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Diphenylmethylene hydroxamic acids as selective class IIa histone deacetylase inhibitors

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ABSTRACT

We have identified a series of diphenylmethylene hydroxamic acids as novel and selective HDAC class IIa inhibitors. The original hit, *N*-hydroxy-2,2-diphenylacetamide (**6**), has sub-micromolar class IIa HDAC inhibitory activity, while the rigidified oxygen analogue, *N*-hydroxy-9*H*-xanthene-9-carboxamide (**13**), is slightly more selective for HDAC7 with an IC_{50} of 0.05 μM. Substitution of **6** allows for the modulation of selectivity and potency amongst the class IIa HDAC isotypes.

The dynamic and reversible acetylation of lysine residues on the N-terminal tails of histones is tightly regulated and controlled by histone acetyltransferases (HATs) and deacetylses (HDACs).[1] Based on their specificity and catalytic mechanisms, eukaryotic HDACs are divided into two main groups; group I, the zinc-dependent amidohydrolases, and group II, NAD^+-dependent enzymes (also designated as HDAC class III, or sirtuins 1–7). Group I consists of eleven subtypes, HDAC1-11, sharing close catalytic site homology. Group I is further subdivided into class I (HDAC1, 2, 3 and 8), class IIa (HDAC4, 5, 7, and 9), class IIb (HDAC 6, and 10), and class IV (HDAC11), the last of which shares properties with both class I and class II enzymes.[2]

HDAC class I enzymes are ubiquitously expressed, and are predominantly nuclear enzymes. They are reported to regulate differentiation, proliferation, cell cycle, protein turnover, and apoptosis.[3] Inhibitors of HDAC class I enzymes have demonstrated benefits in cancer therapy either as approved drugs (SAHA, or Zolinza®/Vorinostat),[4a] or as agents in different stages of clinical development, such as MGCD0103,[4b] and LBH589 amongst others.[4c–e]

HDAC class II enzymes are expressed in a limited number of cell types, and either shuttle between the nucleus and cytoplasm (class IIa), or are mainly cytoplasmic (class IIb).[5a,b] The N-terminus of class II HDACs is involved in gene regulation through its interaction with other proteins.[2c,5c] HDAC6, a member of the class IIb enzymes, deacetylates α-tubulin,[5d] however the cellular substrates of the class IIa HDACs are not well characterized.[5b] In addition, the function of HDAC class IIa is not as well defined, but they may play an essential role as transcriptional regulators of various developmental and differentiation processes.[2c]

A few reports have appeared in the literature describing selective HDAC6 inhibitors[6] such as **1**,[6a–c] **2**,[6d] **3**[6e] and **4**[6f] (Fig. 1). However, it was not until the report of Jones and co-workers[7a,b] and the identification of the unnatural trifluoroacetamide lysine substrate, Boc-L-Lys-MCA, that a reliable classification of HDAC class II inhibitors was feasible. Consequently, the trifluoromethyl ketone class of compounds, such as **5** (Fig. 1) have emerged as selective class II HDAC inhibitors.[7c–e]

In order to understand the function of the different HDAC enzymes, there is still a dire need for class and isoform selective HDAC inhibitors. Despite the reports of selective HDAC class II and HDAC class IIb selective inhibitors, to the best of our knowledge, there is no report of HDAC class IIa selective inhibitors. Our initial strategy for the discovery of HDAC class IIa selective inhibitors involved the screening of a variety of small hydroxamic acids with diverse topology utilizing acetyl-Boc-lysine for detection of class I inhibitory activity, and Boc-Lys-(ε-trifluormethylacetyl)-AMC for the detection of class IIa inhibitory activity. From this exercise, lead compound **6** (Fig. 2) was determined to have class IIa activity in the high nanomolar range. Although reported to have weak activity for

