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Benzofused hydroxamic acids: Useful fragments for the preparation of histone deacetylase inhibitors. Part 1: Hit identification

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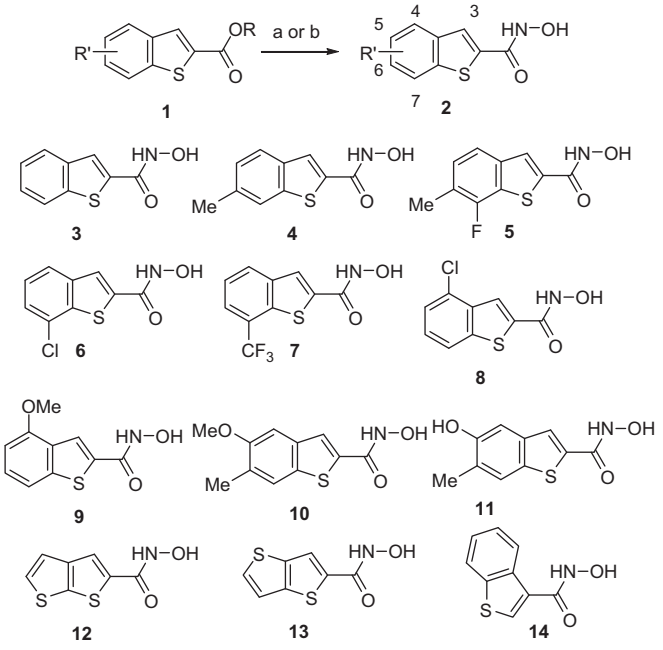
ABSTRACT

In the search for a new class of histone deacetylase inhibitors, we prepared a series of simple benzofused hydroxamic acids to find an anchoring fragment of minimal molecular weight. These initial hits, all belonging to the benzothiophene class, showed very good ligand efficiencies. Following these findings, a classical fragment growing approach was performed to increase binding affinity and cytotoxicity.

Histone deacetylases (HDACs) are zinc dependent hydrolases that mediate chromatin remodelling and gene expression by removal of acetyl groups from histone lysine residues. Histone hyperacetylation, induced by HDAC inhibitors, correlates with modulation of gene expression, cell-cycle arrest, differentiation and apoptosis in tumor cells and in recent years, HDACs have become important targets for the treatment of a number of cancers.[1] Currently there are several HDAC inhibitors in clinical trials[2] and two on the market.[3] Based on their homology to the yeast HDACs, mammalian HDACs can be categorized into four classes. Mechanistically, Classes I, II and IV are distinct from Class III in their co-factor requirement, in that the former require zinc in the active site to mediate deacetylation catalysis, while the latter is NAD^{+2} dependent. Virtually all of the known inhibitors target the Zn^{2+} catalytic domain of Class I, II and IV HDACs and are only slightly selective or non-selective. They fall into several distinct structural classes: hydroxamic acids, such as SAHA, aminobenzamides, short chain fatty acids, cyclic peptides.[4]

As part of an internal project aimed at the identification of new and proprietary classes of HDAC inhibitors, we decided to apply the fragment approach philosophy.[5] This involves first finding a small fragment able to show binding affinity in our test and then to evolve this fragment. We targeted molecular interactions only with the zinc atom and the active site cavity, planning to optimize pharmacokinetic and pharmacodynamic properties of the selected hits via systematic functionalization.



Scheme 1. Reagents and conditions: (a) NaOH, H_2O/THF and then NH_2OH, HOAt, EDAC, DIPEA; (b) NH_2OH, NaOH, MeOH.

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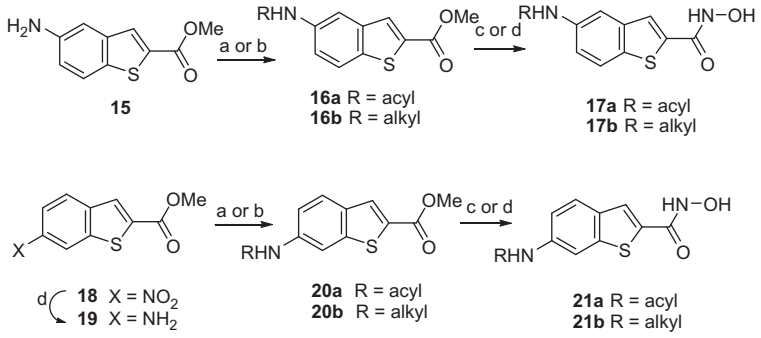
Table 1
IC_{50} values for compounds **3**–**14** and ligand efficiencies (LEs) calculated according to Ref. 6

