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Succinimide Hydroxamic Acids as Potent Inhibitors of Histone Deacetylase (HDAC)

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Abstract—A series of succinimide hydroxamic acids was prepared and evaluated in vitro for HDAC inhibition and tumor cell antiproliferation. While the original macrocyclic analogue **6** was quite potent in both assays, several appropriately substituted non-macrocyclic succinimides, such as **23**, were equipotent.
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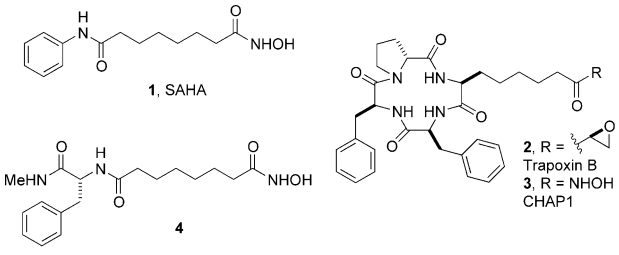
Histone deacetylases alter the acetylation status of the amino terminal region of histone proteins complexed with DNA in the nucleosome; the extent of histone acetylation determines the accessibility of DNA within chromatin and thereby influences gene expression.[1] The inappropriate recruitment of HDACs provides at least one mechanism by which oncogenes can alter gene expression in favor of excessive proliferation and makes inhibition of HDACs a potential target for the development of small molecule anticancer agents.[2]

The human histone deacetylases fall into two distinct classes, the HDACs and the SIRTs.[3] The HDACs are divided into two major sub-classes based on sequence homology, the class I HDACs (HDAC1, HDAC2, HDAC3, HDAC8, and HDAC11) which are related to the yeast histone deacetylase Rpd3 and the class II HDACs (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, HDAC10) which are related to Hda1.[4] All of the HDACs possess a highly conserved zinc-dependent catalytic domain while the class II HDACs are roughly twice as large as those of class I. The SIRTs, or surtuins, are a family of NAD-dependent deacetylases which display significant sequence divergence from class I and II HDACs.



Several families of potent ($IC_{50} < 100$ nM), small-molecule HDAC inhibitors have been reported in the recent literature[5] and include: SAHA (**1**), one of several reversible inhibitors bearing a hydroxamic acid and an aromatic terminus separated by a hydrophobic spacer (lit. IC_{50} 10 nM);[6] trapoxin B (**2**), an irreversible inhibitor from the cyclic tetrapeptide family which has an epoxyketone at the terminus of the hydrophobic chain (lit. IC_{50} 0.1 nM);[7] and CHAP1 (**3**), a hybrid analogue of the cyclic peptide and hydroxamic acid classes (lit. IC_{50} 2 nM).[8] The nature of the terminal group of the hydroxamic acids is an important determinant of inhibitory activity as exemplified by hydroxamic acid **4**, which despite being structurally similar to **1** is a much weaker HDAC inhibitor (IC_{50} 1.4 μM).[9] Based on a comparison of amino acid sequences around the active site of the individual human HDACs, it has been suggested that the terminus of these inhibitors should interact with a 'selectivity-determining region' and that proper substitution could lead to subtype selective inhibitors.[10]

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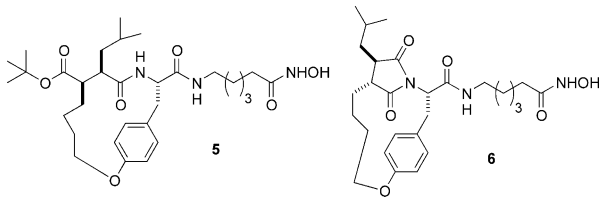
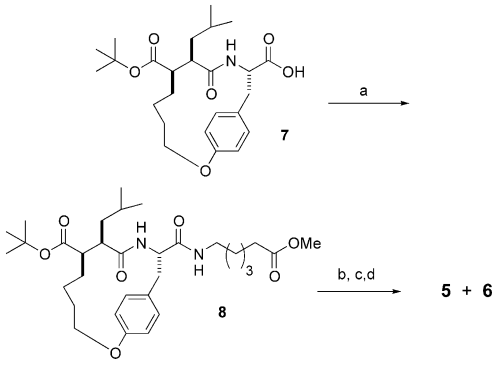