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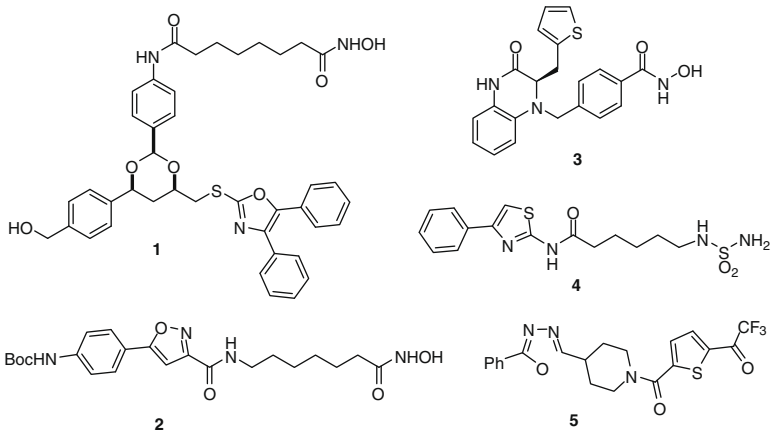


Figure 1. Examples of HDAC6 selective inhibitors.

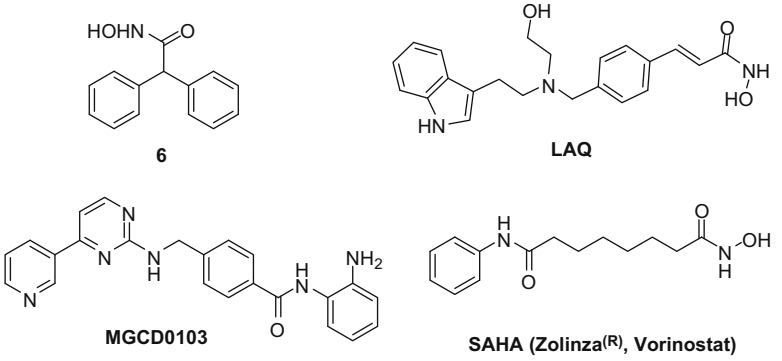


Figure 2. Examples of HDAC inhibitors with different selectivity profile.

HDAC8,[8] this compound was previously unknown as an HDAC class IIa inhibitor. Using both the acetyl-Boc-Lysine and Boc-Lys-(ε-trifluormethylacetyl)-AMC substrates, an HDAC Class I and IIa activity profile was generated comparing lead **6** to several known HDAC inhibitors with different selectivity profiles (Fig. 2, and Table 1).

The unique aspect of **6** appears to be the bifurcated diphenyl moiety, and it was decided that initial analogs should focus on three main areas of interest; namely, the modification of the site of bifurcation, rigidification of the diphenyl system and substitution on the phenyl rings.

Various commercial acids were available with a degree of variation around the branch point of compound **6**. These acids were either converted directly to the hydroxamic acid by coupling with hydroxylamine using BOP reagent in pyridine, compounds **7**, **8**, **9**, and **10** (see Table 2), or were first converted to the methyl or ethyl ester and subsequently transformed to the hydroxamic acid, compound **11**, using established protocols employing potassium hydroxide and hydroxylamine.[9]

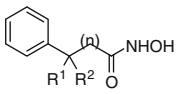
Rigidification of the diphenyl system was investigated as we considered analogs of **6** with the phenyl rings linked through the *ortho* position by a carbon (**12**), oxygen (**13**), or nitrogen atom (**14**), Table 3. The carbon and oxygen acids were commercially available, while the nitrogen analog required synthesis from acridine-9-carboxylic acid as detailed in Scheme 1.

Table 1
Selectivity of lead **6** on HDAC isoforms[a]

MG	HDAC isoforms IC_{50} (μM)				
	1[b]	**2**[b]	**4**[c]	**5**[c]	**7**[c]
6[d]	**>10**	**6.6**	**0.75**	**0.14**	**0.39**
LAQ	0.008	0.01	0.50	0.19	2.24
MGCD0103	0.2	0.18	>10	>10	>10
SAHA	0.14	0.20	>10	1.30	>10

[a] Values are average of at least two determinations. Standard deviations were within 30% of the IC_{50} values.
[b] The substrate used is acetyl-Boc-Lysine.
[c] The substrate used is Boc-Lys-(ε-trifluormethylacetyl)-AMC.
[d] Compound **6** was not active against HDAC6 (IC_{50} >10 μM); HDAC8 IC_{50} = 66 μM, as reported in Ref. 8.