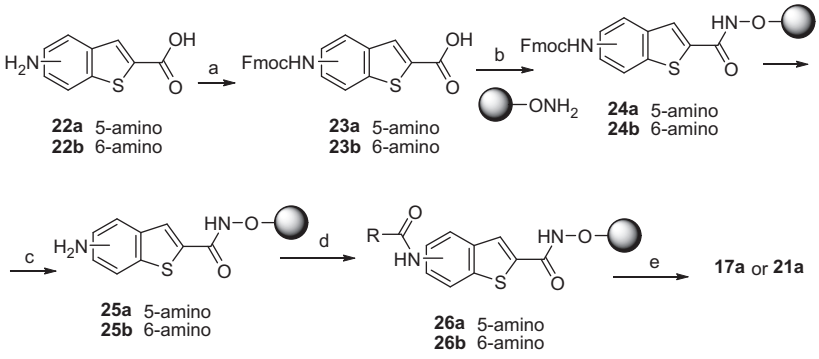
Compd	IC_{50} (μM)	LE
3	2.17 ± 0.65	0.59
4	1.76 ± 0.52	0.56
5	0.458 ± 0.169	0.58
6	2.91 ± 0.70	0.54
7	9.19 ± 3.73	0.40
8	11.63 ± 1.40	0.29
9	3.79 ± 1.31	0.49
10	1.35 ± 0.42	0.40
11	0.989 ± 0.151	0.54
12	1.08 ± 0.28	0.68
13	0.557 ± 0.185	0.71
14	76.13 ± 26.53	0.43

From the data reported in literature and a visual inspection of the various X-ray structures of HDACs complexed with various inhibitors,[6,7] a structural feature caught our attention: two phenyl alanine side chains (Phe 152 and Phe 208 in HDAC-8) form part of the wall of the binding cavity and are almost parallel. We reasoned that these two aromatic rings could be used to gain binding energy from π–π interactions via the insertion of an aromatic fragment.

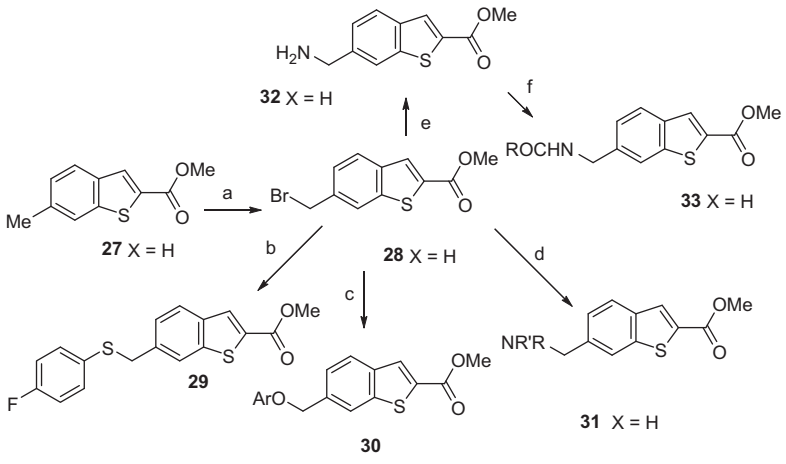
Given these assumptions we hypothesised that benzofused derivatives containing a hydroxamic acid were the initial fragments of choice. A docking of benzothiophene-2-hydroxamic acid into the active site of HDAC-8 showed that the hydroxamic acid moiety could complex the Zn atom without any steric clash with the heterocycle's sulfur atom, and that the benzene ring of the benxothiophene was able to sandwich between the two phenyl alanine aromatic side chains. These positive docking results



Scheme 2. Reagents and conditions: (a) EDAC, HOAt, RCO_2H; (b) RCHO, $NaBH_3CN$; (c) KOH, NH_2OH–HCl, MeOH/THF; (d) NaOH, H_2O/THF and then NH_2OH–HCl, EDAC, HOAt, DMF.

