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Potent Histone Deacetylase Inhibitors Derived from 4-(Aminomethyl)-*N*-hydroxybenzamide with High Selectivity for the HDAC6 Isoform

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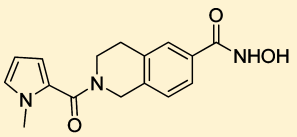
Supporting Information

ABSTRACT: A screen for HDAC6 inhibitors identified acyl derivatives of 4-(aminomethyl)-*N*-hydroxybenzamide as potent leads with unexpected selectivity over the other subtypes. We designed and synthesized constrained heterocyclic analogues such as tetrahydroisoquinolines that show further enhanced HDAC6 selectivity and inhibitory activity in cellular assays. Selectivity may be attributed to the benzylic spacer more effectively accessing the wider channel of HDAC6 compared to other HDAC subtypes as well as hydrophobic capping groups interacting with the protein surface near the rim of the active site.



5a
HDAC6 IC_{50} = 36 nM
Nuclear extract/HDAC6 = 500
AcTub IC_{50} = 210 nM

INTRODUCTION

The histone deacetylases (HDAC) constitute a family of amidohydrolases that remove acetyl groups from the ε-amino group of lysine residues and can be subdivided into four groups termed class I (types 1, 2, 3, 8), IIa (4, 5, 7, 9), IIb (6, 10), and IV (11), of which the class I, II, and IV enzymes are Zn^{2+} dependent metalloamidases.[1] Unlike other HDACs, HDAC6 resides predominantly in the cytosol and has two functional catalytic domains as well as a carboxy-terminal zinc-finger domain that binds ubiquitinated proteins.[2] Substrates for HDAC6 include the cytoskeletal proteins α-tubulin and cortactin and the chaperone Hsp90.[3,4] Thus, HDAC6 mediates a wide range of cellular functions ranging from microtubule-dependent transport, membrane remodeling and chemotactic motility and cellular adhesion to sensing ubiquitin levels, as well as regulating chaperone levels and activity, all of which are important in tumorigenesis, tumor growth, and metastasis.[5−8] Recently, HDAC6 has been linked to autophagy, an alternative pathway for protein degradation that compensates for deficiencies in the activity of the ubiquitin proteasome system arising from treatment with a proteasome inhibitor.[9−12] HDAC6 binds ubiquitin, or ubiquitin-like conjugated proteins, which would otherwise induce proteotoxic stress and transports them as ternary complexes with dynein to the microtubule organizing center (MTOC). These perinuclear complexes are then sequestered into the aggresome[6] and degraded by fusion with lysosomes as part of autophagy. Hence, targeting both the proteasome-dependent pathways and the aggresome pathway in tumor cells is expected to cause greater accumulation of polyubiquitinated proteins with consequent cell stress and apoptosis. Indeed, the HDAC6 selective inhibitor tubacin is reported to induce selective hyper-acetylation of α-tubulin over histones in multiple myeloma cells[13−15] and to potentiate proteasome inhibitor-induced apoptosis.[16]

Numerous HDAC inhibitors have been reported[17] that are based on Zn^{2+} complexing agents including thiols, acylated *ortho*-phenylene diamines, and, most commonly, hydroxamic acids (Figure 1). Pan HDAC or class I selective HDAC inhibitors such as trichostatin are most common, and some, such as suberoylanilide hydroxamic acid (SAHA) or vorinostat, have been shown to have therapeutic benefits.[18−22] To investigate further potential synergistic effects of HDAC6 and proteasome inhibitors, we required inhibitors with significant selectivity for the HDAC6 isoform, at least comparable to tubacin and with improved physical properties. Despite their metabolic liabilities, hydroxamic acids exhibit unique binding energetics[23] whereby the uncharged form predominates in aqueous solution and the conjugate base is formed in the active site of the enzyme. As a result, these chemotypes pay a much smaller desolvation penalty on binding than species that are already ionized in the aqueous phase. The HDAC inhibitors shown in Figure 1 comprise a hydroxamic acid Zn^{2+} chelator, spacer (in the case of vorinostat, the aliphatic chain), and capping group (acylaniline). We report here a series of *C*-arylhydroxamic acids that potently inhibit the HDAC6 isoform with unusually high selectivity. While this work was in progress, Kozikowski and co-workers reported a highly potent HDAC6 inhibitor NOC-7 with moderate subtype selectivity[24] and a compound with high HDAC6 selectivity, denoted tubastatin A,[25] related to the compounds described herein (Figure 1).

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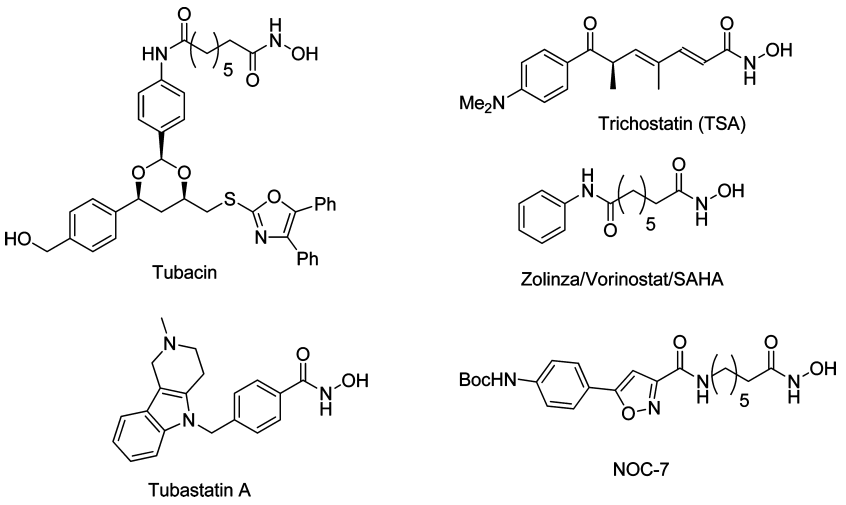

Figure 1. Examples of hydroxamic acid inhibitors of histone deacetylases.

RESULTS AND DISCUSSION

We began our search for HDAC6 selective inhibitors by screening compounds in our screening collection containing functionality known to interact with the metal ion of zinc metalloproteases. The inhibitory activities of these compounds on the rate of deacetylation of the substrate Ac-Arg-Gly-Lys(Ac)-AMC (AMC: 7-aminocoumarin) by HDAC6 were measured, and selectivity was initially assessed from inhibition of the enzymes present in HeLa nuclear extract (NucEx), predominantly the class I HDACs 1 and 2. Numerous hydroxamic acids were included in the screen, most of which exhibited the pan HDAC inhibitory activity shown by vorinostat.[26] In contrast, these screens identified a derivative of 4-aminomethylbenzoic acid (compound **1a**) as a selective inhibitor of HDAC6 in the enzymatic assays (Table 1).

Table 1. HDAC6 Inhibitory Activity and Selectivity of Hydroxamates Derived from 4-Aminomethylaryl Acids

compd	HDAC6[a] IC_{50} (nM)	selectivity NucEx[b]/HDAC6
1a	19	115
1b	79	200
1c	76	84
2	19	202
3	52	123
4	12000	8

[a]Enzymatic data obtained with recombinant HDAC6 or HeLa nuclear extracts using acetylated-Arg-Gly-Lys(Ac)-AMC as substrate with subsequent release of AMC by treatment with trypsin. IC_{50} values are means of two or more determinations. Standard deviation is within 20% of the IC_{50}. [b]Nuclear extract expresses predominantly HDACs 1 and 2.

Hydroxamate **1a** also inhibited the cleavage of the HDAC6 substrate acetyl tubulin in cells (IC_{50} = 0.46 μM) with little inhibitory activity on the hydrolysis of acetyl histone (26% inhibition @ 10 μM). Related compounds were prepared by parallel syntheses as shown in Scheme 1. Thus, acylation of the methyl ester of 4-aminomethylbenzoic acid followed by treatment with hydroxylamine hydrochloride in the presence of base gave compounds **1a**–**c**. We also synthesized analogues derived from aminomethyl-heteroaryl carboxylic acids such as thiophene **2** and thiazole **3** that proved to be HDAC6 selective (Table 1). In contrast, isomeric thiazole **4** showed no significant HDAC inhibitory activity, presumably because the ring nitrogen adjacent to the hydroxamate interferes with coordination to active site zinc. On the basis of crystal structures[27,28] and homology models[29] of HDAC complexes, hydroxamate anions have been shown to interact with active site zinc in a bidentate fashion, with two Asp and one His residue of the active site completing the primary coordination sphere[23] at the end of an 11 Å deep channel.[30] The spacer projects along the channel with the capping groups participating in additional binding interactions with the rim of the active site of the protein. We reasoned that the selectivity of compounds **1**–**3** might derive, in part, from the differing "width" of the (benzylic) spacer as compared to long chain aliphatic series, and we sought to explore this hypothesis by configurationally restraining such analogues in fused ring systems.

The first examples of fused ring analogues were prepared according to Scheme 2 by acylation of tetrahydroisoquinoline-6-carboxylic acid methyl ester followed by conversion to the hydroxamic acid as described previously to give compounds **5a**–**e**. Isoindoline homologues were obtained starting from the bis-benzylic bromide **6**,[31] which was cyclized by reaction with *p*-methoxybenzylamine and deprotected by hydrogenolysis to the secondary amine **7b**, which was acylated and then transformed to hydroxamic acids **8**.