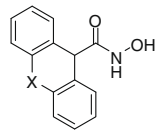
Table 2
SAR around the branch point of lead **6** on HDAC class IIa isoforms



Compd	*n*	R^1	R^2	HDAC class IIa isoform IC_{50} (μM)[a,b]		
				4	**5**	**7**
6	0	Ph	H	0.75	0.14	0.39
7[c]	0	Me	H	>10	>10	>10
8	0	CH_2Ph	H	>10	>10	>10
9[d]	1	Ph	H	>10	>10	>10
10	0	Ph	Me	4.5	1.2	4.8
11	0	Cy	H	>10	>10	>10

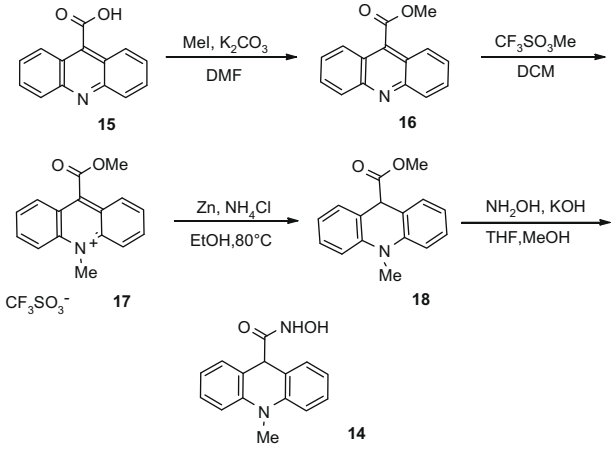
[a] Values are average of at least two determinations. Standard deviations were within 30% of the IC_{50} values.
[b] Compounds showed no inhibition against HDAC1 and 2 when screened at 20 μM inhibitor concentration.
[c] Ref. 15.
[d] Ref. 16.

Table 3
SAR of rigid analogs of lead **6** on HDAC class IIa isoforms[a]



Compd	X	HDAC class IIa isoforms IC_{50} (μM)		
		4	**5**	**7**
6	H	0.75	0.14	0.39
12	CH_2	2.32	1.46	0.26
13	O	0.25	0.11	0.05
14	NMe	1.91	0.47	0.35

[a]Values are average of at least two determinations. Standard deviations were within 30% of the IC_{50} values.



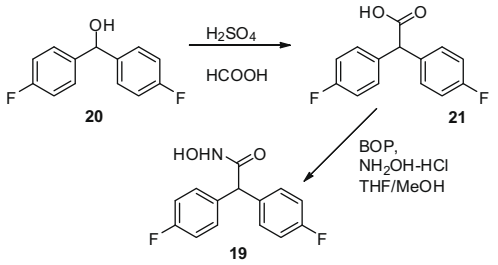
Scheme 1. Synthesis of the rigid nitrogen analogue **14**.

Initial methylation of acid **15** was effected through the use of methyl iodide to give ester **16**. Quartenization of the nitrogen did not occur as expected, and it was necessary to use methyl triflate to afford **17**, which upon reduction of the central ring with zinc and ammonium chloride gave the dihydroacridine **18**. Finally, ester **18** was transformed with hydroxylamine and potassium hydroxide to hydroxamic acid **14** (Scheme 1).