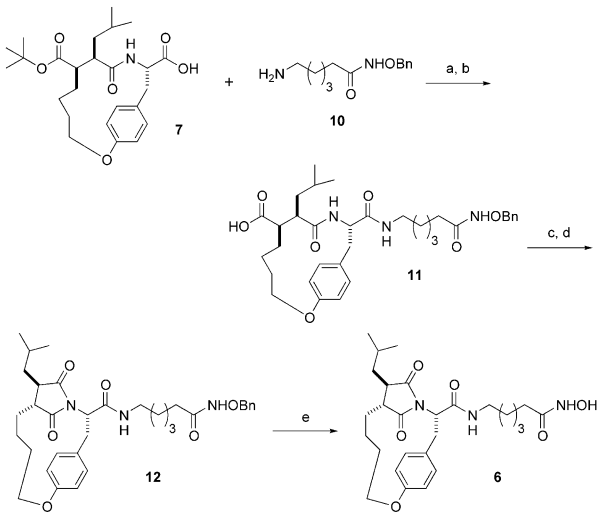
In an effort to prepare novel and less peptidic analogues of **3**, which could be HDAC subtype selective, we sought to replace the cyclic tetrapeptide of **3** with the peptidomimetic core of Abbott's macrocyclic matrix metalloproteinase inhibitors.[11] The synthetic route chosen for the initial target macrocycle **5** not only gave the desired product but also the related succinimide hydroxamic acid **6** as a significant by-product. While it was determined that **5** was only a modest inhibitor of HDAC (IC_{50} 2.1 μM), compound **6** was suprisingly potent (IC_{50} 38 nM).[9a] The efficient synthesis and characterization of **6** as well as the SAR of simplified analogues is reported here.

The preparation of **5** and **6** (Scheme 1) began with the coupling of methyl 6-aminocaproate and carboxylic acid **7**, which was prepared using published procedures.[11] When methyl ester **8** was saponified with LiOH or NaOH at room temperature, a 1:1 mixture of inseparable carboxylic acids were isolated and coupled with *O*-benzyl hydroxylamine. The *O*-benzyl hydroxamates were partially separated by column chromatography and deprotected separately to give hydroxamic acids **5** and **6**. The structural assignment of **6** was made based on ^1H and ^{13}C NMR and later confirmed by X-ray crystallography of a related intermediate.[12,13] The macrocyclic succinimide presumably arises from the intramolecular attack of the *tert*-butyl ester by the internal amide nitrogen.[14]

An improved synthesis of **6** is shown in Scheme 2. Amine **10**, which had been prepared in two steps from commercially available *N*-(*tert*-butoxycarbonyl)-6-aminocaproic acid, was coupled with macrocycle **7** to give carbocyclic acid **11** after *tert*-butyl ester removal. This acid was converted to the corresponding methyl ester and, after some experimentation, it was found that treatment of this intermediate with one equivalent of



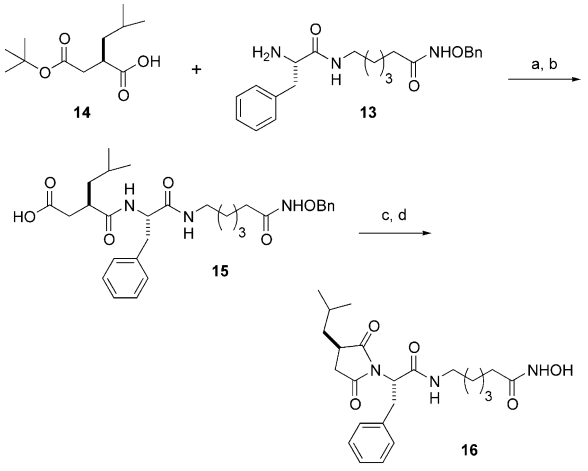
Scheme 1. Reagents and conditions: (a) EDC, HOBt, NMM, methyl 6-aminocaproate hydrochloride, DMF, 95%; (b) LiOH, 1:1 THF/MeOH, 78%; (c) EDC, HOBt, NMM, H_2NOBn, DMF; flash chromatography, 51% total yield; (d) H_2, Pd/C, THF, 86% for **5**, 91% for **6**.



