New Cysteine Derivatives with Antiproliferative Activity on Melanoma Cells

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Abstract: Here we describe the rational design, computer-aided virtual ligand docking and synthesis of 19 nonpeptidic compounds designed to inhibit histone deacetylases and kill melanoma cells. Compounds were derived from cysteine, fused at the S-terminus to 4-butanoyl hydroxamate, and at the N-terminus to 4-(dimethylamino)benzoic acid. The latter was extended by coupling to amines to form a small library of prospective anti-cancer compounds. Four compounds were cytotoxic at sub-micromolar concentrations against cells of a particularly aggressive human melanoma (MM96L), and nine compounds showed selectivities of ≥5:1 for killing human melanoma instead of normal human fibroblast cells. The most active compounds were shown to cause hyperacetylation of histones due to inhibition of histone deacetylases. Further refinement of these compounds may produce an anti-tumor drug suitable for treating melanoma.

Key Words: Histone deacetylase, melanoma, cancer, anti-cancer, inhibitor, drug design.

INTRODUCTION

The incidence of melanoma continues to rise in most developed countries, and is one of the main causes of death and morbidity from cancer [1]. Malignant melanoma is an aggressive form of skin cancer and is characteristically resistant to all current forms of cancer therapy with average survival rates being very low for metastatic melanoma [2, 3]. Melanoma consequently has a large socioeconomic and psychological impact on patients and families as it is not strongly age-related, and so affects most age groups [4]. Although anti-cancer drugs generally kill susceptible cancer cells through induction of apoptosis, melanoma cells tend to have an intrinsic resistance to apoptosis [5, 6] and by reprogramming their proliferation and survival pathways during melanoma progression, they become readily resistant to a variety of chemotherapeutic drugs [7].

Histone deacetylase (HDAC) inhibitors are a new class of compounds that have shown promising anti-tumour potential due to their ability to arrest cell growth, induce cell differentiation, and in some cases, induce apoptosis of cancer cells [8]. The first histone deacetylase inhibitors known to differentiate cancer cells in cell culture were compounds like butyrate, retinoic acid, HMBA, ABHA, SBHA, SAHA, Scriptaid, and Sirtinol [9-13]. These were all of low potency, not very selective *in vivo,* and the resulting differentiation was usually reversible. More potent HDAC inhibitors that have shown more promising anti-cancer properties *in vitro* include Trichostatin (TSA) (**1**) [14, 15], Trapoxin B (**2**) [16] Fig. (**1**), Apicidin [17] and analogues [18-22].

HDAC inhibitors have a range of effects on melanoma cells including the induction of a differentiated phenotype (e.g. increased dendritic morphology) [11, 23], cell cycle effects (e.g. upregulation of $p21^{WAF1/Cip1}$ [24] and induction of a G_2/M cell cycle arrest [25]), and induction of apoptotic cell death [25, 26]. Predominantly, all melanoma cell lines undergo apoptosis following treatment with HDAC inhibitors [27], with some showing more sensitivity to the inhibitors compared with normal melanocytes. Current HDAC inhibitors in clinical trials for treatment of cancer are regarded as broad spectrum HDAC inhibitors with moderate anti-cancer activity and well tolerated toxicities. Ultimately, it would be clinically desirable to have much more potent and selective anti-cancer drugs that are less toxic to normal cells and even better tolerated by the patient.

This work describes the design, computer-aided virtual docking and synthesis of some new anti-cancer compounds that were designed to reproduce and modify the protein surface-binding interactions made by highly potent and naturally occurring HDAC inhibitors, such as TSA (**1**) and Trapoxin B (**2**) Fig. (**1**).

RESULTS AND DISSCUSSION

Compound Design And Ligand Docking

Known HDAC inhibitors generally consist of a zinc binding group tethered through a short spacer unit to one or more enzyme surface binding groups. Compounds **8-26** were designed to combine structural features of both **1** and **2**. Thus, scaffold **3** was constructed *in silico* to contain a core cysteine residue extending three methylene units from the sulphur atom and terminating in a hydroxamic acid moiety. The core cysteine residue was further functionalized through formation of amide bonds with 4-(dimethylamino)benzoic acid (TSA like head group) and 4-(aminomethyl)benzoic acid (Trapoxin B like phenylalanine group) to complete the core scaffold **3**. Scaffold **3** was then further elaborated through a selection of amines to expand the enzyme surface

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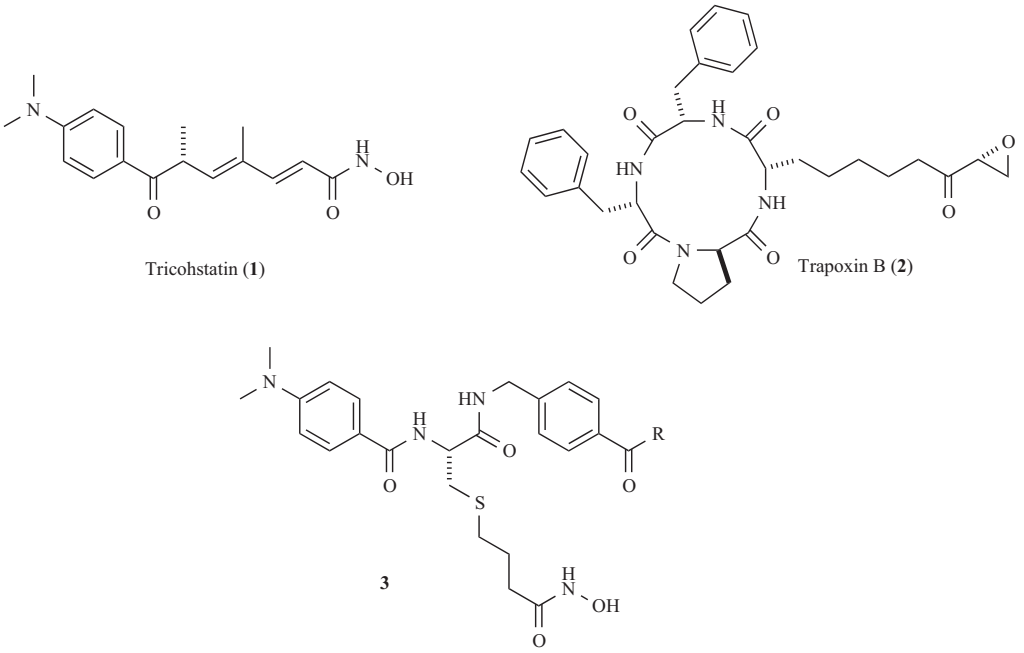


Fig. (1). Structures of Trichostatin (TSA) (**1**), Trapoxin B (**2**) and cysteine derived scaffold (**3**).

binding group and provide acyclic Trapoxin B (**2**) like mimics.

