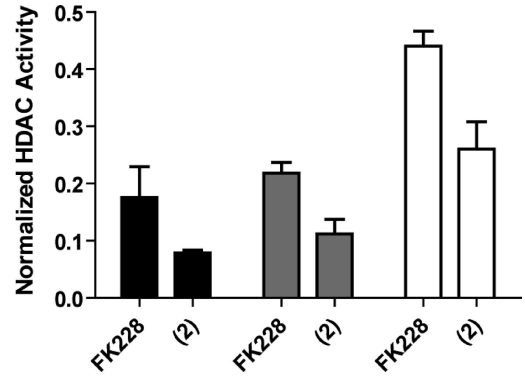

Figure 4. Comparative profiling of FK228 and the largazole thiol (**2**). Dose-ranging studies of FK228, largazole, and **2** were performed against Class I HDAC proteins using the described kinetic, homogeneous assay (see Supporting Information). As a comparative measure of potency, compounds were studied in triplicate at a standard concentration (0.6 nM). Averaged data are presented for inhibition of HDAC1 (black), HDAC2 (gray), and HDAC3/NCoR2 (white). Error bars reflect one standard deviation from the mean.

Table 1. HDAC Inhibitory Activity (K_i, nM) of Largazole (**1**) and Largazole Thiol (**2**) As Compared to Pharmaceutical Hdac Inhibitors

compound	HDAC1	HDAC2	HDAC3	HDAC6
1	20	21	48	>1000
2	0.07	0.07	0.17	25
FK228[a]	0.12	0.14	0.28	35
SAHA	10	10	15	9

[a] The FK228 sample used in this study was synthesized in these laboratories[12a] and purified by PTLC to homogeneity.

Detailed studies of FK228 isoform selectivity by our laboratories previously identified a strong bias favoring the Class I enzymes, HDAC1, HDAC2 and HDAC3, over the Class IIb enzyme, HDAC6.[12b] Similarly, the active depsipeptide largazole thiol (**2**) exhibits substantial potency against HDAC1, HDAC2 and HDAC3 in the picomolar range (Table 1). Indeed, this degree of inhibitory potency against HDAC1, HDAC2 and HDAC3 is, to the best of our knowledge, unprecedented. Only FK228 itself has HDACi potency approaching that of **2**.

Comparative profiling of FK228 and **2** demonstrates superior inhibitory potency of the thiol derivative against HDAC1, HDAC2, and HDAC3 (Figure 4). The comparatively diminished potency of largazole itself in these homogeneous assays supports the hypothesis of pro-drug activation of largazole, the specific catalysis of which is the focus of our immediate follow-up studies.

We have initiated studies aimed at determining the potential utility of largazole as an HDAC inhibitor-based therapeutic agent. This work comprises studies of physiochemical properties, metabolic stability in serum and liver microsomes, and pharmacokinetics in rodents. Immediately, we have initiated studies to determine the antineoplastic effects of **1** and **2** on cultured human cancer cells. Predicting a potent antiproliferative effect of largazole on the basis of the biochemical potency for Class I HDACs as described above, we selected a panel of malignant melanoma cell lines for study due to the typically extreme chemoresistance of this tumor. As demonstrated in Figure 5, largazole exhibits submicromolar inhibitory effect on melanoma

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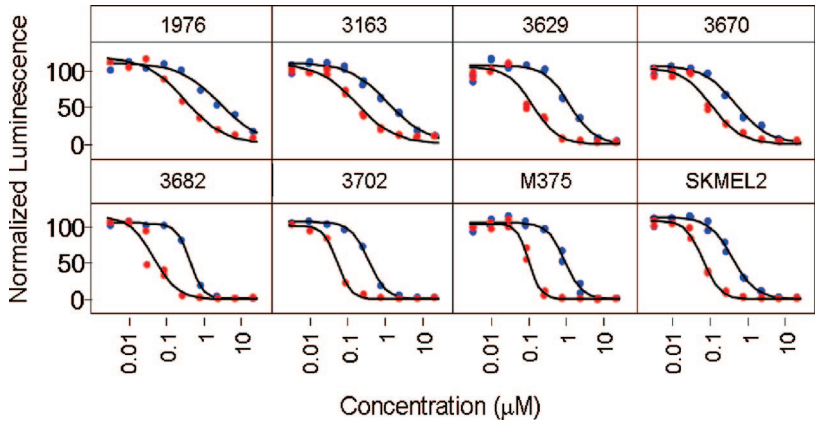