Scheme 2. Reagents and conditions: (a) EDC, HOBt, NMM, DMF; (b) TFA, CH_2Cl_2, 93% two steps; (c) $iPrO_2CCl$, Et_3N, DMF, 0 °C; MeOH, 93%; (d) KO*t*Bu, THF, 76%; (e) H_2, Pd/C, THF, 94%.

KO*t*Bu at room temperature for 2 h gave the desired succinimide **12** in 70–80% isolated yield. *O*-Benzyl deprotection then gave hydroxamic acid **6**. Treatment of carboxylic acid **11** under coupling conditions (EDC, HOBt, NMM or DBU) did not give succinimide formation.

Simplified succinimide hydroxamic acids that lacked the macrocyclic ring were prepared as shown in Scheme 3. Amine **13**, which was prepared from amine **10** and *N*-(*tert*-butoxycarbonyl)-L-phenylalanine in two steps, and succinate **14**[15] were coupled and the adduct was deprotected to give carboxylic acid **15**. Early experimentation and literature precedent[16] indicated that the acyclic succinamides should cyclize much more readily than the macrocyclic amides; however, while treatment of **15** with EDC, HOBt and NMM in DMF did give succinimide cyclization, the yields were low (30–40%) and the reactions were capricious. Substituting the stronger base DBU for NMM and heating (50 °C for 2–3 h) dependably gave cyclization in good yield (70–75%) regardless of succinate



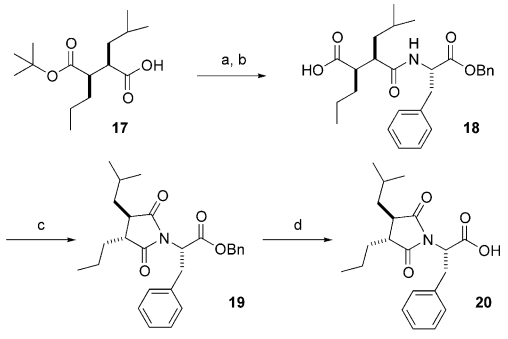
Scheme 3. Reagents and conditions: (a) EDC, HOBt, NMM, DMF; (b) TFA, CH_2Cl_2, 75% two steps; (c) EDC, HOBt, DBU, 50 °C, DMF, 70%; (d) H_2, Pd/C, THF, 96%.

substitution. Debenzylation gave the desired product, hydroxamic acid **16**.

In order to easily prepare tether and hydroxamate modified analogues of the monocyclic succinimides, a synthetic route to succinimide carboxylic acids such as **20** was developed and is shown in Scheme 4. Succinate **17**[15,17] and L-phenylalanine benzyl ester, prepared from *N*-(*tert*-butoxycarbonyl)-L-phenylalanine in two steps, were coupled and the product was deprotected to give carboxylic acid **18**. Cyclization as before (EDC, HOBt, DBU) followed by *O*-debenzylation gave the desired intermediate **20**, which could be coupled with aliphatic amines in high yield. The route to the analogous macrocyclic carboxylic acid **9** was not successful.