Seven-membered homologues, viz. isomeric benzazepines **9** and **10**, were synthesized starting from the previously reported[32] amines **13** and **14** (Scheme 3). Thus, Schmidt rearrangement of 6-bromotetralone using a modification of the published procedure[32] afforded a mixture of the inseparable isomeric lactams **11** and **12**, which were reduced to the corresponding amines **13** and **14** then Boc protected. Derivatives **15** and **16** were then separated by chromatography, and each isomer was then subjected individually to lithiation and quenching with methyl chloroformate to give esters **15** and **16**, which were characterized and converted to hydroxamic acids by treatment with hydroxylamine hydrochloride in the presence of base as described previously.

Analogues in the thiophene series were accessed as illustrated in Schemes 4 and 5. Thus, 3-fluoro-4-formylpyridine was condensed with methyl 2-mercaptoacatate[33] to give **17** which was quaternized, hydrogenated, and demethylated to give the tetrahydro derivative **18**.[34] The 5-aza series derived from

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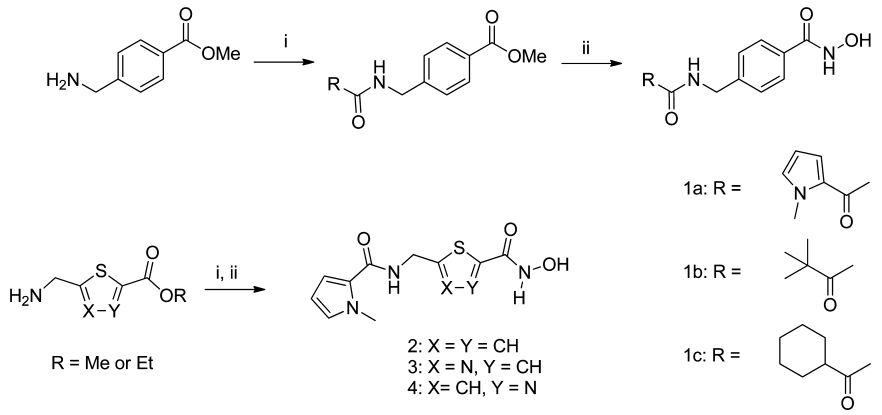
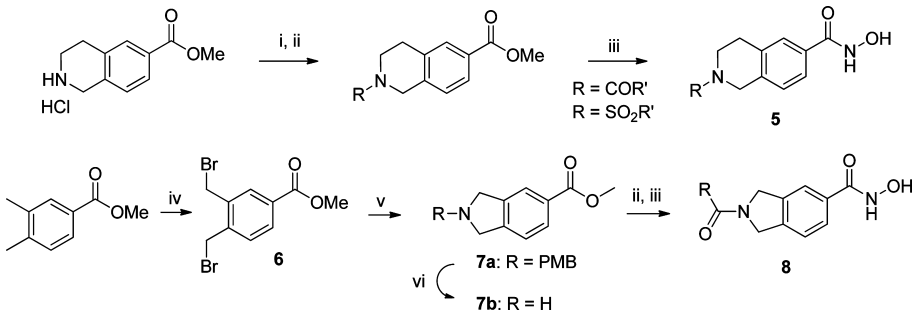
Scheme 1. Synthesis of 4-(Acylaminomethyl)-*N*-hydroxybenzamides[a]



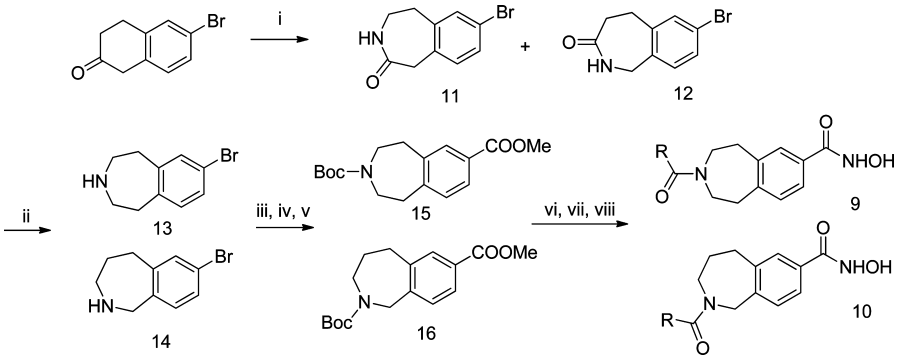
[a]Reagents and conditions: (i) RCOOH, HBTU, *N*-methylmorpholine, DMF; (ii) $NH_2OH{\cdot}HCl$, MeOH, KOH, 70 °C 4 h.

Scheme 2. Synthesis of Tetrahydroisoquinolines and Isoindolines[a]



[a]Reagents and conditions: (i) TFA, CH_2Cl_2; (ii) RCOOH, HBTU, *N*-methylmorpholine, dichloroethane or RSO_2Cl, DMAP, DMF; (iii) $NH_2OH{\cdot}HCl$, MeOH, KOH, 70 °C, 4 h; (iv) NBS, CCl_4, 12 h, 90%; (v) *para*-methoxybenzylamine, THF; (vi) Pd−C, HCl, MeOH.

Scheme 3. Synthesis of Tetrahydro-1*H*-benzazepines[a]



[a]Reagents and conditions: (i) $MeSO_3H$, NaN_3, 0−25 °C, 2 h; (ii) BH_3SMe_2, DME, 80 °C; (iii) Boc_2O, Et_3N, THF; (iv) silica gel chromatography; (v) (a) *t*-BuLi, THF, −78 °C, (b) ClCOOMe, THF, 25 °C; (vi) TFA, DCM; (vii) RCOOH, HATU, DIPEA, DMF; (viii) $NH_2OH{\cdot}HCl$, KOH, MeOH.

heterocycle **21** was prepared starting with Vilsmier formylation of Boc-piperidone[35] to give **20**. Condensation of **20** with methyl 2-mercaptoacatate[36] and deprotection afforded amine **21**, which was acylated and transformed to the corresponding hydroxamic acids as described previously.

The HDAC6 enzyme and cellular inhibitory activities as well as selectivity data for the configurationally constrained series are listed in Table 2. Tetrahydroisoquinoline **5a** exhibited slightly reduced inhibitory activity for HDAC6 in the enzymatic assay compared to the acyclic analogue **1a** but improved selectivity with respect to inhibition of class I enzymes present in nuclear extract as well as improved cellular activity (Ac-Tub assay). Further improvements to the selectivity were observed with analogues **5b−e**, in which the *N*-acetylpyrrole capping group was replaced by alternative hydrophobes albeit with small losses in HDAC6 inhibitory potency. The two examples of five-membered homologues were found to be less active and selective than their six-membered counterparts (compare **8a** with **5a** and **8b** with **5b**). The enzymatic and cellular HDAC6 inhibitory activities and selectivity of the seven-membered ring compounds **9a−b** and **10a−b** were lower than for series **5** and **8** and could not be compensated by further changes to the

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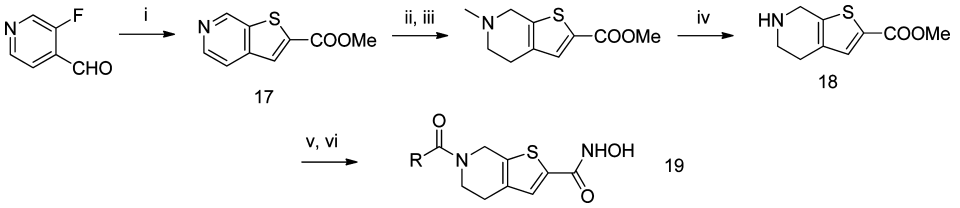
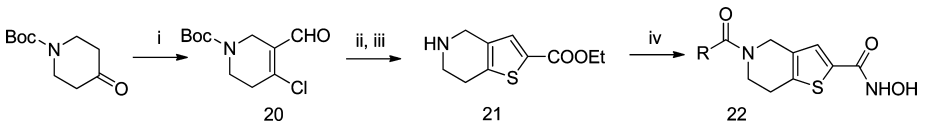
Scheme 4. Synthesis of Tetrahydrothieno[2,3-*c*]pyridines[a]



[a]Reagents and conditions: (i) $HSCH_2COOMe$, K_2CO_3, DMF, 82%; (ii) MeI, MeCN, 40 °C; (iii) H_2, PtO_2, MeOH, 95%; (iv) (a) MeCH(Cl)OCOCl, DIPEA, dioxane 0−70 °C, 3 h, (b) MeOH, reflux; (v) RCOOH, TBTU, DIPEA, DMF, DCM; (vi) $NH_2OH{\cdot}HCl$, KOH, MeOH, 80 °C, 2 h.

Scheme 5. Synthesis of Tetrahydrothieno[3,2-*c*]pyridines[a]



[a]Reagents and conditions: (i) $POCl_3$, DMF, DCM, 0 °C; (ii) $HSCH_2COOEt$, Et_3N, THF, reflux; (iii) TFA; (iv) RCOOH, HATU, Et_3N, DMF; (v) $NH_2OH{\cdot}HCl$, KOH, MeOH.

capping group (exemplified by **9e−f** and **10e−f**). Consistently, the most potent HDAC6 inhibitors, in both the enzymatic and cellular assays, were derived from tetrahydrothienopyridines (series **19** and **22**) with all the tabulated compounds showing IC_{50} values <50 nM. For example, the pyrrole capped compounds **19a** and **22a** are more potent than the analogous compounds shown in Table 2, including **1a** and **5a**. The improved potency was more dramatic in the case of the cellular data where **19a** and **22a** show IC_{50} values of 5 and 18 nM, respectively, while **5a** and **1a** exhibit IC_{50} values of 210 and 460 nM, respectively. However, compounds in series **19** and **22** were also more active inhibitors of class I HDACs as shown by the relatively low 14- and 30-fold selectivity ratios, respectively (compared to 500-fold for tetrahydroisoquinoline **5a**).

