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Synthesis of 7200 Small Molecules Based on a Substructural Analysis of the Histone Deacetylase Inhibitors Trichostatin and Trapoxin

Scott M. Sternson, Jason C. Wong, Christina M. Grozinger, and Stuart L. Schreiber*

Howard Hughes Medical Institute (HHMI), Institute of Chemistry and Cell Biology (ICCB), Department of Chemistry & Chemical Biology, Harvard University, Cambridge, Massachusetts 02138

sls@slsiris.harvard.edu

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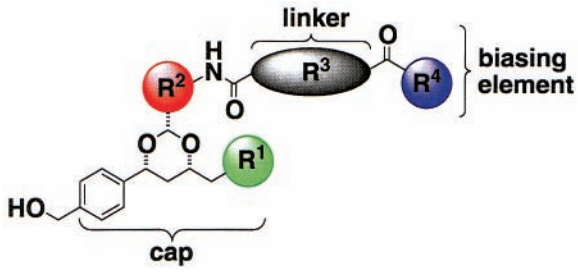
ABSTRACT



Seventy-two hundred potential inhibitors of the histone deacetylase (HDAC) enzyme family, based on a 1,3-dioxane diversity structure, were synthesized on polystyrene macrobeads. The compounds were arrayed for biological assays in a "one bead-one stock solution" format. Metal-chelating functional groups were used to direct the 1,3-dioxanes to HDAC enzymes, which are zinc hydrolases. Representative structures from this library were tested for inhibitory activity and the 1,3-dioxane structure was shown to be compatible with HDAC inhibition.

Post-translational modification of proteins through acetylation and deacetylation of lysine residues has a critical role in regulating their cellular functions.[1] While small molecule probes for specific protein kinases and phosphatases exist, probes of histone acetyl transferases and deacetylases are limited as a result of their lack of selectivity. Here we report a step toward our goal of identifying selective small molecule inhibitors of histone deacetylases (HDACs) by preparing thousands of such compounds using the one bead-one stock solution format[2] for synthesis.

HDACs are zinc hydrolases that modulate gene expression through deacetylation of the *N*-acetyl lysine residues of histone proteins and other transcriptional regulators.[3] HDACs participate in cellular pathways that control cell shape and differentiation, and an HDAC inhibitor has been shown effective in treating an otherwise recalcitrant cancer.[4] Nine human HDACs have been characterized[5] and two inferred;[6] these members fall into two related classes (class I and II). No small molecules are known that selectively target either the two classes or individual members of this family.[7]

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The natural products trapoxin[8] (TPX) and trichostatin A[9] (TSA) (Figure 1) are potent inhibitors and useful probes of

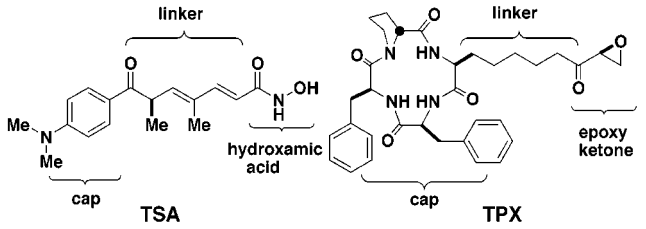

Figure 1. HDAC inhibitors trichostatin (TSA) and trapoxin (TPX). Analysis of these natural products and other HDAC inhibitors reveal a substructural organization consisting of a metal-binding functional group, a linker region, and a cap region.

HDACs, but their lack of selectivity among family members limits their ability to dissect the functions of individual members. The absence of atomic resolution structures of human HDACs complicates a structure-based solution to this problem; therefore, we have undertaken a screening-based approach. On the basis of sequence alignments of HDACs and structural analyses of natural HDAC inhibitors (Figure 1), including TPX and TSA, we conceived a synthetic pathway leading to 7200 1,3-dioxanes, all biased toward HDAC inhibition. Representative compounds that resulted from this pathway were shown to inhibit two different human HDACs; the varied structures of the dioxanes and their tendency to inhibit HDACs suggest that suitable screening methods may identify the sought after, specific inhibitors.