The compounds from this work were tested in an in vitro assay against a partially purified nuclear HDAC preparation from K562 erythroleukemia cells;[18] immunocharacterization indicated that deacetylase activity from this preparation was predominantly from the class I HDACs, HDAC1 and HDAC2. Promising compounds were then assessed for their effect on cell proliferation in an AlamarBlue assay after 72 h exposure using human HT1080 fibrosarcoma cells and human MDA435 breast carcinoma cells.[19] In order to verify that the observed antiproliferative effects were HDAC mediated, selected inhibitors were evaluated for their ability to cause hyperacetylation of nuclear histones and induce $p21^{waf1/cip1}$ expression.[20]

In vitro characterization indicated that macrocyclic succinimide **6** compared very well to **1**, having greater deacetylase inhibitory activity than the reference inhibitor in the nuclear HDAC assay and superior cellular activity against the two cell lines tested (Table 1). In order to determine the pharmacophore of this class of compounds, an analogue lacking the macrocycle was prepared; the resulting monocyclic analogue **16** was quite potent against the nuclear extracted HDACs (99 nM) and still had significant cellular activity (670 nM, MDA435). The notable potency and the significantly easier preparation of these analogues led us to conduct most of the SAR studies on the monocyclic series. Further simplification of **16** by removal of the succinimide *iso*-butyl moiety (**21**) gave an even larger decrease in activity with a corresponding loss in antiproliferative activity. Replacement of the succinimide with an unsubstituted five-membered ring lactam (**22**) gave a compound with no measureable HDAC activity below 5 μM. Conversely, a second, vicinal substituent on the succinimide (methyl, *n*-propyl, *iso*-butyl) generally led to an increase in inhibitory activity when compared to **16** (e.g., **23**). Additional succinimide substitution did not give further improvement (**24**). Replacement of the succinimide with phthalimide or dimethylhydantoin led to a 5- to 10-fold loss in activity versus **16**.



Scheme 4. Reagents and conditions: (a) EDC, HOBt, NMM, L-phenylalanine benzyl ester, DMF; (b) TFA, CH_2Cl_2, 88% two steps; (c) EDC, HOBt, DBU, 50 °C, DMF, 71%; (d) H_2, Pd/C, MeOH, 92%.

Regarding the amino acid residue, it was found that the nature of the side chain was very important to enzymatic activity. Removal of the benzyl moiety resulted in a very weak inhibitor (**25**) while its replacement with an *iso*-butyl also gave weak enzymatic activity (IC_{50} 6 μM, compound not shown); replacement with an L-cyclohexylalanine residue regained some of the potency (**26**). The use of *p*-methoxy- or *p*-ethoxy-L-phenylalanine gave significant potency, both enzymatically and cellularly, as exemplified by monocyclic succinimide **27** which has comparable activity to macrocycle **6**. Unlike **6**, for which the analogue epimeric at the amino acid center is equipotent, the analogue of **16** prepared from D-phenylalanine was significantly less potent (IC_{50} 770 nM).

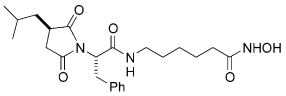
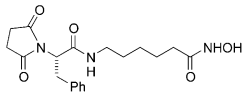
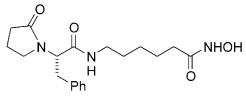
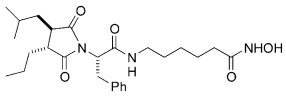
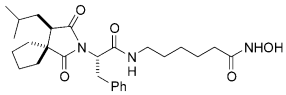
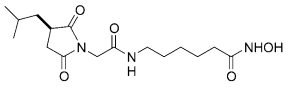
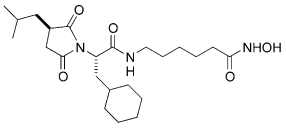
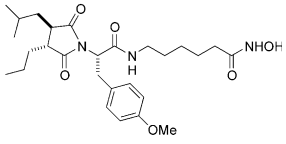
Examination of tether length between the hydroxamic acid and succinimide of **23** indicated that both the one-methylene homologated or shortened analogues were less potent enzymatically (IC_{50}'s of 232 and 1600 nM, respectively). Replacement of the hydroxamic acid moiety in **16** or **23** with a methyl ester, *O*-benzyl hydroxamate or carboxylic acid gave inactive compounds.