To probe subtype selectivity in more detail, several compounds were assessed across the full panel of individual HDACs and their inhibitory activities are compared with previously reported HDAC6 selective compounds in Table 3. These data confirm the significant improvement in selectivity of **5a** as compared to the acyclic analogue **1a** observed in the nuclear extract assay. Moreover, compound **5a** shows a selectivity profile considerably better than those reported in the literature for both vorinostat[26] and tubacin[16,25] and, in addition, shows negligible inhibition of matrix metalloproteases such as MMPs 2, 9, and 4 (>100 μM). In fact, the profile is comparable to that of tubastatin,[25] with HDAC8 the only isoform for which any significant inhibitory activity was observed (ca. 50-fold selectivity). This general selectivity profile was also observed for compounds **5b**, **8a**, **8b**, and **10a** (Table 3). Other properties of compounds in this series bode well for their use as biological tools and for potential further development, exemplified by compound **5a**, which showed high aqueous solubility (2 mM at pH 7.5)[37] and high Caco-2 permeability (22×10^{-6} cm s^{-1} at pH 7.4 with efflux ratio = 1.5).

Although the crystal structure of HDAC6 has not been reported, homology models of HDAC6 and HDAC1 have been employed to explain the selectivity of tubastatin A[25] and tubacin[29] in terms of the interactions of capping groups with the rim of the catalytic channels. We also developed a homology model of HDAC6, the catalytic channel of which is shown in Figure 2, left panel, and compared with that of HDAC7 (Figure 2, right panel) obtained from the published crystal structure.[27] While the HDACs are highly conserved,[29] some differences in the loop regions have been noted[38] which affect the geometry of the active site with the channel of HDAC6 that leads to the catalytic Zn^{2+} being wider and shallower than that of HDAC 7 (as well as the other subtypes). In consequence, HDAC6 can readily accommodate hydroxamic acids with benzylic spacers such as compound **5b**, whereas significant steric clashes are observed when **5b** is docked into the crystal structure of HDAC7 (Figure 2). In contrast, the flexibility of hydroxamic acids derived from long chain aliphatic spacers facilitates access to the active site channels of all the subtypes, accounting for the pan HDAC inhibitory activity of vorinostat and related compounds. The more subtle SAR trends summarized in Table 2 may be attributed to varying effectiveness of interaction between the hydrophobic capping group and amino acid residues in the rim of the catalytic channel. For example the *t*-butyl capping group of **5b** forms a near optimal interaction with the rim of the HDAC6 channel as shown in Figure 2, left panel).

CONCLUSION

A screen of our compound repository for HDAC6 inhibitory activity and selectivity against other HDAC subtypes identified derivatives of 4-(aminomethyl)-*N*-hydroxybenzamide as potent inhibitors of HDAC6 with improved selectivity compared to previously reported hydroxamates. Optimization of this chemotype in the case of fused ring analogues led to several subseries with further improved selectivities (up to 1000-fold vs nuclear extract and 40-fold vs HDAC8) compared to previously reported HDAC6 selective agents. Selectivity may be attributed to the benzylic spacer more effectively accessing the wider channel of HDAC6 compared to other HDAC subtypes as well as hydrophobic capping groups interacting with the protein surface near the rim of the active site. These biological tools are being investigated further to probe the function of HDAC6 in proteotoxic stress and its interactions between the ubiquitin proteasome system and aggresome pathways for protein degradation.

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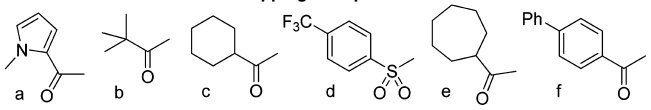
Table 2. HDAC6 Inhibitory Activity and Selectivity of Fused Ring Hydroxamates

Capping Groups



ring system	capping group	HDAC6 IC_{50} (nM)[a]	selectivity NucEx[b]/ HDAC6	Ac-Tub IC_{50} (nM)[c]
1	a	19	115	460
5	a	36	500	210
	b	52	1500	430
	c	50	730	540
	d	60	610	1300
	e	54	650	340
8	a	40	690	340
	b	89	340	560
9	a	130	480	1510
	b	120	340	580
	e	120	340	2000
	f	59	340	2600
10	a	260	>380	1480
	b	490	>200	3680
	e	340	>200	2900
	f	120	60	3090
19	a	28	14	5.0
	b	35	71	158
	c	20	50	50
22	a	24	30	18
	b	47	64	177
	c	36	47	162

[a]Enzymatic data obtained with recombinant HDAC6 or HeLa nuclear extracts using acetylated-Arg-Gly-Lys(Ac)-AMC as substrate with subsequent release of AMC by treatment with trypsin. IC_{50} values are means of two or more determinations. Standard deviation is within 20% of the IC_{50}. [b]Nuclear extract (NucEx) expresses predominantly HDACs 1 and 2. [c]Cellular data: Ac-tubulin accumulation was determined by immunofluorescence with an anti-Ac-tubulin antibody in cytosol. Ac-Lys accumulation in nuclear histones was measured by immunofluorescence in HeLa cells with all compounds showing <20% inh @ 20 μM.

EXPERIMENTAL SECTION

General. NMR spectra were recorded on a Bruker 300 MHz Avance1 or 400 MHz Avance2 using residual solvent peaks as the reference. Compound purity was determined by integration of the diode array UV trace of LC-MS chromatograms acquired on an Agilent 1100 LC interfaced to a micromass Waters Micromass Zspray mass detector (ZMD). Analyses were conducted using a water–MeCN gradient containing 0.1% formic acid (FA); all compounds were determined to be >95% pure. High-resolution mass spectra were recorded using a QSTAR XL quadrupole time-of-flight mass spectrometer (Applied Biosystems) coupled to an 1100 series HPLC system (Agilent Technologies). For each analysis, approximately five scans were summed and the centroid m/z value of the protonated monoisotopic molecular ion $[M + H]^+$ was recorded.

Synthetic Procedures. *N-(4-(Hydroxycarbamoyl)benzyl)-carboxamides (**1a–c, 2, 3, 4**).* A solution of methyl 4-aminomethylbenzoate (40 mg, 0.14 mmol) in DMF (3 mL) was treated with the appropriate carboxylic acid (0.15 mmol) followed by HBTU (57 mg, 0.15 mmol) and *N*-methylmorpholine (33 μL, 0.3 mmol) in a deep-well polypropylene plate. The wells were sealed and the plate shaken at ambient temperature for 16 h then concentrated under reduced pressure in a Genevac. The residues were partitioned between $CHCl_3$–THF (3:1) and aqueous 1N $NaHCO_3$ and the organic layer collected and combined with further extracts of the aqueous layer. After evaporation, the residue in each well was dissolved in anhydrous MeOH (2.5 mL) and treated with hydroxylamine hydrochloride (40 mg, 0.56 mmol) and powdered KOH (80 mg, 1.4 mmol) and heated at 70 °C for 4 h. The reaction mixtures were concentrated under reduced pressure and the residues treated with DMSO (1.4 mL), filtered, and the solution of crude product subjected to preparative HPLC on a C-18 column eluting with 10–60% MeCN–H_2O to give **1a** (7 mg, 19%). ^{1}H NMR (300 MHz, CD_3OD): δ 7.70 (m, 2H), 7.41 (m, 2H), 6.82 (m, 1H), 6.79 (dd, J = 4.0, 1.7 Hz, 1H), 6.05 (dd, J = 3.9, 2.6 Hz, 1H), 4.52 (s, 2H), 3.88 (s, 3H). ^{13}C NMR (100 MHz, CD_3OD, ppm) δ 168.04, 164.48, 144.96, 132.18, 129.56, 128.40, 128.31, 126.53, 114.19, 108.33, 43.32, 36.88. HRMS (ESI^+): calculated for $C_{14}H_{15}N_3O_3$ $[M + H]$ 274.1186; found 274.1182.

Compounds **1b–c** and **2–3** were prepared similarly from the respective methyl esters and compound **4** from the corresponding ethyl ester.

***1b** (9 mg, 26%).* ^{1}H NMR (400 MHz, CD_3OD ppm): δ 8.21 (br, s, 1H), 7.71 (d, J = 9.0 Hz, 2H), 7.36 (d, J = 9.0 Hz, 2H), 4.42 (m, 2H), 1.25 (s, 9H). ^{13}C NMR (100 MHz, CD_3OD) δ 181.48, 168.04, 144.81, 132.26, 128.31, 128.27, 43.72, 39.72, 27.88. HRMS (ESI^+): calculated for $C_{13}H_{18}N_2O_3$ $[M + H]$ 251.1390; found 251.1385.

***1c** (6 mg, 16%).* ^{1}H NMR (400 MHz, DMSO-d_6, ppm): δ, 8.24 (s, 1H), 7.67 (d, J = 9 Hz, 2H), 7.27 (d, J = 9 Hz, 2H), 4.08 (s, 2H), 2.16 (m, 1H), 1.69–1.57 (m, 4H), 1.36–1.15 (m, 6H). ^{13}C NMR (100 MHz, CD_3OD, ppm) δ 175.27, 165.59, 143.14, 129.14, 127.05, 126.77, 44.89, 41.47, 29,21, 25.42, 25.24. HRMS (ESI^+): calculated for $C_{15}H_{20}N_2O_3$ $[M + H]$ 277.1547; found 277.1541.