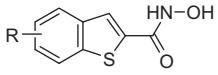


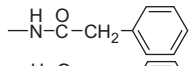
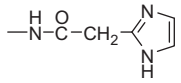
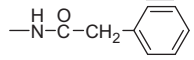
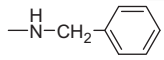
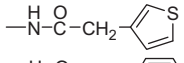
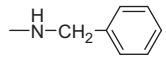
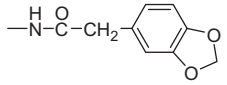
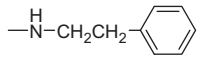
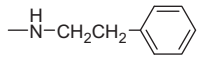
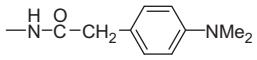
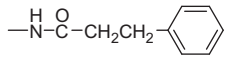
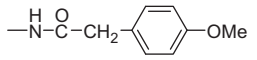
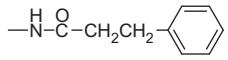
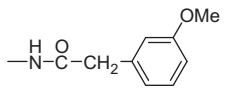
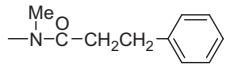
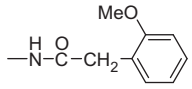
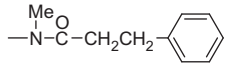
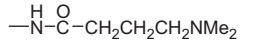
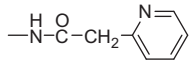
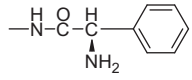
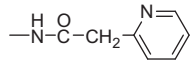
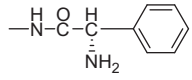
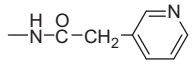
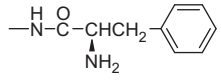
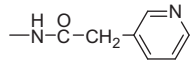
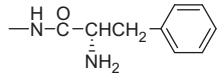
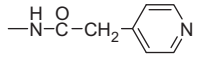
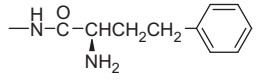
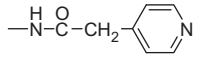
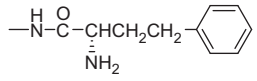
Scheme 3. (a) Fmoc-*N*-hydroxysuccinimide; (b) DIPC, HOAt, pyridine, hyroxylamine chlorotrityl resin; (c) 20% piperidine in DMF; (d) DIPC, HOAt, pyridine; (e) TES, TFA, DCM.



Scheme 4. (a) NBS, CCl_4; (b) *p*-F-thiophenol; (c) ArOH, Cs_2CO_3; (d) NHRR'; (e) NaN_3, DMF and then H_2, Pd/C, THF; (f) RCO_2H and coupling agent.

Table 2
HDAC inhibition of amines **34**–**39** and amides **40**–**63**, and cytotoxicity on HCT-116 cells



Compd	R		Inh at (%)		IC_{50}	Compd	R		Inh at (%)		IC_{50}
			0.1 μm	1.0 μm	HCT-116				0.1 μm	1.0 μm	HCT-116
34	5		48	82	1.3	**50**	6		24	78	nt
35	6		71	85	0.10						
36	5		28	68	nt	**51**	6		73	87	0.11
37	6		58	79	1.3	**52**	6		55	78	nt
38	5		28	61	nt						
39	6		32	73	nt	**53**	6		57	82	0.50
40	5		28	69	nt	**54**	6		75	84	0.49
41	6		42	76	2.6	**55**	6		65	83	0.6
42	5		27	67	nt	**56**	6		54	81	0.55
43	6		21	63	nt	**57**	6		50	79	2.4
44	5		39	74	nt	**58**	6		70	85	0.5
45	6		59	78	0.48	**59**	6		69	85	0.77
46	5		49	7	17	**60**	6		41	77	0.34
47	6		62	83	3.0	**61**	6		54	81	5.0
48	5		49	78	8.6	**62**	6		44	78	0.32
49	6		66	84	1.8	**63**	6		71	85	3.4

prompted us to form a collection of aromatic bicyclic compounds containing a thiophene and a carboxylic acid, available commercially, and in our chemical archives, and to synthesize the corresponding hydroxamic acids (Scheme 1, compounds **3**–**14**).[8]

The benzothiophene hydroxamic acids **3**–**14** were prepared from the corresponding carboxylic acids through coupling with hydroxylamine in the presence of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDAC) and 7-aza-1-hydroxybenzotriazole (HOAt), and then profiled using partially purified HDAC enzyme obtained from HeLa cells.[9] The IC_{50} values and ligand efficiencies (calculated using the method of Rees co-workers.[10]) for these initial fragments are reported in Table 1.

These data clearly showed that the 2 position was better than the 3 position from the point of view of the hydroxamic acid moiety, and that while some substituents were tolerated in the 4 and 7 positions, it seemed that there was a limit to their size, with the 7 position appearing the more sensitive of the two. Functionalisation of the 5 and 6 positions (singularly or together) was tolerated and this prompted us to use these positions for the introduction of new functional groups aimed at finding new interactions within the active site. This was carried out either via the introduction of amino groups at the 5 and 6 positions of scaffold **3**, or via the modification of the methyl group in compounds **4** and **5**. The subject of communication is the study done on **3** and **4**, which were selected as starting point for the easiness of modification. The information obtained was then used for the synthesis of the 7-fluorobenzothiophene derivatives, which will be the subject of a subsequent report.