A structural rationale for HDAC inhibition is suggested from the X-ray crystal structure of TSA-bound HDAC-like protein (HDLP), an HDAC ortholog from the thermophilic bacterium *Aquifex aeolicus*.[10] In this structure, the hydroxamic acid of TSA penetrates a narrow, hydrophobic channel and chelates a buried zinc ion. The substructural organization of most HDAC inhibitors can be rationalized in light of the HDLP structure. Inhibitors typically possess metal-binding functionality and a cap substructure that interacts with amino acids at the entrance of the *N*-acetyl lysine binding channel. The cap and the metal-binding functionality are connected by a linker, often a 5−6 atom hydrocarbon chain.[11] Synthetic molecules incorporating these substructural elements are likely to inhibit HDAC enzymes.

Comparison of amino acid sequences around the active site was used to infer structural differences between individual HDAC family members that could be exploited in the design of selective inhibitors. Most of the amino acids that contact TSA in the HDLP structure are conserved across all HDACs. However, this conservation diverges for amino acids at the solvent-exposed rim of the channel, indicating that this is a selectivity-determining region (Figure 2). The

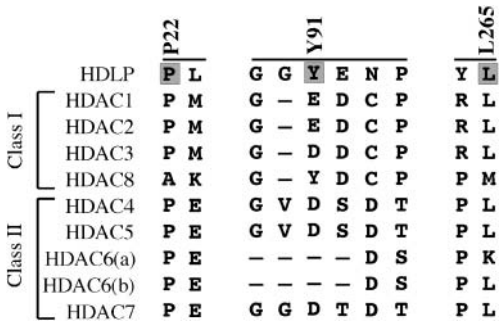

Figure 2. Sequence comparison of residues on the rim of the *N*-acetyl lysine binding channel. Amino acids in HDLP that contact TSA are boxed in gray. The numbering is based on the HDLP sequence.

most significant sequence differences are observed between class I and class II HDACs. The sequence diversity in the rim of the *N*-acetyl lysine binding channel suggests that selective inhibitors may be identified from collections of compounds having varied cap groups, since these groups would be expected to interact with the rim residues. It is also of note that an Arg265Pro single nucleotide polymorphism has been recently identified in the rim region of HDAC3,[12] providing the structural rationale for polymorph-specific design of HDAC inhibitors. By synthesizing molecules that possess diversity elements targeted toward regions predicted to be structurally divergent, discovery of selective inhibitors may be possible.

Our synthetic plan (Scheme 1) generates diversity in the cap region of the small molecules by using the split-pool synthesis technique. The chain length for the hydrocarbon linker ranges from 3 to 6 methylene groups so that the orientation of the cap relative to the enzyme channel is varied.[13] Literature precedence[11] and confirmatory HDAC