***2** (20 mg, 14%).* ^{1}H NMR (400 MHz, DMSO-d_6, ppm): δ, 10.72 (br, 1H), 8.28 (m, 1H), 7.81 (s, 1H), 7.04 (s, 1H), 6.57 (m, 1H), 6.53 (m, 1H), 6.41 (dd, J = 3.8, 1.6 Hz, 1H), 5.62 (s, 1H), 4.13 (m, 2H), 3.43 (s, 3H). ^{13}C NMR (100 MHz, CD_3OD, ppm) δ 164.25, 149.82, 129.67, 129.41, 126.77, 126.35, 114.38, 108.37, 101.42, 96.04, 38.82, 36.88. HRMS (ESI^+): calculated for $C_{12}H_{13}N_3O_3S$ $[M + H]$ 280.0750; found 280.0747.

***3** (7 mg, 9%).* ^{1}H NMR (400 MHz, CD_3OD ppm): δ 8.11 (s, 1H), 6.84 (m, 2H), 6.08 (dd, J = 3.8, 1.6 Hz, 1H), 4.74 (s, 2H), 3.89 (s, 3H). ^{13}C NMR (100 MHz, CD_3OD, ppm) δ 176.58, 164.43, 161.10, 143.98, 132.93, 130.04, 125.98, 114.79, 108.50, 41.95, 36.91. HRMS (ESI^+): calculated for $C_{11}H_{12}N_4O_3S$ $[M + H]$ 281.0703; found 281.0699.

***4** (8 mg, 20%).* ^{1}H NMR (400 MHz, CD_3OD, ppm) δ 7.58 (s, 1H), 6.90 – 6.69 (m, 2H), 6.06 (dd, J = 8.9, 4.4 Hz, 1H), 4.61 (s, 2H), 3.88 (s, 3H). ^{13}C NMR (100 MHz, CD_3OD, ppm) δ 164.50, 162.77, 159.49, 157.44, 129.63, 126.46, 121.14, 114.34, 108.34, 40.12, 36.86. HRMS (ESI^+): calculated for $C_{11}H_{12}N_4O_3S$ $[M + H]$ 281.0703; found 281.0698.

*General Synthesis of N-Hydroxy-2-(substituted-phenylsulfonyl)-1,2,3,4-tetrahydroisoquinoline-6-carboxamide Analogues (**5a–c, 5e**).* A suspension of methyl 1,2,3,4-tetrahydroisoquinoline-6-carboxylate hydrochloride (47 mg, 0.2 mmol) and the appropriate carboxylic acid (0.23 mmol) in anhydrous 1,2-dichloroethane (1.5 mL) was treated with HBTU (109 mg, 0.28 mmol) and *N*-methylmorpholine (34 μL, 0.3 mmol). The reaction mixtures in a polypropylene deep-well plate were sealed and shaken at ambient temperature for 16 h then concentrated under reduced pressure in a Genevac. The residues were partitioned between $CHCl_3$–THF (3:1) and aqueous 1N $NaHCO_3$ and the organic layer collected and combined with further extracts of the aqueous layer. After evaporation, the residue in each well was dissolved in anhydrous MeOH (2.5 mL) and the presence of the desired methyl ester confirmed by LC-MS analysis before treatment with hydroxylamine hydrochloride (30 mg, 0.44 mmol) and powdered KOH (50 mg, 0.87 mmol) and heating at 70 °C for 4 h. The reaction mixtures were concentrated under reduced pressure and

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Table 3. Comparison of HDAC Isoform Inhibitory Activity of Benzylic Spacer Derived Inhibitors with Reference Compounds

	IC_{50} (μM)[a] isoform										
compd	1	2	3	4	5	6	7	8	10	11	Ac-Tub[b] IC_{50} (μM)
vorinostat[c]	0.119	0.164	0.106	NR	NR	0.090	NR	1.50	0.072	NR	NR
tubacin[d,e]	1.40	6.27	1.27	17.3	3.35	0.004	9.70	1.27	3.71	3.79	2.9
tubastatin[e]	16.4	>30	>30	>30	>30	0.015	>30	0.854	>30	>30	ca. 2.5
1a	5.80	16.0	4.50	15.0	3.30	0.019	4.60	0.69	3.70	ND	0.460
5a	45.0	>50	46.0	>50	>50	0.036	>50	2.10	>50	>50	0.21
5b	>50	>50	>50	>50	>50	0.052	>50	2.10	>50	>50	0.43
8a	>50	>50	>50	>50	>50	0.040	>50	1.30	>50	>50	0.34
8b	>50	>50	>50	>50	>50	0.089	>50	1.30	>50	>50	0.56
10a	>50	>50	>50	>50	>50	0.260	>50	0.890	>50	>50	1.48

[a]Enzymatic data obtained with recombinant HDAC6 or HeLa nuclear extracts using acetylated-Arg-Gly-Lys(Ac)-AMC as substrate with subsequent release of AMC by treatment with trypsin. [b]Cellular data: Ac-tubulin accumulation was determined by immunofluorescence with an anti-Ac-tubulin antibody in cytosol. Ac-Lys accumulation in nuclear histones was measured by immunofluorescence in HeLa cells. [c]Data from refs 24 and 26. [d]Data from ref 16. [e]Data from ref 25; NR, not reported. ND, not determined.

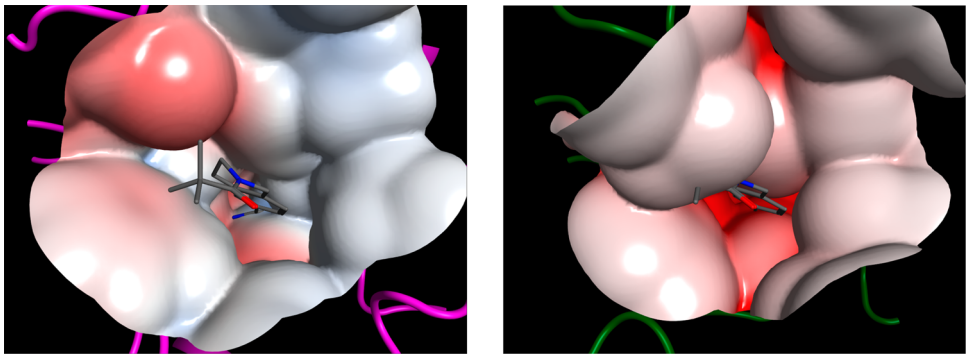

Figure 2. Compound **5b** docked into the active sites of HDAC6 from a homology model (left) and HDAC7 (right) from a crystal structure (PDB: 2PQO, 2.9 Å).[27] For the homology model, HDAC7 (PDB: 2PQO) was used as the primary template and additional crystal structures HDAC2 (PDB: 3MAX 2.0 Å), HDAC8 (PDB: 1T69 2.9 Å), and HDAH (PDB: 1ZZ1 1.57 Å) were used to improve loop modeling. Electrostatic color coding is red positive, blue negative, and gray neutral. See Supporting Information for further details.

the residues treated with DMSO (1.3 mL), filtered, and the solution of crude product subjected to preparative HPLC on a C-18 column eluting with 10−60% MeCN−H_2O to give:

***5a** (20 mg, 32%).* ^{1}H NMR (400 MHz, CD_3OD, ppm) δ 7.57−7.54 (m, 2H), 7.24 (br, 1H), 6.86 (br, 1H), 6.50 (dd, J = 3.8, 1.6 Hz, 1H), 6.12 (dd, J = 3.7, 2.7 Hz, 1H), 4.91 (s, 2H), 3.97 (t, J = 6.1 Hz, 2H), 3.74 (t, 3H), 3.00 (t, J = 6.1 Hz, 2H). ^{13}C NMR (100 MHz, CD_3OD, ppm) δ 167.94, 165.58, 138.40, 136.40, 132.05, 128.68, 128.20, 127.75, 126.19, 126.08, 114.31, 108.27, 35.76, 29.96. HRMS (ESI^+): calculated for $C_{16}H_{17}N_3O_3$ [M + H] 300.1348; found 300.1340.

***5b** (8 mg, 12%).* ^{1}H NMR (400 MHz, CD_3OD, ppm) δ 7.57 (m, 2H), 7.27 (d, J = 5.5 Hz, 1H), 4.82 (s, 2H), 3.91 (t, J = 6.1 Hz, 2H), 2.94 (t, J = 6.1 Hz, 2H), 1.32 (s, 9H). ^{13}C NMR (100 MHz, CD_3OD, ppm) δ 178.96, 168.04, 138.63, 136.27, 132.03, 128.62, 127.73, 126.10, 44.69, 39.96, 29.84, 28.58. HRMS (ESI^+): calculated for $C_{17}H_{19}N_3O_3$ [M + H] 314.1499; found 314.1498.

***5c** (14 mg, 20%).* ^{1}H NMR (400 MHz, CD_3OD, ppm) δ 7.58 (m, 2H), 7.32−7.27 (m, 2H), 4.82 and 4.73 (s, 2H combined, rotamers), 3.82 (m, 2H), 2.99 and 2.89 (s, 2H combined, rotamers), 2.76 (m, 1H), 1.84−1.72 (m, 4H), 1.51−1.23 (m, 6H). ^{13}C NMR (100 MHz, CD_3OD, ppm) δ 177.68, 177.43, 168.00, 138.60, 138.40, 136.73,136.25, 132.20, 131.92, 128.57, 128.41, 127.92, 127.66, 126.15, 45.47, 44.19, 42.08, 41.90, 42.13, 30.70, 30.57, 30.44, 29.33, 27.04, 26.75, 26.70 (doubling of some resonances due to rotamers). HRMS (ESI^+): calculated for $C_{15}H_{20}N_2O_3$ [M + H] 277.1547; found 277.1548.