Cellular assays with T24 bladder carcinoma cells showed that **6** and **23** increased histone acetylation and induced expression of $p21^{waf1/cip1}$, similar to the cellular effects of **1** and **3**.[21]

The X-ray crystal structure of a bacterial HDAC homologue (HDLP)[22] with bound inhibitor **1** suggested that this type of compound inhibits the human HDACs by binding in a tubular hydrophobic pocket and chelating the active site zinc. Presumably, other hydroxamic acids with lipophilic tethers such as **3** and succinimides **6** or **23** bind the HDACs in the same fashion. The data presented in this work is consistent with this notion as chain length is important and the presence of a zinc chelator is crucial for activity. However, because the HDLP has a large deletion at the entrance to the active site pocket, little is known about the specific interactions between the HDACs and the terminus of the hydroxamate inhibitors. While it is clear that the presence and substitution of the succinate moiety of these inhibitors is very important to HDAC activity, molecular modeling has not revealed a consistent mode of binding to the human HDACs.

In conclusion, a novel and potent series of hydroxamic acids has been found that has significant cellular activity against several tumor cell lines. Consistent with known HDAC inhibitors, these compounds demonstrate that subtle adjustments of the succinimide terminus can lead

Table 1. HDAC inhibition and antiproliferation data for SAHA and selected succinimide hydroxamic acids

Compd	Structure	HDAC IC_{50} (nM)[9a,18]	HT1080 proliferation IC_{50} (μM)[19]	MDA435 proliferation IC_{50} (μM)[19]
1		120	2.4	1.9
6		38	0.25	0.15
16		99	2.3	0.67
21		660	14.0	10.0
22		> 5000	ND	ND
23		51	0.40	0.57
24		66	1.5	0.7
25		5000	ND	ND
26		640	ND	ND
27		38	0.53	0.18

to large changes in HDAC inhibitory activity. The isolation of the different isoforms of HDAC is in progress and the selectivity profile of these inhibitors will be reported in due course.

Acknowledgements

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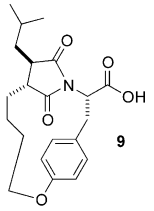
References and Notes

1. (a) Cheung, W. L.; Briggs, S. D.; Allis, C. D. *Curr. Opin. Cell Biol.* **2000**, *12*, 326. (b) Cress, W. D.; Seto, E. *J. Cell. Physiol.* **2000**, *184*, 1.
2. Coombes, R. C.; Vigushin, D. M. *Anti-Cancer Drugs* **2002**, *13*, 1.
3. (a) Gray, S. G.; Ekstrom, T. J. *Exp. Cell. Res.* **2001**, *262*, 75. (b) For a discussion of the SIRT active site, see: Dutnall, R. N.; Pillus, L. *Cell* **2001**, *105*, 161.
4. For the most recent published isolation (HDAC 10), see: Kao, H. Y.; Lee, C. H.; Komarov, A.; Han, C. C.; Evans, R. M. *J. Biol. Chem.* **2002**, *277*, 187.
5. (a) Jung, M. *Curr. Med. Chem.* **2001**, *8*, 1505. (b) Meinke, P. T.; Liberator, P. *Curr. Med. Chem.* **2001**, *8*, 211.
6. (a) The given IC_{50} was determined using immunoprecipitated HDAC1. Richon, V. M.; Emiliani, S.; Verdin, E.; Webb, Y.; Breslow, R.; Rifkind, R. A.; Marks, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 3003.
7. The given IC_{50} was determined in ref 8 against recombinant HDAC1. Kijima, M.; Yoshida, M.; Sugita, K.; Horinouchi, S.; Beppu, T. *J. Biol. Chem.* **1993**, *268*, 22429.
8. The given IC_{50} was determined using recombinant HDAC1. Furumai, R.; Komatsu, Y.; Nishino, N.; Khochbin, S.; Yoshida, M.; Horinouchi, S. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 87.
9. (a) The given IC_{50} was determined using a mixture of HDAC1 and HDAC2 prepared from the nuclear extraction of K562 erythroleukemia cells.[18] (b) Compound **4** is similar to inhibitors reported in: Jung, M.; Hoffmann, K.; Brosch, G.; Loidl, P. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1655.
10. (a) Sternson, S. M.; Wong, J. C.; Grozinger, C. M.; Schreiber, S. L. *Org. Lett.* **2001**, *3*, 4239. (b) Jung, M.; Gerald, B.; Kolle, D.; Scherf, H.; Gerhauser, C.; Loidl, P. *J. Med. Chem.* **1999**, *42*, 4669.
11. Steinman, D. H.; Curtin, M. L.; Garland, R. B.; Davidsen, S. K.; Heyman, R. H.; Holms, J. H.; Albert, D. H.; Magoc, T. J.; Nagy, I. B.; Marcotte, P. A.; Li, J.; Morgan, D. W.; Hutchins, C.; Summers, J. B. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2087.