Virtual docking of compounds **8-26** into the recently determined structure of human HDAC 8 [28, 29] was performed to determine their potential as HDAC inhibitors. The structure of HDAC 8 has been solved with a variety of cocrystallized inhibitors e.g. trichlostatin (TSA, 1T64.pdb) [28] and suberoylanilide hydroxamic acid (SAHA, 1W22. pdb) [29]. The structure of HDAC 8 (1T64.pdb) shows two TSA molecules bound to the protein. One projects its hydroxamic acid group into the catalytic pocket (binding Zn atom) and the dimethyl aniline head group forms a π–π stacking interaction with Tyr100 Fig. (**2**). The second TSA molecule inserts its dimethyl aniline head group into a second nearby hydrophobic pocket (surrounding residues: Pro35, Trp142, Phe152, Tyr111 and Tyr306) with its hydroxamic acid group interacting with the first TSA molecule. Therefore, compounds **8-26** were designed to bind to both the catalytic domain and the second hydrophobic pocket. This was achieved through the use of 4-(aminomethyl) benzoic acid group, which provided a potential π–π stacking interaction with Tyr100 and also provided a linking group to the second hydrophobic pocket. Thus, the R groups of compounds **8-26** were designed to be predominantly hydrophobic to enhance additional hydrophobic interactions with the second binding pocket.

Compounds **8-26** were docked into the HDAC 8 crystal structure (1T64.pdb) [28] using the program GOLD [30], which uses a genetic docking algorithm to discriminate between different binding modes of the same compound. Recently, GOLD was shown to be a good choice for inhibitor docking into metallo-β-lactamases with good correlation between experimental inhibitor affinities and GOLD score [31]. GOLD identified tight binding conformations in which the aliphatic side chain had been inserted into the tubular pocket of the active site with the side groups in contact with the shallow pockets of the enzyme surface and second deeper hydrophobic pocket Fig. (**2**).

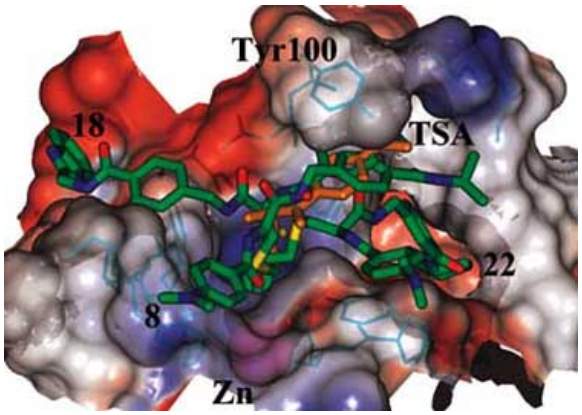
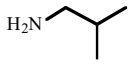
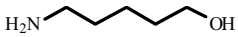
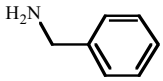
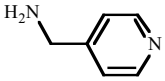
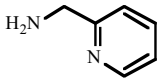
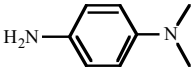
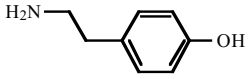
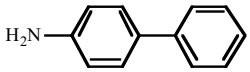
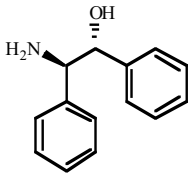
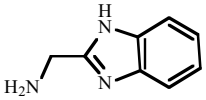
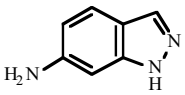


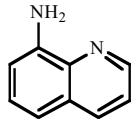
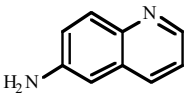
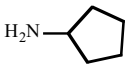
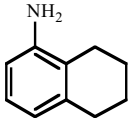
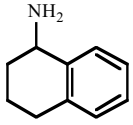
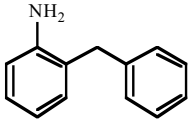
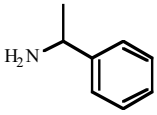
Fig. (2). Active site of Human HDAC 8 with co-crystallized with TSA (orange) [28], representative compounds **8**, and **18** shown accessing shallow pockets on the enzyme surface and **22** binding the deeper second hydrophobic pocket of the enzyme (shown with a hydrophobic surface).

The top ranked conformations determined by GOLD were rescored using LUDI [32] to give an indication of the enzyme binding affinity for each compound. LUDI scores shown in Table **1**, indicate potential binding affinities between 1μM - 1nM for all compounds to HDAC 8. Before

Table 1. Structure-Activity Relationships of Anti-cancer Agents Based on Cysteine Derived Scaffold 3

Compound	R[a]	GOLD fitness[b]	LUDI score[c]	Log $D_{7.0}$[d]	NFF[e] IC_{50} (μM)	MM96L[f] IC_{50} (μM)	Selectivity Index[g]
1				2.5	0.20	0.03	6.7
8		63	1196	1.4	10.1	1.4	7.2
9		80	828	1.7	8.1	1.6	5.0
10		72	959	1.0	17.0	4.3	4.0
11		75	785	2.0	11.8	1.2	10.0
12		77	963	0.8	16.9	3.0	5.6
13		79	1053	0.9	16.9	2.3	7.3
14		86	1117	2.2	2.4	0.5	4.8
15		80	1465	2.0	16.1	3.0	5.4
16		92	1593	4.2	1.1	0.5	2.2
17		70	819	3.0	14.3	2.9	5.0
18		71	795	1.5	15.8	4.0	3.9
19		79	1145	1.7	12.2	1.5	8.1

(Table 1. Contd….)