***5e** (10 mg, 10%).* ^{1}H NMR (400 MHz, CD_3OD) δ 7.59 (t, J = 8.0 Hz, 2 H), 7.32 (m, 1H), 4.67 (s, 2H), 3.87 (m, 2H), 3.01 (m, 1H), 2.91 (m, 2H), 1.67 (m, 12H). ^{13}C NMR (100 MHz, CD_3OD) 178.74, 167.94, 138.67, 138.38, 136.75, 136.28, 132.24, 131.94, 128.58, 128.39, 127.91, 127.63, 126.20, 126.15, 44.38, 43.22, 43.07, 41.26, 32.49, 32.37, 30.62, 29.30, 29.20, 27.57, 27.70 (doubling of some resonances due to rotamers). HRMS (ESI^+): calculated for $C_{18}H_{24}N_2O_3$ [M + H] 317.1860; found 317.1852.

*N-Hydroxy-2-((4-(trifluoromethyl)phenyl)sulfonyl)-1,2,3,4-tetrahydroisoquinoline-6-carboxamide (**5d**).* A solution of methyl 1,2,3,4-tetrahydroisoquinoline-6-carboxylate hydrochloride (50 mg, 0.22 mmol) and *N*,*N*-dimethylaminopyridine (110 mg, 0.9 mmol) in DMF (3.2 mL) was treated with the appropriate sulfonyl chloride (0.22 mmol) and stirred at ambient temperature for 16 h. The solvent was removed under reduced pressure and the residue partitioned between DCE and saturated aqueous $NaHCO_3$ and the organic layer collected and combined with further extracts of the aqueous layer. The organic layer was dried ($MgSO_4$) and the presence of the desired methyl ester confirmed by LC-MS analysis [ES^+ (FA) 400] and treated with hydroxylamine hydrochloride (80 mg, 1.00 mmol) and powdered KOH (200 mg, 4.0 mmol) and heated at 70 °C for 4 h. The solvent was removed under reduced pressure and the residue was treated with DMSO (1.3 mL), filtered, and the solution of crude product subjected to preparative HPLC on a C-18 column eluting with 10−60% MeCN−H_2O to give **5d** (24 mg, 27%). ^{1}H NMR (400 MHz, CD_3OD, ppm) δ 8.08 (m, 2H), 7.88 (m, 2H), 7.50 (m, 2H), 7.20 (m, 1H), 4.40 (s, 2H), 3.44 (m, 1H), 2.95 (m, 2H). ^{13}C NMR (100 MHz, CD_3OD, ppm) δ 167.74, 141.98, 136.82, 135.65, 135.33, 134.99, 132.18, 129.63, 128.75, 127.86, 127.58, 126.07, 123.53, 48.61, 40.44, 29.60. HRMS calculated for $C_{17}H_{15}F_3N_2O_4S$ [M + H] 401.0777; found 401.0771.

*Methyl 2,3-Dihydro-1H-isoindole-5-carboxylate Hydrochloride (**7b**).* A mixture of methyl 3,4-dimethylbenzoate (1.33g, 8.1 mmol), *N*-bromosuccinimide (3.17g, 17.8 mmol), and benzoyl peroxide (0.14g, 0.58 mmol) in CCl_4 (5 mL) was heated under reflux for 1.5 h. After cooling to ambient temperature, the suspension was filtered

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and evaporated to give the crude bis-bromomethyl derivative which was redissolved in THF (30 mL) and treated with Et_3N (2.26 mL, 16.3 mmol), followed by 4-methoxybenzylamine (1.05 mL, 8.13 mmol) in THF (20 mL), and stirred at ambient temperature for 16 h. The reaction mixture was filtered, the solvent evaporated, and the crude product purified by flash chromatography on silica gel (hexane–EtOAc gradient) to give **7a** as a pale-yellow oil (1.19g, 49%). LC-MS ES^+ 298. ^1H NMR (400 MHz, $CDCl_3$, ppm) δ 7.89 (dd, J = 7.8, 1.5 Hz, 1H), 7.84 (s, 1H), 7.74 (d, J = 8.7 Hz, 2H), 7.31 (d, J = 8.7 Hz, 2H), 7.22 (d, J = 7.2 Hz, 2H), 6.89 (d, J = 8.7 Hz, 2H), 3.93 (s, 3H), 3.89 (s, 4H), 3.85 (s, 2H), 3.82 (s, 3H).

A solution of **7a** (0.82 g, 2.76 mmol) in MeOH (25 mL) was treated with 20% Pd on carbon (0.19 g) and conc HCl (0.41 mL) and stirred under an atmosphere of hydrogen gas for 48 h. The reaction mixture was filtered and the solvent evaporated to give **7b** (0.53 g, 91%). LC-MS ESI^+ 179. ^1H NMR (400 MHz, DMSO-d_6, ppm) δ 9.71 (br, s, 2H), 8.01 (s, 1H), 7.96 (d, J = 8.0 Hz, 1H), 7.55 (d, J = 7.7 Hz, 2H), 4.56 (d, J = 6.0 Hz, 4H), 3.87 (s, 3H).

*N-Hydroxy-2-(1-methyl-1H-pyrrole-2-carbonyl)isoindoline-5-carboxamide (**8a**).* A suspension of **7b** (40 mg, 0.19 mmol) and *N*-methylpyrrole-2-carboxylic acid (26 mg, 0.21 mmol) in DCM (2.5 mL) was treated with HATU (78 mg, 0.21 mmol) and *N*-methylmorpholine (0.1 mL, 0.91 mmol) and stirred at ambient temperature for 16 h. The resulting solution was washed with saturated aqueous $NaHCO_3$ and the organic layer dried ($MgSO_4$) and evaporated. The residue was redissolved in MeOH (3 mL) and treated with hydroxylamine hydrochloride (39 mg, 0.56 mmol) and powdered KOH (100 mg, 1.8 mmol) and heated at 70 °C for 2 h. The solvent was removed under reduced pressure, and the residue was suspended in DMSO, filtered, and subjected to preparative HPLC on a C-18 column eluting with 10–60% MeCN–H_2O to give **8a** (27 mg, 50%). ^1H NMR (400 MHz, CD_3OD, ppm) 7.72 (m, 2H), 7.45 (br, 1H), 6.88 (t, J = 2.1 Hz, 1H), 6.81 (dd, J = 3.8, 1.6 Hz, 1H), 6.17 (dd, J = 3.8, 2.6 Hz, 1H), 5.13, (br, 2H), 4.99 (br, 2H), 3.85 (s, 3H). ^{13}C NMR (100 MHz, CD_3OD, ppm) δ 167.88, 164.57, 133.32, 128.91, 127.78, 126.19, 124.09, 122.66, 115.73, 108.28, 55.83, 53.56, 36.73. HRMS (ESI^+): calculated for $C_{15}H_{15}N_3O_3$ [M + H] 286.1192; found 286.1183. Compound **8b** was prepared similarly (13 mg, 27%). ^1H NMR (400 MHz, CD_3OD) δ 7.60 (s, 1H), 7.58 (d, J = 7.9 Hz, 1H), 7.32 (d, J = 7.7 Hz, 1H), 5.05 (br, 2H), 4.74 (br, 2H), 1.25 (s, 9H). ^{13}C NMR (100 MHz, CD_3OD, ppm) δ 179.16, 167.94, 133.27, 127.71, 123.95, 122.63, 55.78, 54.35, 40.28, 27.81. HRMS (ESI^+): calculated for $C_{14}H_{18}N_2O_3$ [M + H] 263.1390; found 263.1385.

*Synthesis of Carboxymethylbenzazepines **15** and **16**.* The Schmidt rearrangement of 6-bromo-2-tetralone to give the mixture of lactams **11** and **12** was conducted using a modification of the method of Schultz and co-workers[32] as follows:

A solution of 6-bromo-2-tetralone (10 g, 0.044 mol) in $MeSO_3H$ (50 mL) was cooled in an ice bath and treated with sodium azide (3.61g, 0.055 mol) with stirring. The reaction mixture was allowed to warm to ambient temperature and stirred for a further 2 h then poured into a cold solution of 1 M aqueous KOH (800 mL) and extracted thoroughly with ethyl acetate. The extracts were washed with brine and evaporated to give a mixture of **11** and **12**, which was used without further purification. Reduction to amines **13** and **14**, Boc protection, and separation was also conducted as described.[32]

*2-tert-Butyl 7-Methyl 4,5-Dihydro-1H-benzo[c]azepine-2,7(3)-dicarboxylate (**16**).* A solution of the *N*-Boc-derivative of bromo compound **14** (0.37g, 1.13 mmol) in anhydrous THF (4 mL) was added dropwise to a solution of *tert*-butyllithium (0.92 mL of 1.6 M solution in pentane) in anhydrous THF (15 mL) at −78 °C. After 10 min, methylchloroformate (1.75 mL, 22.7 mmol) was added and the reaction mixture allowed to warm to ambient temperature and stirred for 30 min. The solvent was evaporated under reduced pressure and the residue partitioned between ethyl acetate (50 mL) and water (10 mL). The organic layer was combined with further extracts of the aqueous phase, washed with brine, dried ($MgSO_4$), and evaporated to give an oil that was purified by chromatography on silica gel eluting with 0–10% EtOAc in hexane to give **16** (0.14g, 41%). ^1H NMR (400 MHz, $CDCl_3$, ppm): δ 7.83–7.80 (m, 2H), 7.24 (d, J = 7.6 Hz, 1H), 4.45 (br, 1H), 4.40 (br, 1H), 3.90 (s, 3H), 3.68 (br, 2H), 3.00 (br, 2H), 1.78 (m, 2H), 1.37 (s, 9H).