12. All compounds were characterized by ^{1}H NMR, mass spectroscopy and elemental analysis.
13. An X-ray crystal structure was obtained of carboxylic acid **9**, which was isolated in low yield from a macrocycle synthetic route similar to that shown in Scheme 4. Carboxylic acid **9** was coupled with amine **10** and the product was debenzylated to give authentic hydroxamic acid **6**.



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14. Cyclizations of succinamide esters under basic conditions have been reported but only for acyclic systems with unhindered esters (i.e., methyl, benzyl). For an example, see: Fujii, N.; Nomizu, M.; Futaki, S.; Otaka, A.; Funakoshi, S.; Akaji, K.; Watanabe, K.; Yajima, H. *Chem. Pharm. Bull.* **1986**, *34*, 864.
15. Beckett, R. P.; Crimmin, M. J.; Davis, M. H.; Spavold, Z. *Synlett* **1993**, 137.
16. Bodansky, M.; Martinez, J. *J. Org. Chem.* **1978**, *43*, 3071.
17. Curtin, M.L; Garland, R. B.; Davidsen, S. K.; Marcotte, P. A.; Albert, D. H.; Magoc, T. J.; Hutchins, C. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1443.
18. Yoshida, M.; Kijima, M.; Akita, M.; Beppu, T. *J. Biol. Chem.* **1990**, *265*, 17174.
19. Procedure for cell growth inhibition assays: HT1080 or MDA-435 cells were seeded at 5×10^3 cells/well in a 96-well tissue culture 18 h prior to addition of compound or vehicle (DMSO) in complete growth medium (10% serum). After 72 h exposure to compound the cells were washed with PBS. AlamarBlue (modified MTT) was added in serum free medium or PBS (10% AlamarBlue in 100 μL) and allowed to incubate for 4 h at 37 °C. Cell number was measured by determining the fluorescence at 488/520 nm in an f_{max} fluorescence plate reader (Molecular Devices, San Diego, CA, USA). Treated cells were compared to vehicle treated cells to determine growth inhibition.
20. (a) Hyperacetylation of histone H4 was induced in T24 bladder carcinoma cells after 4 h exposure to HDAC inhibitors. Hyperacetylated histone H4 was analyzed by Western blot using an anti-acetylated H4 antibody (UBI, Lake Placid, NY) and 5 μg/lane of acid-extracted nuclear histones; (b) The expression of $p21^{waf1/cip1}$ was induced in T24 cells by a 24 h exposure to HDAC inhibitors. The induction of $p21^{waf1/cip1}$ was determined by Western blot analysis using a $p21^{waf1/cip1}$ monoclonal antibody (UBI) and 100 μg/lane of cellular lysate.
21. Sambucetti, L. C.; Fischer, D. D.; Zabludoff, S.; Kwon, P. O.; Chamberlin, H.; Trogani, N.; Xu, H.; Cohen, D. *J. Biol. Chem.* **1999**, *274*, 34940.
22. Finnin, M. S.; Donigian, J. R.; Cohen, A.; Richon, V. M.; Rifkind, R. A.; Marks, P. A.; Breslow, R.; Pavletich, N. P. *Nature* **1999**, *401*, 188.