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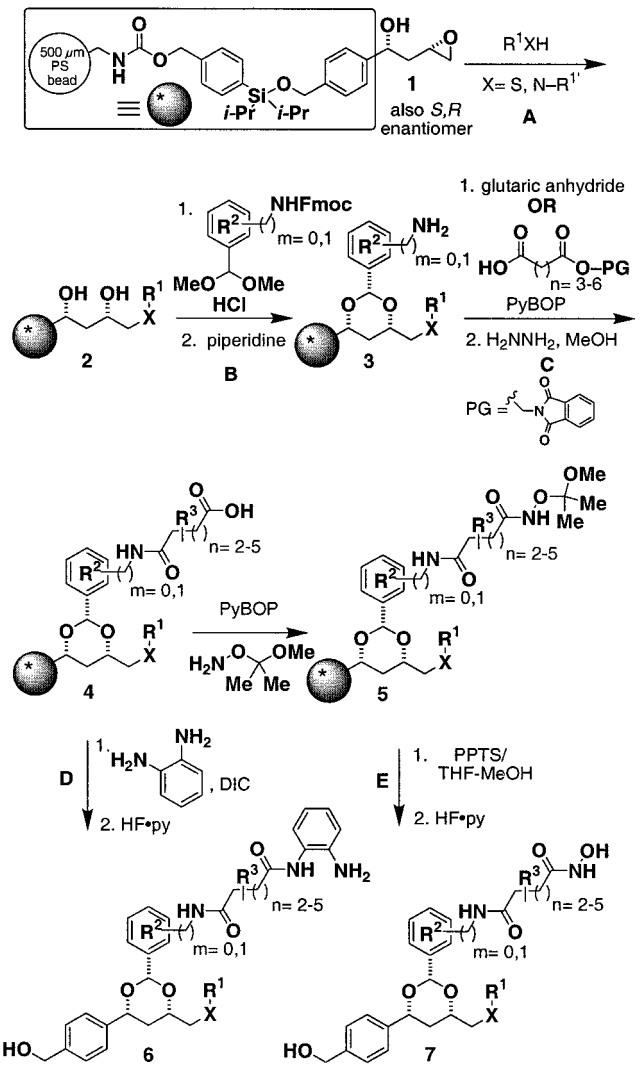
(13) Unsaturated linkers would presumably reduce the entropic penalty of binding this narrow channel. However, because the structural features required for selective inhibition are not known, the simplest set of linkers was chosen for use in this initial screening library. Unsaturated linkers will be tested during optimization of selective inhibitors.

Scheme 1



inhibition studies with a model compound led us to avoid shorter linkers (see below). Three metal-binding functionalities previously demonstrated to inhibit HDAC1 and likely adopting different binding orientations were used: carboxylic acid, *o*-aminoanilide, and hydroxamic acid. The 1,3-dioxane is a rigid core that can be synthesized stereoselectively and with enormous structural diversity.[2a]

To simplify structure determination of synthetic products, we used an adaptation[2b,c] of the encoding strategy reported by Still and co-workers[14] entailing covalent $[Rh(OCOCPh_3)_2]_2$-mediated attachment of electrophoric diazoketone tags to individual, high capacity polystyrene synthesis beads. These tags can be removed oxidatively after the synthesis and analyzed by GC.

Two portions of a silane-derivatized polystyrene resin[2a] were tagged with two diazoketones, and then enantiomeric γ,δ-epoxy alcohols were attached to afford modified polystyrene support (**1**). The resin was pooled, split, encoded for the subsequent reactions with 50 combinations of six diazoketones, and reacted with 50 nucleophile building blocks to generate 100 1,3-diols (**2**) in high purity (Figure 3). The solid-supported 1,3-diols were pooled and split into

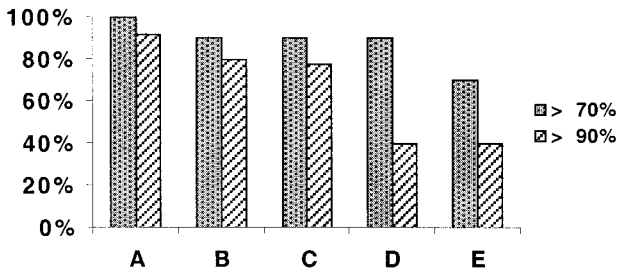


Figure 3. Percentage of synthetic intermediates that are >90% pure and >70% pure (LC). Letters refer to reactions labeled in Scheme 1.