*3-tert-Butyl 7-Methyl-4,5-dihydro-1H-benzo[d]azepine-3,7(2H)-dicarboxylate (**15**).* **15** was prepared similarly. ^1H NMR (400 MHz, $CDCl_3$, ppm) δ 7.82–7.79 (m, 2H), 7.24 (d, J = 7.6 Hz, 1H), 3.90 (s, 3H), 3.55 (br, 4H), 2.95 (br, 4H), 1.48 (s, 9H).

*N-Hydroxy-2-pivaloyl-2,3,4,5-tetrahydro-1H-benzo[c]azepine-7-carboxamide (**10b**).* A solution of **16** (0.44g, 0.145 mmol) in DCM (5 mL) was cooled in an ice bath and treated with 4 M HCl in dioxane (1.5 mL). The reaction mixture was allowed to warm to ambient temperature, stirred for 1 h, and the solvent removed to give the corresponding hydrochloride salt. LC-MS (FA) ES^+ 206. A solution of trimethylacetic acid (15.5 mg, 0.152 mmol), HATU (58 mg, 0.52 mmol), and Et_3N (60 μL, 0.43 mmol) in DCM (2 mL) and DMF (0.2 mL) was treated with a solution of the amine hydrochloride (35 mg, 0.145 mmol) in DMF (0.5 mL). The reaction mixture was stirred at ambient temperature for 6 h and partitioned between DCE and saturated aqueous $NaHCO_3$ solution. The aqueous phase was further extracted with DCE and the combined organic layers dried ($MgSO_4$) and evaporated. The crude ester was treated with MeOH (2.0 mL), KOH (56 mg, 1.0 mmol), and hydroxylamine hydrochloride (30 mg, 0.43 mmol) and heated at 80 °C for 1 h. The reaction mixture was then neutralized by addition of acetic acid, evaporated, and the crude product dissolved in DMSO and subjected to reverse phase chromatography on a C-18 column eluting with 20–45% MeCN in water to give **10b** (19 mg, 45%) as a white solid. ^1H NMR (400 MHz, CD_3OD, ppm) δ 7.55 (m, 1H), 7.49 (dd, J = 7.8, 1.8 Hz, 1H), 7.39 (d, J = 7.8, 1h), 4.57 (br, 2H), 4.01 (br, 2H), 3.08 (br, 2H), 1.86 (br, 2H), 1.21 (s, 9H). ^{13}C NMR (100 MHz, CD_3OD, ppm) δ 178.62, 168.11, 143.90, 143.21, 132.56, 131.25, 128.90, 125.75, 53.60, 53.45, 39.95, 35.49, 30.64, 28.73. HRMS calculated for $C_{16}H_{22}N_2O_3$ [M + H] 291.1709; found 291.1706.

The following compounds were prepared and purified similarly:

***10a** (42%).* ^1H NMR (400 MHz, CD_3OD, ppm) δ 8.08 (s, 1H), 7.57 (s, 1H), 7.48 (m, 2H), 6.75 (m, 1H), 6.28 (m, 1H), 6.06 (m, 1H), 4.78 (s, 2H), 4.00 (br, 2H), 3.11 (br, 2H), 1.88 (br, 2H). ^{13}C NMR (100 MHz, CD_3OD, ppm) δ 167.95, 143.87, 143.07, 132.88, 130.87, 129.28, 126.92, 125.90, 112.63, 108.13, 35.63, 35.21, 30.65. HRMS calculated for $C_{17}H_{19}N_3O_3$ [M + H] 314.1499; found 314.1497.

***10e** (21%).* ^1H NMR (400 MHz, DMSO-d_6, ppm) δ 11.13 (d, J = 13.7 Hz, 1H), 8.98 (s, 1H), 7.69–7.15 (m, 3H), 4.65 (s, 1H), 4.49 (s, 1H), 3.76 (m, 2H), 2.98 (m, 2H), 2.88–2.59 (m, 1H), 1.84–1.27 (m, 14H). ^{13}C NMR (100 MHz, DMSO-d_6) 175.4, 164.0, 141.8, 131.6, 129.5, 128.3, 127.8, 124.4, 51.2, 49.0, 34.3, 31.1, 29.2, 27.7, 27.0, 26.0. HRMS (ESI^+): calculated for $C_{19}H_{26}N_2O_3$ [M + H] 331.2016; found 331.2012.

***10f** (27%).* ^1H NMR (400 MHz, CD_3OD, ppm) δ 7.53 (m, 12H), 4.64 (s, 1H), 4.06 (m, 1H), 3.80 (s, 2H), 3.13 (m, 3H), 1.98 (s, 1H), 1.81 (m, 2H). ^{13}C NMR (100 MHz, CD_3OD, ppm) δ 172.73, 167.72, 143.96, 143.89, 142.65, 141.35, 136.01, 132.94, 131.25, 130.05, 129.23, 128.98, 128.61, 128.24, 128.07, 125.98, 62.69, 54.70, 51.62, 35.83, 35.49, 30.50, 28.28, 20.05 (multiple signals due to rotamers). HRMS (ESI^+): calculated for $C_{24}H_{22}N_2O_3$ [M + H] 387.1703; found 387.1701.

***9a** (20%), White Solid.* ^1H NMR (400 MHz, CD_3OD, ppm) δ 8.06 (s, 1H), 7.52 (m, 2H), 7.25 (d, J = 7.8 Hz, 1H), 6.78 (m, 1H), 6.36 (m, 1H), 6.07 (m, 1H), 3.84 (m, 4H), 3.56 (s, 3H), 3.06 (m, 4H). ^{13}C NMR (100 MHz, CD_3OD, ppm) δ 168.01, 166.18, 145.61, 142.06, 131.94, 130.96, 129.22, 127.66, 126.60, 126.52, 113.52, 108.27, 37.57, 35.41. HRMS calculated for $C_{17}H_{19}N_3O_3$ [M + H] 314.1499; found 314.1498.

***9b** (20%).* ^1H NMR (400 MHz, CD_3OD, ppm) δ 8.07 (s, 1H), 7.51 (m, 2H), 7.23 (d, J = 7.2 Hz, 1H), 3.75 (m, 4H), 3.02 (m, 4H), 1.25 (s, 9H). ^{13}C NMR (100 MHz, CD_3OD, ppm) δ 179.23, 168.07, 145.67, 142.04, 131.82, 130.94, 129.24, 126.43, 62.87, 40.17, 37.84, 37.73, 29.04. HRMS calculated for $C_{16}H_{22}N_2O_3$ [M + H] 291.1709; found 291.1712.

***9e** (17%).* ^1H NMR (400 MHz, CD_3OD, ppm) δ 7.53 (m, 2H), 7.25 (t, J = 8.3 Hz, 1H), 3.93 (m, 4H), 3.11- 2.91 (m, 4H), 2.84 (m, 1H), 1.87–1.35 (m, 12H). ^{13}C NMR (100 MHz, CD_3OD) 179.1,

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168.0, 145.5, 141.9, 131.1, 129.4, 126.5, 45.5, 42.8, 38.3, 37.3, 32.7, 29.3, 27.8. HRMS (ESI$^+$): calculated for $C_{19}H_{26}N_2O_3$ [M + H] 331.2016; found 331.2015.

***9f** (11%).* ^{1}H NMR (400 MHz, CD_3OD, ppm) δ 7.84−7.16 (m, 12H), 3.91 (m, 2H), 3.63 (m, 2H), 3.16 (m, 2H), 3.00 (m, 2H). ^{13}C NMR (100 MHz, CD_3OD) δ 173.40, 167.99, 143.99, 141.39, 136.19, 131.06, 130.05, 129.34, 128.99, 128.42, 128.28, 128.09, 126.59, 51.14, 45.62, 37.96, 37.12. HRMS (ESI$^+$): calculated for $C_{24}H_{22}N_2O_3$ [M + H] 387.1703; found 387.1705.

*Methyl Thieno[2,3-c]pyridine-2-carboxylate (**17**).* A solution of 3-fluoro-4-pyridinecarboxaldehyde (0.80 mL, 8.0 mmol) in DMF (15 mL) was cooled to 0 °C and treated with potassium carbonate (1.22 g, 8.83 mmol) and methyl 2-mercaptoacetate (0.75 mL, 8.43 mmol). After 30 min at 0 °C, the reaction mixture was allowed to warm to room temperature and stirred for 48 h. Water was added, and the precipitate that formed on cooling to 0 °C was collected and washed with cold water, affording **17** (1.55 g, 82%) as a white solid. LCMS: (FA) ES$^+$ 194. ^{1}H NMR (DMSO-d_6, 300 MHz, ppm) δ 9.37 (s, 1 H), 8.56 (d, J = 5.4 Hz, 1 H), 8.26 (d, J = 0.6 Hz, 1 H), 7.97 (dd, J = 5.4 Hz, 1.2 Hz, 1 H), 3.92 (s, 3 H).