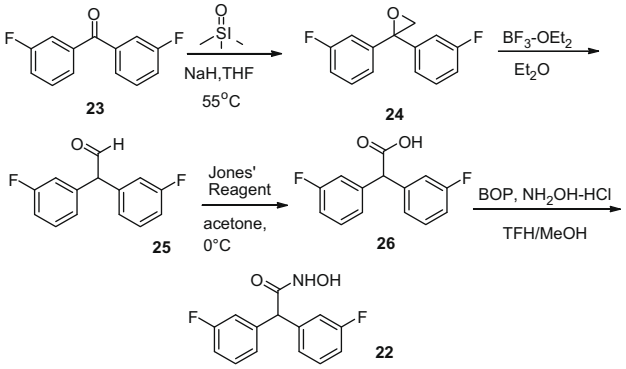
Next, the effect of substitution on the phenyl rings of **6** on the HDAC activity and selectivity was investigated. In order to avoid the introduction of a chiral center, these initial substitutions were limited to those that would afford the molecule a plane of symmetry. Somewhat surprisingly, considering the apparent simplicity of the scaffold of **6**, the literature demonstrated a relative paucity of procedures on how to build the bifurcated system effectively. As a result, it was necessary to employ a disparate set of conditions to construct the starting materials that were not commercial.

For the synthesis of the *p*-fluoroanalogue **19**, the difluro-benzhydrol **20** was commercial and could be converted to the corresponding acid **21** employing a mixture of concentrated sulfuric acid and formic acid.[10] Acid **21** was then transformed to hydroxamic acid **19** via BOP coupling with hydroxylamine HCl (Scheme 2).

The *meta*-fluoro substituted isomer **22** required a somewhat more involved procedure. Initially, the *m*-difluro benzophenone **23** was treated with trimethylsulfoxonium iodide while heating to afford epoxide **24**. Epoxide **24** was quickly converted to the corresponding aldehyde **25** via a 5 min treatment with $BF_3{\cdot}OEt_2$, which was in turn transformed to acid **26** through the use of Jone's reagent. Hydroxamic acid **22** was obtained utilizing the usual BOP and hydroxylamine hydrochloride coupling (Scheme 3).



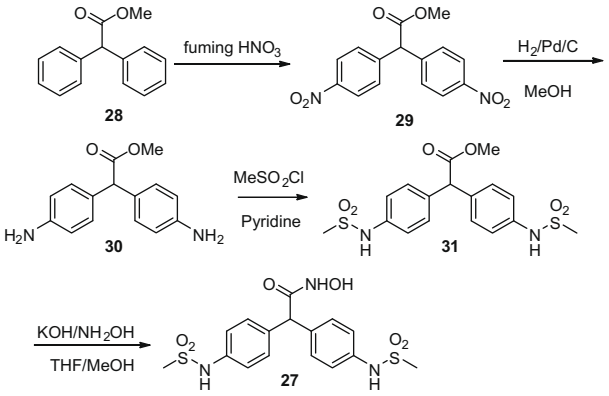
Scheme 2. Synthesis of *p*-F-substituted analogue **19**.



Scheme 3. Synthesis of *m*-F-substituted analogue **22**.

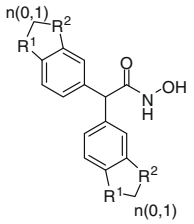
For *p*-methylsulfonamide substituted analogue **27**, the procedure began with nitration of methyl 2,2-diphenylacetate **28**, employing a solution of fuming nitric acid[11] to exclusively give the *para* substitution product **29**. The nitro groups of **29** were then reduced with palladium on carbon under hydrogen to produce aniline **30**, which was reacted with methyl sulfonyl chloride in pyridine to yield **31**. Finally, ester **31** was converted to **27** with potassium hydroxide and hydroxyl amine (Scheme 4). Compounds **32** and **33** were accessible from the corresponding commercial acids which were converted to their respective esters and were then taken to the desired hydroxamic acids (Table 4).

All compounds prepared for this study were profiled against HDAC class IIa, class IIb, and class I enzymes (data not shown).[12] SAR around the branch point of 6 confirmed our initial belief that it was this moiety that gave the molecule its unique HDAC class IIa activity/selectivity profile (Table 2). Replacement of one of the phenyl rings with a methyl (**7**) or ethyl (not shown) group reduced the activity against HDAC class IIa enzymes. In addition, displace-



Scheme 4. Synthesis of *p*-substituted sulfonamide **27**.