The 5- and 6-aminobenzothiophene-2-carboxylate methyl esters **15** and **19** (the latter prepared by catalytic hydrogenation of the commercially available 6-nitro derivative **18**) were submitted to reductive amination using cyanoborohydride[11] to obtain amines

16b and **20b**, or coupled with an appropriate carboxylic acid (EDAC, HOAt) to obtain the corresponding amides **16a** and **20a**. Treatment of **16a/b** and **20a/b** with a methanol solution of NaOH and hydroxylamine hydrochloride, or submission to basic hydrolysis followed by coupling with hydroxylamine, afforded the hydroxamate derivatives **17a/b** and **21a/b** (Scheme 2).

Subsequently, we developed a solid phase method for the synthesis of benzothiophene amides **17a** and **21a** and this allowed us to speed up the SAR process (Scheme 3). The free amine of 5- or 6-amino benzothiophene-2-carboxylic acids was protected with an Fmoc group through treatment with Fmoc-*N*-hydroxysuccinimide to produce acids **23a/b**, which were loaded onto hydroxylamine chlorotrityl resin using standard conditions.

Treatment with a 20% piperidine solution in dimethylformamide (DMF) released the amino group, which was then coupled to a series of carboxylic acids. The use of pyridine as a base in the subsequent coupling reaction was necessary, since with diisopropylethyl amine (DIPEA) consistent amounts of a side product, deriving from acylation of the hydroxamate nitrogen, were observed. Finally, cleavage from the resin with a trifluoroacetic acid (TFA)/triethylslane (TES) solution in dichloromethane (DCM), afforded the corresponding hydroxamic acids, in many cases pure enough to be tested.

An entry to 6-benzyl derivatives of thiophene-2-carboxylate was obtained by radical bromination of methyl derivative **27** with *N*-bromosuccinimide (NBS), followed by nucleophilic substitution with an aromatic thiol (**29**), a phenol (**30**), a secondary amine (**31**) or the azide ion which, after catalytic reduction gave primary benzylamine **32** (Scheme 4).

Coupling with an appropriate carboxylic acid under standard conditions gave amide derivatives **33.**

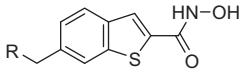
In a first set of compounds (**34**–**39**), a non-functionalized phenyl ring was linked to the benzothiophene in both the 5 and 6 positions. The distance of the ring from the heterocyclic nucleus was modulated with carbon chains of different lengths, while the use of amines, amides and methylamides modulated slightly the geometry of the side chain versus the heteroaromatic moiety.

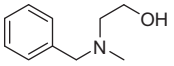
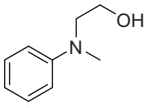
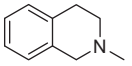
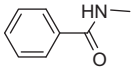
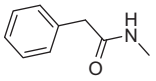
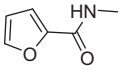
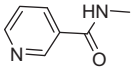
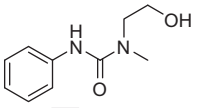
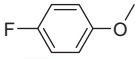
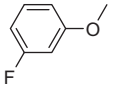
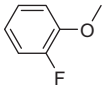
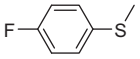
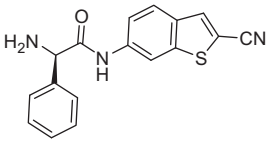
The newly prepared compounds were evaluated for their ability to inhibit HDAC in a two point experiment (1.0 and 0.1 μM). Those having a inhibition >40% at a concentration of 0.1 μM were also tested for their cytotoxicity on HCT-116 cells.[12] From this data three things were clear: first that the functionalization in the 6 position of the benzothiophene scaffolds generally afforded higher potency than the same substituent in the 5 position; second that the amides, if not methylated, were clearly superior to amines, and third that among the amides, phenylacetamide was best.

Taking advantage of this information, a second set of compounds was prepared: all the isomers of pyridyl acetic acid were linked at the amino group in position 5 (**44**, **46** and **48**) and 6 (**45**, **47** and **49**) of the benzothiophene nucleus, with the aim of increasing the aqueous solubility, which for **34**–**43** was not satisfactory. The binding affinities were generally good for all six molecules, although there was a drop in cellular activity, very likely due to decreased cell membrane permeability. Solubility remained low, ranging from 0.004 to 0.05 mg/mL, and this we hypothesised was due to π-stacking.