*Methyl 6-Methyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-2-carboxylate (**18**).* A solution of methyl thieno[2,3-*c*]pyridine-2-carboxylate **17** (370 mg, 1.9 mmol) and methyl iodide (0.34 mL, 5.5 mmol) in acetonitrile (7.0 mL) was stirred at 40 °C for 6 h. Volatiles were then removed under reduced pressure and the residue treated with methanol (10 mL) and platinum dioxide (100 mg, 0.4 mmol) and stirred under an atmosphere of hydrogen gas (balloon pressure) for 4 h. The catalyst and solvent were then removed and the residue partitioned between EtOAc and saturated aqueous $NaHCO_3$ and the aqueous layer extracted with EtOAc three additional times. The combined organic phases were washed with saturated aqueous $NaHCO_3$, water, and brine, dried over anhydrous $MgSO_4$, and concentrated to give methyl 6-methyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-2-carboxylate (0.400 g, 95%) as an off-white solid. ^{1}H NMR ($CDCl_3$, 300 MHz, ppm) δ 7.50 (s, 1 H), 3.85 (s, 3 H), 3.64 (m, 2 H), 2.73 (m, 4 H), 2.48 (s, 3 H). LCMS: (FA) ES$^+$ 212.

Methyl 6-methyl-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-2-carboxylate (9.84 g, 46.6 mmol) in 1,4-dioxane (400 mL) was treated with *N*,*N*-diisopropylethylamine (81.1 mL, 0.47 mol). The solution was cooled to 0 °C and treated dropwise with α-chloroethyl chloroformate (50.2 mL, 0.47 mol). The reaction mixture was allowed to warm to ambient temperature and stirred for 3 h. The solvent was removed under reduced pressure, and the residue obtained was dissolved in methanol (300 mL) and heated to reflux for 3 h. Removal of the solvent under reduced pressure afforded a solid which was partitioned between dichloromethane and saturated aqueous $NaHCO_3$. The aqueous layer was further extracted with dichloromethane three times. The combined organic phases were washed with brine, dried over anhydrous $MgSO_4$, and concentrated. Purification by flash chromatography on silica gel (50% EtOAc−hexanes to 1% triethylamine−99% EtOAc to 1% MeOH−1% triethylamine−98% EtOAc) afforded **18** (5.08 g, 55%). LCMS: (FA) ES+ 198. ^{1}H NMR ($CDCl_3$, 300 MHz, ppm) δ 7.50 (s, 1 H), 4.04 (s, 2 H), 3.86 (*s*, 3 H), 3.12 (*t*, J = 6.0 Hz, 2 H), 2.66 (*t*, J = 6.0 Hz, 2 H).

*6-(2,2-Dimethylpropanoyl)-N-hydroxy-4,5,6,7-tetrahydrothieno-[2,3c]pyridine-2 carboxamide (**19b**).* To a solution of HBTU (38.4 mg, 0.101 mmol) and trimethylacetic acid (10.4 mg, 0.101 mmol) in methylene chloride (2.0 mL) was added triethylamine (28 μL, 0.203 mmol). The reaction mixture was stirred at room temperature for 30 min. A solution of methyl 4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-2-carboxylate (20.0 mg, 0.101 mmol) in methylene chloride added, and the solution was then stirred at room temperature for 16 h then heated at 36 °C for 1 h. Upon cooling to room temperature, the reaction mixture was diluted with DCM and washed with saturated aqueous $NaHCO_3$. The aqueous layer was extracted with DCM, and the combined organic phases were washed with water, brine, dried over anhydrous $MgSO_4$, and concentrated to afford an oil which was treated with methanol (1.5 mL), hydroxylamine hydrochloride (28 mg, 0.41 mmol), and powdered potassium hydroxide (46 mg, 0.81 mmol). The reaction mixture was heated at 80 °C for 2 h, cooled to room temperature, and the solvent evaporated under reduced pressure. The crude product was dissolved in DMSO and subjected to preparative reverse phase HPLC on a C-18 column eluting with 15−45% MeCN in H_2O to give **19b** (3.51 mg, 12%) as a white solid. ^{1}H NMR (400 MHz, DMSO) δ 7.35 (s, 1H), 4.74 (s, 2H), 3.80 (t, J = 5.7 Hz, 2H), 2.66 (t, J = 5.3 Hz, 2H), 1.22 (s, 9H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 175.50, 159.55, 136.50, 134.58, 134.52, 127.22, 44.00, 42.58, 38.29, 27.95, 25.22. HRMS (ESI$^+$): calculated for $C_{13}H_{18}N_2O_3S$ [M + H] 283.1111; found 283.1107.

*N-Hydroxy-6-(1-methyl-1H-pyrrole-2-carbonyl)-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-2-carboxamide (**19a**).* **19a** was prepared similarly. Yield: 20%. ^{1}H NMR (DMSO-d_6, 400 MHz, ppm) δ 7.37 (*s*, 1 H), 6.94 (m, 1 H), 6.44 (m, 1H), 6.06 (m, 1H), 4.83 (*s*, 2 H), 3.86 (*t*, J = 5.9 Hz, 2 H), 3.68 (s, 3H), 2.51 (t, J = 5.9 Hz, 2 H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm) δ 162.43, 159.69, 136.07, 134.70, 127.26, 126.80, 124.67, 112.23, 106.70, 99.50, 45.62, 35.04, 25.35. HRMS (ESI$^+$): calculated for $C_{14}H_{15}N_3O_3S$ [M + H] 306.0912; found 306.0901.

*6-(Cyclohexylcarbonyl)-N-hydroxy-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-2-carboxamide (**19c**).* **19c** was prepared similarly and purified by preparative HPLC on a C-18 column eluting with 20−50% MeCN−H_2O. Yield: 36%. ^{1}H NMR (CD_3OD, 300 MHz, ppm): δ 7.30 (s, 1 H), 4.75 (s, 2 H), 3.83 (t, J = 6.0 Hz, 2 H), 2.65 (m, 3 H), 1.74 (m, 6 H), 1.41 (m, 4 H). ^{13}C NMR (100 MHz, CD_3OD, ppm) δ 177.44, 163.15, 138.47, 138.07, 136.89, 135.87, 129.35, 129.21, 46.00, 44.14, 42.15, 41.86, 41.06, 30.73, 30.48, 27.48, 27.02, 26.72, 26.63, 26.07 (multiple signals due to rotamers). HRMS (ESI$^+$): calculated for $C_{15}H_{20}N_2O_3S$ [M + H] 309.1267; found 309.1265.

*Ethyl 4,5,6,7-Tetrahydrothieno[3,2-c]pyridine-2-carboxylate (**21**).* *N*-Boc protected piperidone was chloroformylated using a Vilsmer−Hack reaction to afford compound **20**, which was treated with ethylmercaptoacetate in the presence of excess triethylamine to give **21**.[22,23] Removal of the Boc-protecting group under acidic conditions yields was conducted as follows:

A solution of 5-*tert*-butyl 2-ethyl-6,7-dihydrothieno[3,2-*c*]pyridine-2,5(4*H*)-dicarboxylate (1.00 g, 3.21 mmol) in DCM (15 mL) was treated with trifluoroacetic acid (1.48 mL, 19.3 mmol) dropwise at 0 °C. The solution was allowed to warm to ambient temperature then stirred for 1 h. The reaction mixture was partitioned between saturated aqueous $NaHCO_3$ solution (10 mL) and DCM (10 mL) and the aqueous phase extracted further with DCM. The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, and concentrated to give ethyl 4,5,6,7-tetrahydrothieno[3,2-*c*]pyridine-2-carboxylate **21** (0.547 g, 81%) as an oil. LCMS: (FA) ES$^+$ 212.

*N-Hydroxy-5-pivaloyl-4,5,6,7-tetrahydrothieno[3,2-c]pyridine-2-carboxamide (**22b**).* A solution of HATU (104.0 mg, 0.274 mmol) and trimethylacetic acid (28.0 mg, 0.274 mmol) in methylene chloride (2.0 mL) was treated with triethylamine (104 μL, 0.203 mmol) and stirred at room temperature for 30 min. A solution of ethyl 4,5,6,7-tetrahydrothieno[3,2-*c*]pyridine-2-carboxylate (**21**) (20.0 mg, 0.101 mmol) in DMF (0.5 mL) was next added and the solution stirred at ambient temperature for 16 h. The reaction mixture was diluted with DCM and washed with saturated aqueous $NaHCO_3$. The aqueous layer was extracted with further with DCM, and the combined organic phase was washed with water, brine, dried over anhydrous $MgSO_4$, and concentrated to afford an oil which was treated with methanol (2.0 mL), hydroxylamine hydrochloride (52.1 mg, 0.75 mmol), and powdered potassium hydroxide (84.2 mg, 1.5 mmol). The reaction mixture was heated at 80 °C for 3 h and the solvent evaporated under reduced pressure. The residue was dissolved in DMSO (1.4 mL) and purified by preparative reverse phase HPLC on a C-18 column eluting with 15−40% MeCN in H_2O to give a white solid (16 mg, 24%). ^{1}H NMR (DMSO-d_6, 400 MHz, ppm): δ 11.13 (*s*, 1 H), 9.08 (s, 1H), 7.37 (*s*, 1 H), 4.56 (*s*, 2 H), 3.83 (*t*, J = 5.8 Hz, 2 H), 2.83 (*t*, J = 5.4 Hz, 2 H), 1.22 (*s*, 9 H). ^{13}C NMR (100 MHz, DMSO-d_6, 100 MHz, ppm) δ 175.69, 159.57, 137.69, 134.37, 133.39, 125.60, 44.50, 42.75, 38.29, 27.97, 25.05. HRMS (ESI$^+$): calculated for $C_{13}H_{18}N_2O_3S$ [M + H] 283.1111; found 283.1108.