Table 4
SAR of substituted analogs of lead 6 on HDAC class IIa isoforms[a]



Compd	R^1	R^2	*n*	HDAC class IIa isoforms IC_{50} (μM)		
				4	**5**	**7**
6	H	H	0	0.75	0.14	0.39
19	F	H	0	1.10	0.28	1.76
22	H	F	0	0.7	0.09	0.44
27	$NHSO_2Me$	H	0	>10	>10	>10
32	O	CH_2	1	0.21	0.11	0.11
33	Cl	H	0	0.96	0.21	1.4

[a] Values are average of at least two determinations. Standard deviations were within 30% of the IC_{50} values.

ment of the branched site with a methylene unit (**8** and **9**), or replacement of the phenyl ring with a cyclohexyl group (**10**) completely destroyed activity. However, addition of a methyl group to the branch point (**11**) appears to be tolerated, albeit with an approximate one log decrease in activity.

Rigidification of **6**, while tolerated somewhat, was generally detrimental or not beneficial to activity. This effect was not uniform across the class IIa isoforms and did lead to a modification of the selectivity profile. Linking of the phenyl rings with either a carbon (**12**) or nitrogen (**14**) had the effect of reducing HDAC 4 and 5 inhibitory activities by several fold, while HDAC7 activity was almost unchanged. Alternatively, linking with oxygen (**13**) left HDAC 4 and 5 inhibitory activities basically unchanged while giving a modest sixfold increase to potency against HDAC7 (Table 3). While compound **13** was slightly superior to our initial lead (**6**), the scaffold of **6** is more versatile to build the SAR and was used to generate more analogs.

Substitution on the phenyl rings appears to be fairly well-tolerated and can be used to modulate HDAC class IIa selectivity (Table 4). When fluoro groups are added at the *para* position (**19**), HDAC 4 and 5 inhibitory activities were slightly decreased while HDAC 7 inhibitory activity was reduced by fivefold. The same trend was observed when the bulkier chloro groups were used instead of fluoro (**33**); but when this group was changed to the more polar methyl sulfonamide, compound **27**, all activity was lost. As the fluorines are shifted to the *meta* position (**22**), the activity was improved over the *para*, but when compared to the parent compound only a very modest enhancement for HDAC 5 inhibitory potency was observed. Some improvement in the HDAC class IIa inhibitory activity was observed, more noticeably against HDAC 4 and 7, when the phenyl groups were replaced with dihydrobenzofuran moieties (**32**).

Table 5
HDAC class I and IIa cellular activity for selected HDAC inhibitors[a]

Compd	293T Class I IC_{50} μM[b]	293T Class IIa IC_{50} μM[c]
6	>50	4.6
10	>50	16
13	>50	3.0
19	>50	3.5
32	>50	0.9
27	>50	>50

[a] Values are average of at least two determinations. Standard deviations were within 30% of the IC_{50} values.
[b] Acetyl-Boc-Lysine substrate.
[c] Boc-Lys-(ε-trifluormethylacetyl)-AMC.

Schuetz et al.[13] studying the crystal structure of cdHDAC7 enzyme, reported that the catalytic domain of class IIa HDACs have a unique active site topology and an enlarged active site pocket. Our SAR data is in agreement with the crystal structure report and point to the presence of a hydrophobic pocket in the active site of class IIa HDACs that is absent in class I and class IIb enzymes. This pocket accommodates binding of compounds with aromatic groups (compare **6** and **11**) suggesting that pi-stacking may play a role in conferring the activity and selectivity of these class IIa inhibitors.