Compound	R[a]	GOLD fitness[b]	LUDI score[c]	Log $D_{7.0}$[d]	NFF[e] IC_{50} (μM)	MM96L[f] IC_{50} (μM)	Selectivity Index[g]
20		75	1119	3.8	1.4	0.56	2.4
21		86	1262	2.4	5.6	2.2	2.5
22		79	781	2.0	5.3	1.2	4.4
23		77	1060	3.8	3.8	1.2	3.2
24		64	1238	3.1	1.7	0.9	1.9
25		82	1361	4.4	2.3	1.0	2.3
26		67	972	2.1	11.6	1.4	8.2

a) R = amines coupled *via* amide bond to **3**; b) GOLD fitness score [30]; c) LUDI scoring function [32]; d) Log $D_{7.0}$ = calculated distribution coefficient for all dissociative compound species between octanol and water [33]; e) NFF = neonatal foreskin fibroblasts; f) MM96L = melanoma cells; g) Selectivity Index = IC_{50} (NFF)/ IC_{50} (MM96L).

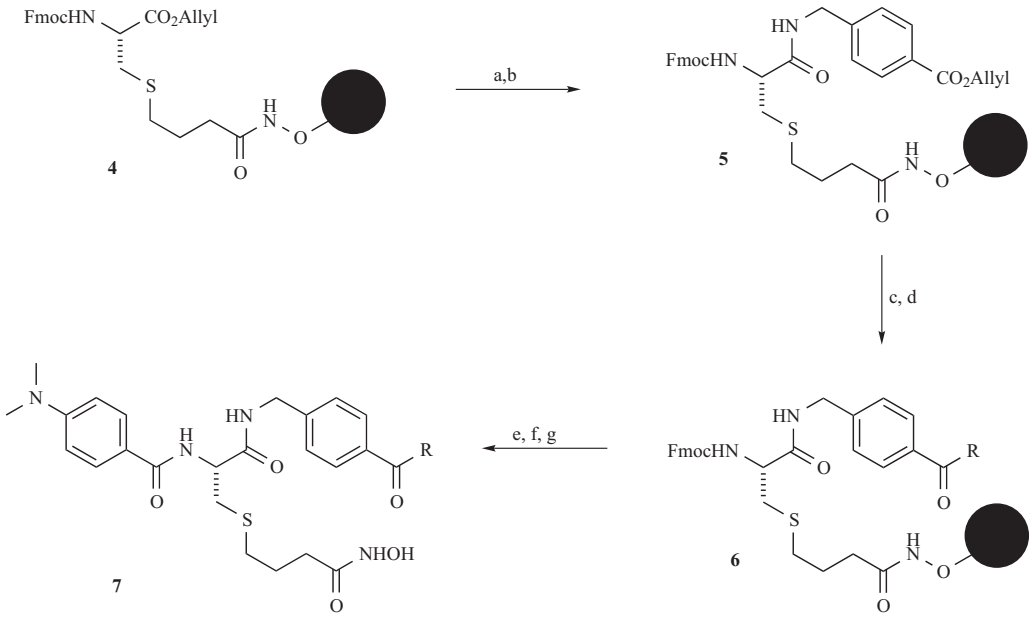
synthesis of the cysteine analogs was performed, the predicted lipophilicities were calculated *in silico* at pH 7 (Log$D_{7.0}$ being the total ionizable species in octanol/water partition coefficient) using PALLAS [33]. There were two compounds with a Log *D* 0-1, 14 compounds with Log *D* 1-3, and four compounds with Log *D* 3-5, with only the latter two categories expected to penetrate cell membranes.

Solid Phase Synthesis

The synthesis of compounds **8-26** was undertaken as indicated in Scheme (**1**). Synthesis of the protected cysteine derivative onto N-hydroxylamine TCP resin was based on a previous report [34]. After fixing the cysteine derivative to resin, the allyl ester was removed by treatment with Pd(0) in the presence of an allyl scavenger (DMBA), and the free acid was coupled to 4-(aminomethyl)benzoic acid. A variety of amines were then coupled to the resulting free acid group providing extended side chain derivatives. Deprotection of the N-terminal amine, followed by the coupling of activated 4-(dimethylamino)benzoic acid gave compounds **8-26** in >80% purity after cleavage from resin. All compounds were then purified to ≥ 95% by reversed phase HPLC with 84% average yield.

Antiproliferative Activity Against MM96L Melanoma Cells

Cytotoxicity of 19 cysteine-derived hydroxamic acids **8-26** was determined by clonogenic survival of human cancer cells (MM96L, melanoma) and human normal cells (NFF, neonatal foreskin fibroblasts) as described previously [34]. Despite the high scores observed in virtual docking computational experiments indicating that the compounds have potential to be potent inhibitors of HDAC 8, this had not been translated into enhanced anti-melanoma potency *in vitro*. Seven compounds had potencies against MM96L cells in the range of 2-5 μM, 8 compounds in the range of 1-2 μM and compounds **14**, **16**, **20** and **24** were submicromolar (0.54, 0.51, 0.56 and 0.93 respectively). Interestingly, three of these



Scheme (1). (a) $Pd(Ph_3)_4$, DMBA (b) allyl 4-(aminomethyl)benzoate, HBTU, DIPEA, DMF (c) $Pd(Ph_3)_4$, DMBA (d) R-amine, HBTU, DIPEA, DMF (e) Piperidine, DMF (f) 4-(dimethylamino)benzoic acid, HBTU, DIPEA, DMF (g) 5% TFA in DCM.

derivatives **16**, **20** and **24** had log *D* values above 3, which may indicate a better ability to penetrate cell membranes and thus reach HDAC enzymes. Also, compounds **14** and **9** contain R substituents 4-dimethylaminobenzylamine and 4-biphenylamine respectively, which appear in many reported HDAC inhibitors [35], indicating a preference of the enzyme surface for these substituents.

CytoSelectivity

Several compounds from Table **1**, exhibited selectivity in their cell killing of MM96L melanoma cells versus normal NFF cells. Compounds displaying ≥ 5-fold selectivity were **8**, **9**, **11**, **12**, **13**, **15**, **17**, **19**, and **26**. Five of these derivatives **8**, **11**, **13**, **19**, and **26** were more selective than TSA, with compound **11** being 10 times more selective in killing MM96L cells. Interestingly, none of these more selective inhibitors had IC_{50} values ≤ 1 μM.