six portions that were reacted with Fmoc-amino dimethylacetal building blocks under HCl catalysis to form 600 Fmoc-amino-1,3-dioxanes (**3**). The resin was tagged with six combinations of three diazoketones to encode the ketalization reactions. To encode the subsequent reactions, the resin was pooled and split into four portions and reacted with four combinations of three diazoketones. After Fmoc removal, these pools were reacted with TESCl to protect free hydroxyls incorporated from the nucleophile building blocks. The amino-1,3-dioxane resin was reacted with four diacid building blocks: pyridine-activated glutaric anhydride or PyBOP-activated monophthalimidomethylester diacids.[15] Treatment with hydrazine generated 2400 carboxyamides (**4**). One-third of these carboxyamide beads was set aside for screening experiments. The high purity of these reaction products indicates that the phthalimidomethylester is well-suited for carboxylic acid protection in solid-phase organic synthesis, where ester hydrolysis can be difficult as a result of the poor aqueous swelling properties of polystyrene resins. The remaining carboxyamides were split into two portions. One portion was reacted with diisopropylcarbodiimide (DIC) and phenylenediamine to generate 2400 *o*-aminoanilides (**6**). Reaction of the remaining portion of resin **4** with *O*-2-methoxypropanehydroxylamine in the presence of PyBOP generated 2400 protected hydroxamic acids (**5**). The 2-methoxypropane protecting group was essential for this reaction as *O*-TBDMS protection gave impure reaction products, and *O*-allyl and *O*-THP protecting groups were not sufficiently labile to be removed under conditions compatible with every synthesized compound. Treatment of resin **5** with PPTS generated 2400 hydroxamic acids (**7**).

The purity of the reaction products at each synthetic step was determined by LC-MS analysis of the crude material cleaved from single beads (Figure 3). Consistent with model

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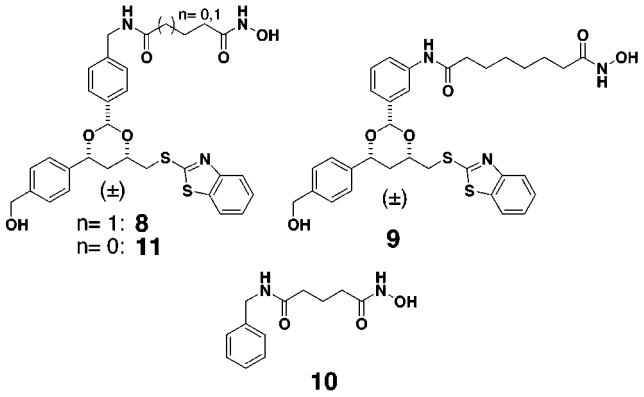
Table 1. HDAC-Inhibitory Activity (IC50 ± SE, μM)



compound	HDAC1	HDAC6
8	1.2 ± 0.5	0.9 ± 0.2
9	1.7 ± 1.2	1.1 ± 0.1
10	1.5 ± 0.2	0.38 ± 0.04

reactions,[16] the purity of reactions A−C was high. However, the purity of the *o*-aminoanilides and hydroxamic acids, while acceptable, was less than in model studies.[16] These results illustrate the difficulty of optimizing the final steps of a split-pool synthesis when many synthetic intermediates are required to react uniformly. Regardless of purity, however, for every reaction product analyzed (50 out of 50), GC analysis of the electrophoric tags allowed their structures to be inferred. In each instance, the mass of the structure inferred was consistent with the LC-MS data.

The 7200 polystyrene beads were arrayed and cleaved in the one bead-one stock solution format[2] to generate 7200 stock solutions. We are now developing high throughput assays to test each stock solution for inhibitory activity against individual HDACs and against cellular HDAC activity. These include protein-binding assays using small molecule microarrays,[17] in vitro histone deacetylase inhibition assays, and cytoblot assays[18] using anti-acetyl lysine antibodies. To demonstrate the ability of the synthesized compounds to inhibit HDAC, we synthesized two compounds (**8** and **9**) representative of the molecules in the library and measured IC50s against HDAC1 and HDAC6 (Table 1). Calculated IC50s of ~1 μM were similar to the IC50s of the substructure **10**, indicating that the 1,3-dioxane portion of the molecules is not detrimental to HDAC inhibition. In contrast, a compound (**11**) with a hydrocarbon linker shorter than those used in the library synthesis weakly inhibited HDAC1 and HDAC6 (IC50s > 50 μM). A full analysis of the inhibitory activity of all individual library members against HDAC1, HDAC4, and HDAC6 is underway.

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Note Added after ASAP: This Letter was released ASAP on 11/30/01 with an error in the structure of compound **4** in Scheme 1. The print and final Web version (12/05/01) are correct.

Supporting Information Available: Building blocks used, experimental procedures, and LC-MS characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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(16) See Supporting Information for LC traces from model reactions.

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