Figure 5. Antiproliferative effects of largazole (red) and largazole thiol (blue). Effects on cell viability were evaluated using a panel of human malignant melanoma cell lines, using the standard, surrogate measurement of ATP content (Cell TiterGlo; Promega) in 384-well plate format. Replicate measurements were normalized to vehicle-only controls and IC_{50} calculations were performed by logistic regression (Spotfire DecisionSite). Largazole demonstrates a potent antiproliferative effect (IC_{50} = 45−315 nM) compared to largazole thiol (IC_{50} = 360−2600 nM).

cell proliferation. Importantly, **1** has a consistent, superior potency (IC_{50} = 45−315 nM) compared to **2** (IC_{50} = 360−2600 nM).[18]

Conclusion

In conclusion, we have completed an efficient total synthesis of largazole (**1**) in eight linear steps and 37% overall yield, and its active metabolite, the largazole thiol (**2**), in seven linear steps. This serves to corroborate the structure assigned by Luesch and co-workers.[1] The synthesis recorded here provided 12 mg of **1** and 19 mg of **2** on the first pass and should be readily scaleable to gram quantities. This amount of synthetic material has allowed for further investigation of the biological activity of this potential cancer chemotherapeutic.

We have demonstrated that largazole is, in fact, a pro-drug, which must be converted to its active form, free thiol **2**. The combination of cap group and zinc-binding motif present in this thiol provides the most potent and selective HDACi reported to date. The octanoyl residue in largazole apparently serves a dual role, imparting better cell-permeability and allowing facile presentation of the free thiol within the cell. We attribute the observed inverse difference in cytotoxicity to the superior cell-permeability of the thioester **1** as compared to the thiol **2**. These studies reveal that numerous opportunities exist for capitalizing on the masked thiol pro-drug manifold inherent in **1**. The data presented here should prove useful in the design and development of potent and therapeutically active agents that target inhibition of HDACs. Since FK228, FR901375, and spirucho-statin mask the common and key 3-hydroxy-7-mercaptohept-4-enoic acid unit as a reductively labile disulfide,[19] other protect-and-release strategies for exploiting this potent zinc-binding arm in the context of new molecular scaffolds are readily apparent. In addition, the molecular scaffold of largazole provides yet another macrocyclic template[20] from which a myriad of potentially active and isoform-selective HDAC inhibitors might be designed and synthesized. Studies along these lines are under investigation in these laboratories.

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Supporting Information Available: Complete experimental details for the synthesis of largazole (**1**) and largazole thiol (**2**) and for the HDAC inhibition assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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(18) We note that there are significant discrepancies between what is reported in ref 1b and our results reported here with respect to the biochemical HDAC inhibitory data obtained and supporting mechanism of action. The biochemical data provided herein reflect activity in highly robust, miniaturized homogeneous assays, with Z' calculations compatible with high-throughput screening. In this assay, we observe high concordance with published kinetic measurements of enzyme inhibition (K_i). Thus, the accuracy of our reported HDAC inhibitory data would be expected to be markedly improved. This is important due to our observation of the unusual, perhaps unprecedented potency of **2** for HDAC1 and the direct comparison provided to FK228. The data offered in ref 1b are IC_{50} data, which have substantial variation between assays and are thus of limited utility and extensibility. We also note that the present synthesis is significantly higher yielding than that reported in ref 1b.

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