Histone Hyperacetylation

The more potent compounds **14**, **16**, **20** and **24** from the series were indirectly tested for inhibition of histone deacetylase by monitoring the acetylation state of histone H4 using Triton-acetic acid-urea gel electrophoresis [23, 36]. The results are shown in Fig. (**3**), with each compound causing substantial hyperacetylation of H4 lanes 2-5. The known HDAC inhibitor SBHA, included for comparison (though at higher concentration), showed similar levels of hyperacetylation indicated by the mobility shift of histone H4. Clearly visible in untreated cells is the nonacetylated histone H4 (lane 1, MOCK). Extracts from cells treated with 5 *u*g/mL of **14**, **16**, **20** and **24**, indicated histone H4 in a variety of acetylation states, ranging from nonacetylated to tetraacetylated. Thus, these results support the notion that this compound series inhibits HDACs.

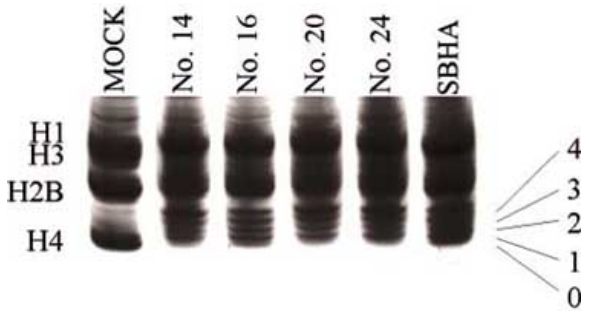


Fig. (3). Acetylation of Histone H4 following treatment. MM96L cells were treated with either no drug (MOCK) or 5 μg/ml of compounds 14, 16, 20 and 24 or 30 μg/ml of suberic bishydroxamic acid (SBHA) for 8 hr, before harvest and analysis of histone H4 acetylation by Triton-acetic acid-urea gel as previously described [23, 36]. Non-acetylated (0), mono-acetylated (1), di-acetylated (2), tri-acetylated (3) and tetra-acetylated (4) histone H4 are indicated.

CONCLUSION

The development of new chemotherapies for the treatment of drug resistant melanoma is an important goal for medicinal chemists. HDAC inhibitors are a promising new class of anti-tumour compounds that cause histones to become hyperacetylated in both normal and tumor cells. They arrest growth and induce differentiation and/or apoptotic cell death in a wide variety of human melanoma cancer cell lines. Most HDAC inhibitors reported to date have shown poor selectivity in killing melanoma cells without affecting normal cells. HDAC inhibitors generally cause G_2/M phase cell cycle arrest in normal cells whereas

most melanoma and tumor cells are deficient in this G_2 phase check point, thus resulting in tumor cell death. Conceivably, the antiproliferative, apoptotic, and differentiating properties of HDAC inhibitors could be made more specific and selective for melanoma and other cancer cells without damaging normal cells.

Herein we have reported a new series of compounds, designed and synthesized from a core cysteine residue, to maximize hydrophobic interactions between the catalytic and second hydrophobic binding pockets of HDAC 8. These compounds were shown to have potent cytotoxicity for melanoma cell lines. Compounds were chosen based on computational docking methods with the expectation that the C-terminal extensions (R, Table **1**) to cysteine would enhance inhibitor interactions with hydrophobic residues at or near the entrance of the active site of HDACs. While this has only been partly successful, with the generation of compounds with sub-micromolar anti-cancer potencies, there was higher selectivity than TSA in their capacity to kill MM96L over normal NFF cells. The capacity of these compounds to inhibit HDAC enzymes was clearly established through an assay for hyperacetylation of histones. Further optimization at the N-, C-, and S-termini of the cysteine used as a scaffold in this work should provide further scope for generating compounds with greater potency and selectivity, through more effective interactions with HDACs or other cellular targets.

EXPERIMENTAL

General Methods

^{1}H NMR spectra were recorded on a Bruker Avance 600 MHz. Analytical HPLC were performed on a Phenomenex Luna 5µ C18(2) 250 x 4.60 mm column run at 1 mL/minute using gradient mixtures of water/0.1% TFA (A) and water (10%)/acetonitrile (90%)/0.1% TFA (B), retention times (RT) reported in minutes. Semi preparative scale rpHPLC separations were performed on a Phenomenex Luna 5µ C18(2) 250 x 21 mm column run at 20 mL/minute using gradient mixtures of (A) and (B), and product fractions lyophilized. Accurate mass determinations were performed on an API QSTAR mass spectrometer using electron impact ionization. Water octanol partition coefficients (Log $D_{7.0}$) were calculated using PALLAS prolog D 2.1.

Chemical Synthesis

A library of compounds was synthesized on resin from **4** as previously described [34].

Removal of the Allyl Ester (General Procedure)

The resin **4** was flow washed with DCM for 2 minutes, and then shaken in DCM (30 mL) for a further 10 minutes. An argon stream was introduced, and the resin and DCM degassed for 5 minutes. DMBA (1.2 g, 7.9 mmol) was added, and bubbling continued for a further minute to ensure thorough mixing. $Pd(Ph_3)_4$ (270 mg, 0.23 mmol) was added to the resin, the flask wrapped in aluminum foil, and after a further 30 seconds of degassing, the argon stream was removed, and the resin shaken gently for 1 hour. The resin was flow washed successively with DCM, DMF, and DCM, before drying under high vacuum to give deprotected **4**.

Coupling of Amines (General Procedure)

Deprotected **4** (0.45 mmol/g, 200mg, 0.09 mmol) was shaken in DMF (1 mL) for 10 minutes, and then DIPEA (122 µL, 0.72 mmol) and 0.5 M HBTU in DMF (360 µL, 0.18 mmol) were introduced and shaking continued for further 5 minutes. Then 4-(aminomethyl)benzoic acid (0.25 mmol) was added, and shaking continued for further 1 hour. After washing the resin well with DMF, cleavage of a small portion of resin and analysis by mass spectroscopy generally indicated a 90% conversion to the amide. Repeating the coupling provided complete conversion in almost all cases. Successive carboxylic acid deprotection and subsequent coupling of a variety of amines provided diversity at the R position of scaffold **3**.

Coupling of 4-(dimethylamino)benzoic Acid (General Procedure)

The resin (1 eq) was shaken in DMF (10 mL/mmol) for 10 minutes, the DMF was removed, and then 1:1 piperidine:DMF (10 mL/mmol) was added. After shaking for 5 minutes, the piperidine:DMF was removed, and the resin washed well with DMF. This procedure was repeated two more times. In a separate flask 0.5 M HBTU (2eq) in DMF was added to a solution of the acid (2 eq) and DIPEA (5 eq) in DMF (10 mL/mmol), and the resulting solution was stirred for 5 minutes before being added in one portion to the resin. The resin was shaken for 1 hour, and then washed well with DMF. Cleavage of a small portion of resin and analysis by mass spectroscopy indicated 100% conversion to the amide.