*N-Hydroxy-5-(1-methyl-1H-pyrrole-2-carbonyl)-4,5,6,7-tetrahydrothieno[3,2-c]pyridine-2-carboxamide (**22a**).* **22a** was

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prepared similarly. ^{1}H NMR (400 MHz, CD_3OD) δ 7.30 (br, 1H), 6.87 (m, 1H), 6.50 (m, 1H), 6.13 (m, 1H), 4.79 (s, 2H), 4.04 (t, 2H), 3.75 (s, 3H), 3.02 (t, 2H). ^{13}C NMR (100 MHz, CD_3OD) 169.68, 165.82, 140.30, 135.00, 134.18, 128.35, 127.23, 126.03, 114.27, 108.32, 47.90, 35.74, 26.44. HRMS (ESI^+): calculated for $C_{14}H_{15}N_3O_3S$ [M + H] 306.0912; found 306.0903.

*5-(Cyclohexanecarbonyl)-N-hydroxy-4,5,6,7-tetrahydrothieno-[3,2-c]pyridine-2-carboxamide (**22c**).* **22c** was prepared similarly (15 mg, 19%). ^{1}H NMR (400 MHz, CD_3OD, ppm) δ 7.32 (s, 1H), 4.67 (s, 2H), 3.87 (s, 2H), 2.96 (s, 1H), 2.82 (m, 1H), 2.74 (m, 1H), 1.74 (m, 4H), 1.57−1.07 (m, 6H). ^{13}C NMR (100 MHz, DMSO-d_6), 174.0, 159.5, 138.0, 134.5, 133.0, 125.6, 45.8, 44.7, 41.9, 29.1, 25.7, 25.6, 25.1. HRMS (ESI^+): calculated for $C_{15}H_{20}N_2O_3S$ [M + H] 309.1267; found 309.1266.

Biological Assays. *HDAC Enzymatic Assays.* Enzymatic data were obtained using recombinant HDAC6 or HeLa nuclear extract effecting deacetylation of Ac-Arg-Gly-Lys(Ac)-AMC substrate with subsequent release of AMC with trypsin in a modification of the procedure of Wegener[39] as follows:

Recombinant HDAC6 (N-GST-tagged, BPS Bioscience, San Diego, CA) was prepared at a concentration of 1 nM in 25 mM Tris-Cl, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, and 0.1 mg/mL BSA at pH 8.0 and was incubated in black 384-well plates with 8 μM Ac-Arg-Gly-Lys(Ac)-AMC substrate (Bachem Biosciences, King of Prussia, PA) and a 10-point 1:3 serial dilution of test compound in DMSO (in duplicate, 2% final DMSO concentration) for 1 h at 30 °C. The reaction was stopped by the addition trichostatin A (Sigma) and bovine trypsin (tosyl phenylalanyl chloromethyl ketone-treated, Sigma) to final concentrations of 1 μM and 10 μg/mL, respectively, in 50 mM Tris-Cl, 137 mM NaCl, 2.7 mM KCl, and 1 mM $MgCl_2$, pH 8.0. After incubation for an additional 30 min at 30 °C, the release of AMC from the deacetylated lysine by trypsin was quantitated on a Pherastar fluorescence plate reader (BMG Labtech, Cary, NC) set at λ_{ex} 340 nm and λ_{em} 460 nm. Concentration−response curves were generated from the fluorescence decrease in test compound-treated samples relative to DMSO-treated controls, and IC_{50} values were calculated using Pipeline Pilot (Accelrys, San Diego, CA) and Condoseo (Genedata, Lexington, MA) software.

Similarly, 30 μg/mL HeLa nuclear extract (Enzo Life Sciences, BML KI-140, containing primarily class I enzymes HDAC 1 and 2 as per manufacturer) was incubated with 20 μM of the acetylated substrate as described above, using a modified reaction buffer consisting of 50 mM Tris-Cl, 137 mM NaCl, 2.7 mM KCl, and 1 mM $MgCl_2$, pH 8.0, and the assay conducted as described previously.

Counter-screen enzyme inhibition assays for the individual isoforms were performed by the Reaction Biology Corporation, Malvern, PA (www.reactionbiology.com). The HDACs 1, 2, 4, 5, 7, 8, 9, 10, and 11 assays used baculovirus expressed recombinant human protein; HDAC1 full length GST tag, HDAC2 and three full length C-terminal 6 X His tagged; HDAC4 aa 627-1085, N-terminal GST tag; HDAC5 aa 657-1123, C-terminal 6 X His tag; HDAC7, aa 518-end, N-terminal GST tag; HDAC8 full length, C-terminal 6 X His tag; HDAC9 aa 604-1066, C-terminal 6 X His tag; HDAC10, aa1-481, N-terminal GST tag; HDAC 11, full length, N-terminal GST tag. The substrate used for HDACs 1, 2, 3, 10, and 11 assays is a fluorogenic, acetylated peptide substrate based on residues 379−382 of p53, i.e., Arg-His-Lys-Lys(Ac). The substrate used for HDAC8 is a fluorogenic, diacetylated peptide substrate based on residues 379−382 of p53, i.e., Arg-His-Lys(Ac)-Lys(Ac). The substrate used for HDACs 4, 5, 7, and 9 assays was fluorogenic, acetyl-Lys(trifluoroacetyl)-AMC. Compounds were dissolved in DMSO and tested in 10-dose IC_{50} mode with 3-fold serial dilution starting at 30 μM in 50 mM Tris-HCl, pH 8.0, 127 mM NaCl, 2.7 mM KCl, and 1 mM $MgCl_2$ containing 1 mg/mL BSA. Plates were sealed and incubated at 30 °C for 2 h. Developer was then added to stop the reaction, and kinetic measurements were recorded for 1.5 h at 15 min intervals on a fluorescence plate reader set at λ_{ex} 360 nm and λ_{em} 460 nm. End point data (after plateau of signal) were used for subsequent data analysis. The control trichostatin A (TSA) was tested in a 10-dose IC_{50} with 3-fold serial dilution starting at 5 μM. IC_{50} values were extracted by curve-fitting the dose/response slopes.

Cellular Immunofluorescence Assays. HDAC6 activity was determined by measuring K40 hyperacetylation of α-tubulin with an acetylation selective monoclonal antibody. Selectivity against class I HDAC activity was determined similarly using an antibody that recognizes nuclear acetylation in the immunofluorescence assay as follows:

Cell Culture. HeLa human tumor cells were obtained from ATCC and were maintained in a humidified chamber at 37 °C with 5% CO_2. HeLa cells were grown in Minimum Essential Media (MEM) (Invitrogen), supplemented with 10% FBS, 1% glutamine, 1 mM sodium pyruvate, and 1 mM nonessential amino acids.

Acetylated Tubulin and Acetylated Histone Assays. HeLa cells (8000/well) were grown on 96-well cell culture dishes. The cells were treated with compound diluted in DMSO in 3-fold serial dilutions with concentrations ranging from 20 to 0.003 μM for the acetylated tubulin and acetylated histone for 6 and 24 h, respectively. Compounds at each dilution were added as triplicates across three plates. Cells treated with DMSO (three wells per plate, N = 9; 0.2% final concentration) served as the untreated control, while cells treated with 20 μM tubacin served as the maximum inhibition control (three wells per plate, N = 9). Following treatment, the cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 10 min, and washed in PBS. Fixed cells were treated with blocking reagent (Roche no. 11096176001) for 1 h at room temperature. Cells were stained with antiacetylated tubulin (1:5000; Sigma no. T6793) or antiacetyl lysine (abcam no. ab21623) in blocking reagent for 60 min at room temperature. The cells were washed with PBS and PBS with 0.05% Tween 20 and stained with Alexa 488-conjugated goat antimouse IgG (1:500, Molecular Probes no. A11029) or Alexa 488-conjugated antirabbit IgG (1:500, Molecular Probes no. A11034, and Hoechst (1:50,000; Molecular Probes no. H-3570) in blocking reagent for 1 h at room temperature. The cells were washed with PBS. Immunofluorescent cells were visualized using an Opera high content imaging system (Perkin-Elmer). Images from nine sites per well were captured at 20× magnification. Inhibition of HDAC6 was determined by measuring fluorescent intensity of acetylated tubulin using Pipeline Pilot software. Concentration response curves were generated by calculating the increase of acetylated-tubulin fluorescent intensity in compound treated samples relative to the DMSO and tubacin treated controls, and 50% inhibitory concentrations (IC_{50}) values were determined from those curves.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

IC_{50} curves for enzymatic experiments involving compound **5a**. Experimental procedures for the Caco-2 and turbidometric solubility assays. Description of homology and molecular modeling. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

HDAC, histone deacetylase; MTOC, microtubule organizing center; NucEx, HeLa nuclear extract; Ac-Tub, acyl tubulin; Boc,

tert-butoxycarbonyl; DIEA, *N*,*N*-diisopropylethylamine; DMAP, 4-(dimethylamino)pyridine; HBTU, *O*-benzotriazole, *N*′,*N*′,*N*′,*N*′-tetramethyluronium hexafluorophosphate; DCE, 1,2-dichloroethane

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