We also tested our inhibitors for their ability to inhibit HDAC activity within intact cells. The inhibitory activity of these compounds was measured in 293T cells using acetyl-Boc-Lysine substrate for HDAC class I and Boc-Lys-(ε-trifluormethylacetyl)-AMC substrate for HDAC class IIa (Table 5).[14]

For all examples, inhibition of HDAC class I cellular activity is non-existent as would be expected. Moreover, cellular class IIa inhibitory activity tracks well versus inhibition of individual recombinant class IIa enzymes, suggesting that these inhibitors are cell permeable. Compound **32**, which had the best recombinant class IIa enzymatic inhibitory activity, also showed a fivefold improvement in the class IIa intracellular inhibitory activity versus the lead **6**, with IC_{50} values of 4.6 and 0.9 μM, respectively. Further optimization of this class of inhibitors is ongoing.

In conclusion, we have identified diphenylmethylene hydroxamic acids as a new class of selective class IIa HDAC inhibitors. The original hit, *N*-hydroxy-2,2-diphenylacetamide (**6**) has class IIa HDAC activity in the sub-micromolar range. While the rigidified oxygen analogue, *N*-hydroxy-9*H*-xanthene-9-carboxamide (**13**), is slightly more selective for HDAC7 with an IC_{50} of 0.05 μM. Substitution of **6** is tolerated and allows for the modulation of selectivity amongst the HDAC class IIa isotypes. These inhibitors demonstrated cellular HDAC class II inhibitory activity in accordance with their enzymatic potencies.

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12. (a) For experimental details see: Tessier, P.; Leit, S.; Smil, D.; Deziel, R.; Ajamian, A.; Chantigny, Y.A.; Dominguez, C. International Patent application WO 07/122115, 2008; (b) The enzymatic assay followed the fluorescent signal obtained from the HDAC catalyzed deacetylation of coumarin-labeled lysine. The substrate used for HDAC1, 2, 3, 6, and 8 was Boc-Lys(ε-acetyl)-AMC (Bachem Biosciences Inc.) and Boc-Lys-(ε-trifluormethylacetyl)-AMC (synthesized in-house) for HDAC4, 5, and 7. Recombinant enzymes expressed in baculovirus were used. HDAC1, and 2 were C-terminal FLAG-tagged and HDAC4 (612-1034), HDAC5 (620-1122), HDAC6, and HDAC7 (438-915) are N-terminal His-tagged. The enzymes were incubated with the compounds in assay buffer (25 mM Hepes, pH 8.0, 137 mM NaCl, 1 mM MgCl2 and 2.7 mM KCl) for 10 min at ambient temperature in black 96-well plates. The substrate was added into enzyme-compound mixture and incubated at 37 °C. Reaction was quenched by adding trypsin and TSA to a final concentration of 1 mg/mL and 1 μM, respectively. Fluorescence was measured using a fluorimeter (SPECTRAMAX GeminiXS, Molecular Devices). The 50% inhibitory concentrations (IC_{50}) for inhibitors were determined by analyzing dose–response inhibition curves with GraFit; (c) At the time we were investigating different substrates for determining the activity of HDAC class II enzymes, Jones et al. (Bioorg. Med. Chem. Lett. **2008**, 18, 1814) published their obervations that purified mammalian HDAC class IIa enzymes could not be isolated and are contaminated with other deacetylases. Our results and those of Jones et al. demonstrated that the BOC-ι-Lys(ε-trifluoroacetyl)-MCA is a selective class IIa substrate and allowed the development of assays to measure HDAC class IIa activity even in the presence of class I enzymes for naked enzymes and even in intact cells; (d) Our studies showed that human HDAC4, 5 and 7 processed the Boc-Lys-(ε-trifluormethylacetyl)-MCA substrate at a much higher turn-over rate than HDAC8 did. A plot of the enzyme reactivity (K_{cat}/K_m, μM^{-1} min^{-1}) versus HDAC1, 2, 3, 4, 5, 7, and 8 enzymes utilizing both acetyl-Boc-Lysine and Boc-Lys-(ε-trifluormethylacetyl)-MCA substrates clearly demonstrated the extraordinary specificity of the Boc-Lys-(ε-trifluormethylacetyl)-MCA substrate for HDAC4, 5, and 7 enzymes (class IIa).
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