Cleavage of the Product from the Resin (General Procedure)

The resin was washed well with DCM, and then drained. TFA:water (99:1, 10 mL/mmol) was added, the resin shaken for 20 min, TFA was collected, and the resin was washed with further 10 mL/mmol of TFA. TFA was removed by distillation, and the resulting oil purified by rpHPLC to provide the desired hydroxamic acid generally as a white solid. All hydroxamic acids displayed high field NMR and HRMS consistent with their proposed structures.

Compound 8

^{1}H NMR(d_6-DMSO, 600 MHz) δ; 10.36 (s, 1H), 8.67 (s, 1H), 8.61 (t, J=6.5 Hz, 1H), 8.19 (d, J=8.0 Hz, 1H), 8.12 (m, 1H), 7.77 (m, 4H), 7.31 (d, J=8.0 Hz, 2H), 6.7 (d, J=8.9 Hz, 2H), 4.61 (m, 1H), 4.34 (d, J= 5.4 Hz, 2H), 4.07 (m, 1H), 2.97 (s, 6H), 2.90 (obsc m, 1H), 2.85 (m, 1H), 2.45 (obsc m, 2H), 2.03 (t, J=6.6Hz, 2H), 1.74 (m, 2H), 1.14 (d, J=6.5Hz, 6H); HRMS calcd for $C_{27}H_{37}N_5O_5S$ (MH^+): 544.2588, Found 544.2578; rpHPLC grad RT: 19.64.

Compound 9

^{1}H NMR(d_6-DMSO, 600 MHz) δ; 10.37 (s, 1H), 8.67 (s, 1H), 8.61 (t, J=6.5 Hz, 1H), 8.37 (t, J=5.9 Hz, 1H), 8.19 (d, J=8.0 Hz, 1H), 7.78 (d, J=8.9 Hz, 2H), 7.76 (d, J=8.0, 2H), 7.33 (d, J=8.0 Hz, 2H), 6.72 (d, J=8.9 Hz, 2H), 4.61 (m, 1H), 4.34 (d, J= 5.4 Hz, 2H), 2.97 (s, 6H), 3.06 (t, J=6.0Hz, 2H), 2.90 (obsc m, 1H), 2.85 (m, 1H), 2.45 (obsc m, 2H), 2.03 (t, J=6.6Hz, 2H), 1.74 (m, 2H), 1.84 (m, 1H), 0.87 (d, J=6.6Hz,

6H); HRMS calcd for $C_{28}H_{39}N_5O_5S$ (MH^+): 558.2745, Found 558.2738; rpHPLC grad RT: 20.97.

Compound 10

^{1}H NMR(d_6-DMSO, 600 MHz) δ; 10.37 (s, 1H), 8.67 (s, 1H), 8.61 (t, J=6.5 Hz, 1H), 8.37 (t, J=5.9 Hz, 1H), 8.19 (d, J=8.0 Hz, 1H), 7.78 (d, J=8.9 Hz, 2H), 7.76 (d, J=8.0, 2H), 7.33 (d, J=8.0 Hz, 2H), 6.7 (d, J=8.9 Hz, 2H), 4.61 (m, 1H), 4.34 (d, J= 5.4 Hz, 2H), 3.37 (dt, J=5.2, 6.3 Hz 2H), 3.33 (dt, J= 6.1, 6.7 Hz, 2H), 3.29 (br s, 1H), 2.97 (s, 6H), 2.90 (obsc m, 1H), 2.85 (m, 1H), 2.45 (obsc m, 2H), 2.03 (t, J=6.6Hz, 2H), 1.74 (m, 2H),1.50 (m, 2H), 1.43 (m, 2H), 1.31 (m, 2H); HRMS calcd for $C_{29}H_{41}N_5O_6S$ (MH^+): 588.2850, Found 588.2835; rpHPLC grad RT: 18.46.

Compound 11

^{1}H NMR(d_6-DMSO, 600 MHz) δ; 10.35(s, 1H), 8.96 (m, 1H), 8.66 (s, 1H), 8.61 (t, J=6.5 Hz, 1H), 8.19 (d, J=8.0 Hz, 1H), 7.78 (d, J=8.9 Hz, 2H), 7.76 (d, J=8.0, 2H), 7.33 (d, J=8.0 Hz, 2H), 7.29 (m, 4H), 7.21 (m, 1H), 6.70 (d, J=8.9 Hz, 2H), 4.60 (m, 1H), 4.35 (d, J= 5.7 Hz, 2H), 4.34 (d, J= 5.9 Hz, 2H), 2.97 (s, 6H), 2.90 (obsc m, 1H), 2.84 (m, 1H), 2.45 (obsc m, 2H), 2.01 (t, J=6.6Hz, 2H), 1.73 (m, 2H); HRMS calcd for $C_{31}H_{37}N_5O_5S$ (MH^+): 592.2588, Found 592.2569. rpHPLC grad RT: 22.02.

Compound 12

^{1}H NMR(d_6-DMSO, 600 MHz) δ; 10.37 (s, 1H), 9.06 (t, J=6.0Hz, 1H), 8.67 (s, 1H), 8.63 (t, J=6.5 Hz, 1H), 8.49 (d, J=5.8 Hz, 2H), 8.20 (d, J=8.0 Hz, 1H), 7.78 (d, J=8.9 Hz, 2H), 7.76 (d, J=8.0, 2H), 7.33 (d, J=8.0 Hz, 2H), 7.28 (d, J=5.2 Hz, 2H), 6.7 (d, J=8.9 Hz, 2H), 4.61 (m, 1H), 4.34 (d, J= 5.4 Hz, 2H), 2.97 (s, 6H), 2.90 (obsc m, 1H), 2.85 (m, 1H), 2.45 (obsc m, 2H), 2.03 (t, J=6.6Hz, 2H), 1.74 (m, 2H); HRMS calcd for $C_{30}H_{36}N_6O_5S$ (MH^+): 593.2541, Found 593.2533. rpHPLC grad RT: 16.62.