An additional group of compounds was prepared with heteroaryl amides in the 6 position in place of the substituted phenylacetic amides (**50**–**56**): they showed no significant improvements, either in the ex-cell and cell tests, or in aqueous solublity. We were pleasantly surprised to find that the introduction of a simple tertiary aliphatic amine produced a compound (**57**) with reasonable potency and solubility (1.31 mg/mL in aqueous solution at pH 7.4). As a result of this we introduced onto the 6-amino group of the benzothiophene scaffold substituents containing both a free amino and an aromatic group: both enantiomers of phenyl glycine (**58**, **59**), phenylalanine (**60**, **61**) and homophenylalanine (**62**, **63**) were prepared. While the two enantiomers of phenylglycine were equipotent in the enzyme and in the cell test, with both phenylalanine and homophenylalanine the D-isomers were more active on the isolated enzymes and the L-isomers more active in the cell-test. The reason for this difference is as yet unclear (see Table 2)

Table 3
Inhibition of compounds **64**–**76** and cytotoxicity on HCT-116 cells



Compd	R	% Inh at 0.1 μm	% Inh at 1.0 μm	IC_{50} HCT-116
64		52	80	0.83
65		53	76	1.7
66		53	83	1.3
67		93	87	0.25
68		60	88	1.2
69		89	92	0.07
70		72	84	0.73
71		77	91	1.4
72		<20	31	nt
73		23	68	nt
74		22	58	nt
75		<20	24	nt
76		<20	<20	nt

Among the aminomethyl derivatives (**64**–**76**, Table 3), the amides showed better activity than the amines, ethers, thioethers and ureas, with compound **69** showing activity in the nanomolar range in the HCT-116 cell test.

Finally a xenograft efficacy study using HCT-116 tumor cells in CDI mice was carried out with compound **35**. It was dosed to female CDI nude (nu/nu) athymic mice at doses of 50 and 100 mg/kg os ($n = 4$ per group) for 21 days. The control vehicle was 0.5% CMC (10 mL/kg). This study resulted in a TVI of 26% at a dose of 50 mg/kg.

In summary, starting from a selection of small, rigid fragments derived from benzothiophene, we have optimized a series of 5- and 6-substituted benzothiophene-2-hydroxamates that display significant potency in vitro. One of the first derivatives prepared, **35**, was also shown to be efficacious after oral administration in a mouse HCT-116 xenograft study.

This series was further elaborated and the results will be reported in a subsequent paper.

References and notes

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8. While we were well advanced in our work on this series, analogous compounds were published by Merck [*Bioorg. Med. Chem. Lett.* **2007**, *17*, 4562]. Nevertheless, we decided to write the current paper since the SAR data (including in vivo activity data) from the current work is much larger than that in the Merck publication and this may be of interest to readers. Some of the compounds presented in this paper, namely **34**–**39**, **54, 67** and **68**, have already been published. We could have easily deleted them from the present paper, but we think that it is important to report their activity to complete the SAR.
9. The compounds were evaluated for their ability to inhibit isolated HDACs in a commercially available assay (Fluor de Lys Assay System, Biomol International LP, Plymouth Meeting PA), employing SAHA and LAQ-xx as positive controls. The reaction mixture, 50 μL run in a 96 well plates, contains a HeLa cell nuclear extract and a commercial substrate containing acetylated lysine side chains. The substrate and extracts are incubated in the presence of the appropriate concentration of the inhibitor. Deacetylation of the substrate followed by mixing with the provided developer generates a fluorophore, The release of the fluorophore was monitored with a Victor 1420 fluorescent plate reader set at excitation/emission wavelength of 335/460 nm. The activity of compounds was expressed as IC_{50} (drug concentration causing a 50% inhibition of enzymatic activity) and calculated with Easy-fit software application. All the experiment were carried out in triplicate and reported values are the average of those determinations.
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11. Borch, R. F.; Bernstein, M. D.; Dupont Durst, H. . *J. Am. Chem. Soc.* **1971**, *93*, 2897.
12. Colon cancer HCT-116 cells were plated in a 96-well tissue culture plates containing 200 μL of complete medium. After 24 h HDAC inhibitors were added at different concentrations, ranging from 0.1 to 100 μM in quadruplicate. After 5 days, 20 μL of Alamar blue were added to each well and the plates were further incubated for 4 h. The chemical reduction of Alamar Blue in the growth medium is a fluorometric/colorimetric indicator of cellular growth based on the detection of metabolic activity. Fluorescence was monitored in a multilabel counter Victor 1420 at 530 nm excitation and 590 nm emission wavelength. All results were expressed as IC_{50} (drug concentration causing a 50% inhibition of cellular proliferation) calculated using the Easy-fit software.