Compound 13

^{1}H NMR(d_6-DMSO, 600 MHz) δ; 10.37 (s, 1H), 9.05 (t, J=5.8 Hz, 1H), 8.68 (s, 1H), 8.63 (t, J=6.2 Hz, 1H), 8.50 (m, 1H), 8.21 (d, J=8.4 Hz, 1H), 7.78 (d, J=8.9 Hz, 2H), 7.76 (d, J=8.0, 2H), 7.74 (m, 1H), 7.36 (d, J=8.3 Hz, 2H), 7.29 (d, J= 7.9 Hz), 7.25 (m, 1H), 6.7 (d, J=8.9 Hz, 2H), 4.62 (m, 1H), 4.55 (d, J=5.9 Hz, 2H), 4.36 (d, J= 5.8 Hz, 2H), 2.97 (s, 6H), 2.90 (obsc m, 1H), 2.85 (m, 1H), 2.45 (obsc m, 2H), 2.03 (t, J=6.6Hz, 2H), 1.74 (m, 2H); HRMS calcd for $C_{30}H_{36}N_6O_5S$ (MH^+): 593.2541, Found 593.2531; rpHPLC grad RT: 16.72.

Compound 14

^{1}H NMR(d_6-DMSO, 600 MHz) δ; 10.37 (s, 1H), 8.67 (s, 1H), 8.64 (t, J=6.1 Hz, 1H), 8.22 (d, J=8.2 Hz, 1H), 7.87 (d, J=8.1 Hz, 2H) , 7.78 (d, J=8.9, 2H), 7.55 (d, J=9.0 Hz, 2H), 7.37 (d, J=8.0 Hz, 2H), 6.7 (d, J=9.0 Hz, 4H), 4.61 (m, 1H), 4.34 (d, J= 5.4 Hz, 2H), 2.97 (s, 6H), 2.90 (obsc m, 1H), 2.86 (s, 6H), 2.84 (m, 1H), 2.45 (obsc m, 2H), 2.03 (t, J=6.6Hz, 2H), 1.74 (m, 2H); HRMS calcd for $C_{32}H_{40}N_6O_5S$ (MH^+): 621.2854, Found 621.2845; rpHPLC grad RT: 17.60.

Compound 15

^{1}H NMR(d_6-DMSO, 600 MHz) δ; 10.37 (s, 1H), 9.15 (s, 1H), 8.68 (s, 1H), 8.60 (t, J=6.1 Hz, 1H), 8.45 (t, J=5.9 Hz, 1H), 8.20 (d, J=8.0 Hz, 1H), 7.78 (d, J=9.0 Hz, 2H), 7.74 (d, J=8.2, 2H), 7.33 (d, J=8.0 Hz, 2H), 7.01 (d, J=8.3 Hz, 2H) 6.72 (d, J=8.9 Hz, 2H), 6.66 (d, J=8.5 Hz, 2H), 4.61 (m, 1H), 4.34 (m, 2H), 3.51(s, 1H), 2.97 (s, 6H), 2.90 (obsc m, 1H), 2.85 (m, 1H), 2.70 (t, J=7.6 Hz, 2H), 2.45 (obsc m, 2H), 2.03 (t, J=6.6Hz, 2H), 1.74 (m, 2H); HRMS calcd for $C_{32}H_{39}N_5O_6S$ (MH^+): 622.2694, Found 622.2686; rpHPLC grad RT: 20.07.

Compound 16

^{1}H NMR(d_6-DMSO, 600 MHz) δ; 10.37 (s, 1H), 8.68 (s, 1H), 8.66 (t, J=6.0 Hz, 1H), 8.22 (d, J=7.8 Hz, 1H), 7.92 (d, J=8.3 Hz, 2H), 7.87 (d, J=9.0 Hz, 2H), 7.78 (d, J=8.6, 2H), 7.66 (m, 4H), 7.44 (m, 4H), 7.33 (m, 1H), 6.72 (d, J=9.1 Hz, 2H), 4.63 (m, 1H), 4.38 (d, J= 5.8 Hz, 2H), 2.97 (s, 6H), 2.90 (obsc m, 1H), 2.85 (m, 1H), 2.45 (obsc m, 2H), 2.03 (t, J=6.6Hz, 2H), 1.74 (m, 2H); HRMS calcd for $C_{36}H_{39}N_5O_5S$ (MH^+): 654.2745, Found 654.2732; rpHPLC grad RT: 26.67.

Compound 17

^{1}H NMR(d_6-DMSO, 600 MHz) δ; 10.37 (s, 1H), 8.67 (s, 1H), 8.57 (t, J=5.7 Hz, 1H), 8.53 (d, J=9.1 Hz, 1H), 8.19 (d, J=8.0 Hz, 1H), 7.78 (d, J=8.9 Hz, 2H), 7.56 (d, J=8.3, 2H), 7.43 (t, J=8.2 Hz, 6H), 7.26 (m, 4H), 7.20 (m, 1H), 7.17 (m, 1H), 6.72 (d, J=8.9 Hz, 2H), 5.42 (d, J=5.2 Hz, 1H), 5.10 (t, J=8.7 Hz, 1H), 4.89 (m, 1H), 4.60 (m, 1H), 4.30 (d, J= 5.8 Hz, 2H), 2.97 (s, 6H), 2.90 (obsc m, 1H), 2.85 (m, 1H), 2.45 (obsc m, 2H), 2.03 (t, J=6.6Hz, 2H), 1.74 (m, 2H); HRMS calcd for $C_{38}H_{43}N_5O_6S$ (MH^+): 698.3007, Found 698.2990; rpHPLC grad RT: 22.91.

Compound 18

^{1}H NMR(d_6-DMSO, 600 MHz) δ; 10.38 (s, 1H), 9.22 (s, 1H), 8.71 (br s, 1H), 8.64 (t, J=6.0 Hz, 1H), 8.21 (d, J=8.0 Hz, 1H), 7.87 (d, J=8.9 Hz, 2H), 7.77 (d, J=8.0, 2H), 7.59 (br s, 2H), 7.38 (d, J=8.3 Hz, 2H), 7.29 (s, 2H), 6.72 (d, J=9.1 Hz, 2H), 4.77 (d, J=5.2 Hz, 2H), 4.61 (m, 1H), 4.36 (d, J= 5.8 Hz, 2H), 2.97 (s, 6H), 2.90 (obsc m, 1H), 2.85 (m, 1H), 2.45 (obsc m, 2H), 2.03 (t, J=6.6Hz, 2H), 1.74 (m, 2H); HRMS calcd for $C_{32}H_{37}N_7O_5S$ (MH^+): 632.2650, Found 632.2630; rpHPLC grad RT: 18.05.

Compound 19

^{1}H NMR(d_6-DMSO, 600 MHz) δ; 10.37 (s, 1H), 10.3 (s, 1H), 8.68 (d J=1.6Hz, 1H), 8.65 (t, J=6.2 Hz, 1H), 8.26 (s, 1H), 8.22 (d, J=8.1 Hz, 1H), 7.98 (s, 1H), 7.91 (d, J=8.2 Hz, 2H), 7.78 (d, J=8.9 Hz, 2H), 7.75 (m, 1H), 7.68 (d, J=8.5, 1H), 7.42 (d, J=8.2 Hz, 2H), 7.37 (dd, J=1.5, 8.7 Hz, 1H), 6.72 (d, J=9.2 Hz, 2H), 4.62 (m, 1H), 4.38 (d, J= 5.8 Hz, 2H), 2.97 (s, 6H), 2.90 (obsc m, 1H), 2.85 (m, 1H), 2.45 (obsc m, 2H), 2.03 (t, J=6.6Hz, 2H), 1.74 (m, 2H); HRMS calcd for $C_{31}H_{35}N_7O_5S$ (MH^+): 618.2493, Found 618.2480; rpHPLC grad RT: 20.19.

Compound 20

^{1}H NMR(d_6-DMSO, 600 MHz) δ; 10.37 (s, 1H), 8.98 (s, 1H), 8.73 (d, J=6.6 Hz, 1H), 8.70 (s, 1H), 8.65 (m, 1H), 8.42 (d, J=8.0 Hz, 1H), 8.23 (d, J=7.0 Hz, 1H), 7.94 (d, J=7.1 Hz, 1H), 7.78 (d, J=8.9 Hz, 2H), 7.76 (d, J=8.0, 2H), 7.68 (dd, J=8.3, 1.2 Hz, 1H), 7.65 (dd, J=8.3, 4.2 Hz, 1H), 7.59 (t, J=8.0 Hz, 1H), 7.33 (d, J=8.0 Hz, 2H), 6.72 (d, J=8.9 Hz,

2H), 4.64 (m, 1H), 4.41 (d, J=5.4 Hz, 2H), 2.97 (s, 6H), 2.90 (obsc m, 1H), 2.85 (m, 1H), 2.45 (obsc m, 2H), 2.03 (t, J=6.6Hz, 2H), 1.74 (m, 2H); HRMS calcd for $C_{33}H_{36}N_6O_5S$ (MH^+): 629.2541, Found 629.2532; rpHPLC grad RT: 21.94.

Compound 21

^{1}H NMR(d_6-DMSO, 600 MHz) δ; 10.37 (s, 1H), 8.82 (d, J=2.6 Hz, 1H), 8.67 (t, J=6.0 Hz, 1H), 8.55 (s, 1H), 8.34 (d, J=8.8 Hz, 1H), 8.22 (d, J=8.2 Hz, 1H), 8.03 (m, 3H), 7.95 (d, J=8.1 Hz, 2H), 7.78 (d, J=8.9, 2H), 7.77 (m, 1H), 7.51 (m, 1H), 7.44 (d, J=8.0 Hz, 2H), 6.72 (d, J=9.1 Hz, 2H), 4.63 (m, 1H), 4.39 (d, J= 5.4 Hz, 2H), 2.97 (s, 6H), 2.90 (obsc m, 1H), 2.85 (m, 1H), 2.45 (obsc m, 2H), 2.03 (t, J=6.6Hz, 2H), 1.74 (m, 2H); HRMS calcd for $C_{33}H_{36}N_6O_5S$ (MH^+): 629.2541, Found 629.2522; rpHPLC grad RT: 17.94.

Compound 22

^{1}H NMR(d_6-DMSO, 600 MHz) δ; 10.37 (s, 1H), 8.61 (m, 1H), 8.19 (m, 2H), 7.82 (d, J=8.0 Hz,1H), 7.78 (d, J=8.9 Hz, 2H), 7.76 (d, J=8.0, 2H), 7.35 (d, J=7.8 Hz, 2H), 6.71 (d, J=8.9 Hz, 2H), 4.61 (m, 1H), 4.34 (d, J= 5.4 Hz, 2H), 2.97 (s, 6H), 2.90 (obsc m, 1H), 2.85 (m, 1H), 2.45 (obsc m, 2H), 2.03 (t, J=6.6Hz, 2H), 1.74 (m, 2H); HRMS calcd for $C_{29}H_{39}N_5O_5S$ (MH^+): 570.2745, Found 570.2729; rpHPLC grad RT: 21.27.

Compound 23

^{1}H NMR(d_6-DMSO, 600 MHz) δ; 10.37 (s, 1H), 9.65 (s, 1H), 8.67 (d, J=1.6 Hz, 1H), 8.65 (t, J=6.0 Hz, 1H), 8.21 (d, J=8.0 Hz, 1H), 7.89 (d, J=8.2 Hz, 1H), 7.78 (d, J=9.0 Hz, 2H), 7.78 (d, J=8.0, 2H), 7.39 (d, J=8.1 Hz, 2H), 7.11 (m, 2H), 6.97 (d, J=7.6 Hz, 1H), 6.72 (d, J=9.1 Hz, 2H), 4.62 (m, 1H), 4.36 (d, J= 6.1 Hz, 2H), 2.97 (s, 6H), 2.90 (obsc m, 1H), 2.85 (m, 1H), 2.75 (m, 2H), 2.62 (m, 2H), 2.45 (obsc m, 2H), 2.03 (t, J=7.4 Hz, 2H), 1.74 (m, 2H), 1.70 (m, 4H); HRMS calcd for $C_{34}H_{41}N_5O_5S$ (MH^+): 632.2901, Found 632.2890; rpHPLC grad RT: 24.73.

Compound 24

^{1}H NMR(d_6-DMSO, 600 MHz) δ; 10.37 (s, 1H), 8.70 (t, J=7.8Hz, 1H), 8.67 (s, 1H), 8.61 (t, J=6.5 Hz, 1H), 8.19 (d, J=8.0 Hz, 1H), 7.78 (d, J=8.9 Hz, 2H), 7.76 (d, J=8.0, 2H), 7.33 (d, J=8.0 Hz, 2H), 7.13 (m, 4H), 6.70 (d, J=8.9 Hz, 2H), 5.22 (m, 1H), 4.61 (m, 1H), 4.34 (d, J= 5.4 Hz, 2H), 2.97 (s, 6H), 2.87 (m, 1H), 2.85 (m, 1H), 2.76 (m, 2H), 2.45 (obsc m, 2H), 2.03 (t, J=6.8 Hz, 2H), 1.97 (m, 2H), 1.85 (m, 2H), 1.74 (m, 2H); HRMS calcd for $C_{34}H_{41}N_5O_5S$ (MH^+): 632.2901, Found 632.2892; rpHPLC grad RT: 24.13.

Compound 25

^{1}H NMR(d_6-DMSO, 600 MHz) δ; 10.37 (s, 1H), 9.81 (s, 1H), 8.67 (m, 1H), 8.64 (t, J=6.1 Hz, 1H), 8.21 (d, J=7.9 Hz, 1H), 7.78 (d, J=8.8Hz, 2H), 7.76 (d, J=8.0, 2H), 7.36 (m, 3H), 7.26-7.10 (m, 8H), 6.71 (d, J=9.0 Hz, 2H), 4.61 (m, 1H), 4.36 (m, 2H), 4.00 (s, 2H), 2.97 (s, 6H), 2.90 (obsc m, 1H), 2.87 (m, 1H), 2.52 (m, 2H), 2.03 (t, J=7.1 Hz, 2H), 1.74 (m, 2H); HRMS calcd for $C_{37}H_{41}N_5O_5S$ (MH^+): 668.2901, Found 668.2886; rpHPLC grad RT: 25.62.

Compound 26

^{1}H NMR(d_6-DMSO, 600 MHz) δ; 10.37 (s, 1H), 8.73 (d, J=8.0 Hz, 1H), 8.68 (s, 1H), 8.62 (t, J=6.0 Hz, 1H), 8.20 (d, J=8.1 Hz, 1H), 7.82 (d, J=8.3 Hz, 2H), 7.77 (d, J=8.9, 2H), 7.36-7.31 (m, 6H), 7.21 (t, J=7.4 Hz, 1H), 6.72 (d, J=9.0 Hz, 2H), 5.15 (m, 1H), 4.61 (m, 1H), 4.34 (d, J= 5.4 Hz, 2H), 2.97 (s, 6H), 2.90 (obsc m, 1H), 2.85 (m, 1H), 2.45 (obsc m, 2H), 2.03 (t, J=7.4Hz, 2H), 1.74 (m, 2H), 1.46 (d, J=7.0 Hz, 3H); HRMS calcd for $C_{32}H_{39}N_5O_5S$ (MH^+): 606.2754, Found 606.2742; rpHPLC grad RT: 22.80.

Cell Lines and Culture Medium

All cell lines used in this study have been described previously [37, 38]. All cell lines were cultured in 10% heat-inactivated fetal calf serum (CSL, Australia) in RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 3 mM HEPES at 5% CO_2, 99% humidity at 37°C. Primary human fibroblasts were obtained from neonatal foreskins and cultured in the above medium. Routine mycoplasma tests were performed using Hoechst stain [39] and were always negative.

Cell Survival Assay

Cells were plated into 96-well microtitre plates at 5×10^3 cells / well, and allowed to adhere overnight. Compounds were added to culture medium at the indicated concentrations, and plates incubated in the above conditions for 24 hours. Following this incubation period, compounds and media were removed, and replaced with fresh culture medium. Cells were then grown for a further 72 hours before assay using sulforhodamine B (SRB; Sigma, St. Louis, MO) as previously described [40, 41]. Briefly, the culture medium was removed from the 96-well microtitre plates and the plates washed twice with phosphate buffered saline (PBS), before the cells were fixed with methylated spirits for 15 minutes. The plates were then rinsed with tap water and the fixed cells stained with 50 µL / well of SRB solution (0.4% sulforhodamine B (w/v) in 1% (v/v) acetic acid) over a period of 1 hour. The SRB solution was then removed from the wells and the plates rapidly washed two times with 1% (v/v) acetic acid. Protein bound dye was then solubilized with the addition of 100 µL of 10 mM unbuffered Tris, and incubated for 15 min at 25°C. Plates were then read at 564 nm on a VERSA max tuneable microplate reader (Molecular Devices, Sunnyvale, CA).

Ligand Docking

Ligands **8**-**26** were constructed *in silico* using Insight II [32] running on an SGI Octane workstation. The ligands were then flexibly docked into the active site of the HDAC8 crystal structure (1T64.pdb) [28] using the GOLD (Version 2.1) [30] program with a standard parameter setup. One distance constraint (range of 1.5-3.0 Å) was imposed between the hydroxamic acid hydroxyl oxygen atom of the ligand and the active site zinc atom of HDAC8. GOLD was used to generate ten docked conformations for each ligand and to rank their relative binding conformations. Docked conformations were then rescored using the LUDI module within InsightII.

ABBREVIATIONS

ABHA = Azelaic bishydroxamic acid

Ac = Acetyl

BOP = (benzotriazol-1-yloxy)-tris(dimethylamino) phosphonium hexafluorophosphate

DCM = Dichloromethane

DIPEA = Diisopropylethylamine

DMAP = 4-(Dimethylamino)pyridine

DMBA = 1,3-Dimethylbarbituric acid

DMF = Dimethylformamide

DIC = 1,3-Diisopropylcarbodiimide

DMSO = Dimethyl sulfoxide

EtOAc = Ethyl acetate

FCC = Flash column chromatography

Fmoc = 9H-Fluoren-9-ylmethoxycarbamate

Fmoc-OSu = 9-Fluorenylmethyloxycarbonyl-N-hydroxysuccinimide

HATU = O-(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

HMBA = Hexamethylene bisacetamide

HBTU = [(benzotriazolyl)oxy N',N',N',N'-tatramethyluronium hexafluorophosphate

rpHPLC = Reverse phase High Performance Liquid Chromatography

LCMS = Liquid Chromatography coupled- Mass Spectroscopy

LRMS = Low Resolution Mass Spectroscopy

Pro = Proline

SAHA = Suberoylanilide hydroxamic acid

SBHA = Suberic bishydroxamic acid

TFA = Trifluoroacetic acid

THF = Tetrahydrofuran.

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