Brief Report

Novel *N*-hydroxybenzamide histone deacetylase inhibitors as potential anti-cancer agents

Jie Jiao, Hao Fang, Wenfang Xu*

Department of Medicinal Chemistry, School of Pharmaceutical Sciences, Shandong University, Ji'nan, China.

ABSTRACT: Histone deacetylases (HDACs) are a class of Zn^{2+} dependent metalloproteases that play an important role in tumorigenesis. Inhibition of HDACs may be a potential strategy for cancer therapy. This study designed and synthesized a series of novel *N*-hydroxybenzamide histone deacetylase inhibitors based on the structural features of suberoylanilide hydroxamic acid (SAHA), the first HDAC inhibitor that came to market. Preliminary biological evaluation *in vitro* found that most of the inhibitors had satisfactory inhibitory activity (IC_{50} = 1-17 μM) against HDACs and HCT116 tumor cells.

Keywords: HDACs, Inhibitors, *N*-hydroxybenzamide, Antitumor

1. Introduction

Cancer is the most dreaded killer of humans and takes a number of lives every year. In recent years, a class of metalloproteases known as histone deacetylases (HDACs) has been considered as an epigenetic target for cancer therapy. These enzymes play an important role in gene transcription, the cell cycle, differentiation, and tumorigenesis. Inhibitors of these key enzymes are inducers of growth arrest and apoptosis of many tumor cells, so HDAC inhibitors are considered to be a new generation of anti-cancer agents (*1*).

HDACs are involved in modification and remodeling of chromosomal histones by removing acetyl groups from ε-NH_2 of lysine residues in histones through Zn^{2+}-dependent hydrolysis (Figure 1). Deacetylation results in the positive charge density on the *N*-termini of nucleosomal histones increasing, which strengthens the interaction with the negatively charged DNA chain and blocks the access of transcription factors. In tumor cells, HDACs are over-expressed, resulting in deacetylated histones being tightly packed with DNA to form an abnormal "compact structure" of chromatin. In this process, expression of the onco-suppressors $p21^{WAF1}$ and $p27^{KIP1}$ is inhibited and activity of the onco-suppressor p53 is down-regulated, but tumor activators HIF-1 and VEGF are up-regulated. Therefore, the inhibition of HDAC activity is considered a potential strategy for cancer therapy (*2-4*).

Suberoylanilide hydroxamic acid (SAHA), a linear chain hydroxamic acid and the first HDAC inhibitor, was approved by the US FDA in 2006 (*5*). As reported in the literature, there are two rules to the structure-activity relationship (SAR) of SAHA derivatives: 1) introduction of hydrophobic groups to the para-position of the benzene ring results in a higher level of activity, and 2) the optimal chain length between the benzene ring and zinc-binding group (ZBG) is 7-8 atoms (Figure 2) (*6-8*). *N*-hydroxy-4-(3-phenylpropanamido)benzamide (**HPPB**) (*9*) and its cinnamamide analogue provide the molecular formwork for the design of novel *N*-hydroxybenzamide HDAC inhibitors. This design strategy has been shown in Figure 2; ferulic acid, a natural cinnamic acid, was used as the starting material to prepare the **HPPB** derivatives and their cinnamamide analogues.

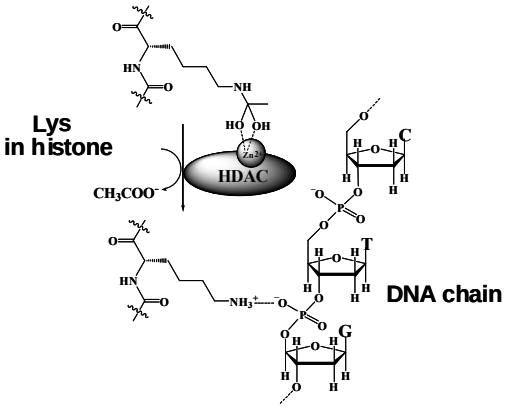


Figure 1. HDACs function of modification of histones.

**Correspondence to:* Dr. Wenfang Xu, The Department of Medicinal Chemistry, School of Pharmacy, Shandong University, 44 West Wenhua Road, Ji'nan 250012, Shandong, R.P. China;
e-mail: xuwenf@sdu.edu.cn

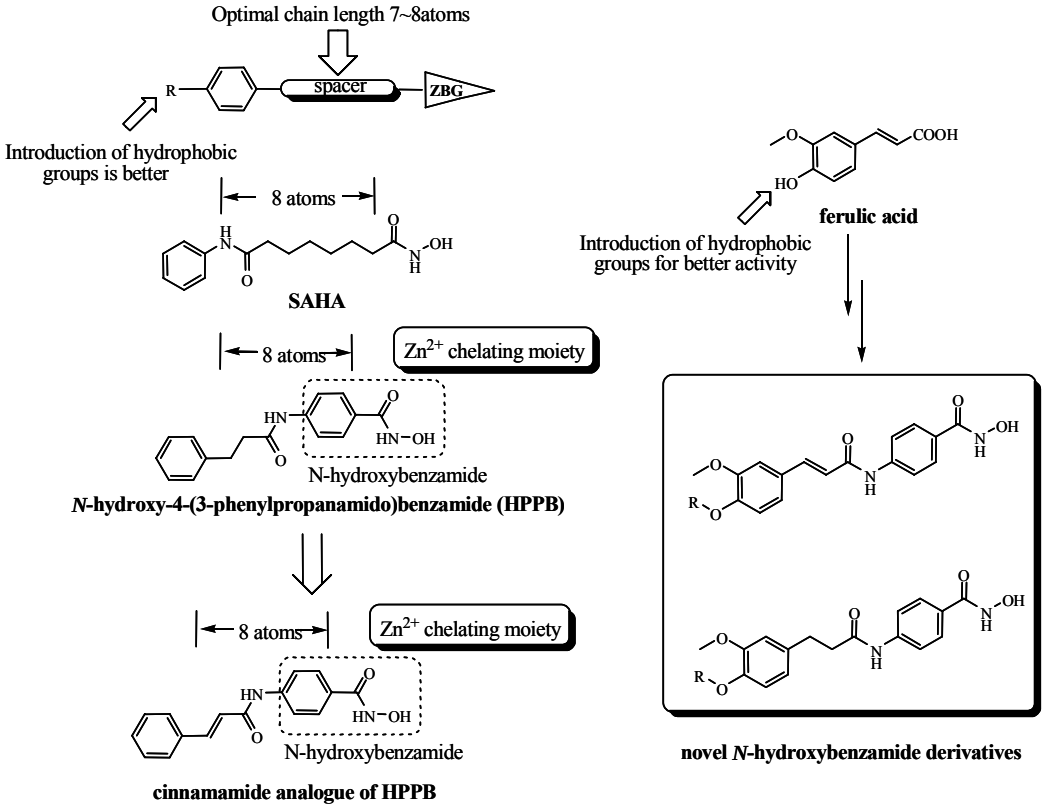


Figure 2. Design of novel *N*-hydroxybenzamide derivatives.

2. Materials and Methods

All material and reagents used in this work are analytical reagents. All reactions were monitored by thin-layer chromatography on 0.25 mm silica gel plates (60GF-254) and visualized with UV light. ^{1}H NMR spectra were determined on a Brucker Avance 300 spectrometer using TMS as an internal standard. ESI-MS were determined on an API 4000 spectrometer. Melting points were determined on an electrothermal melting point apparatus and are uncorrected. The route of synthesis has been shown in Scheme 1.

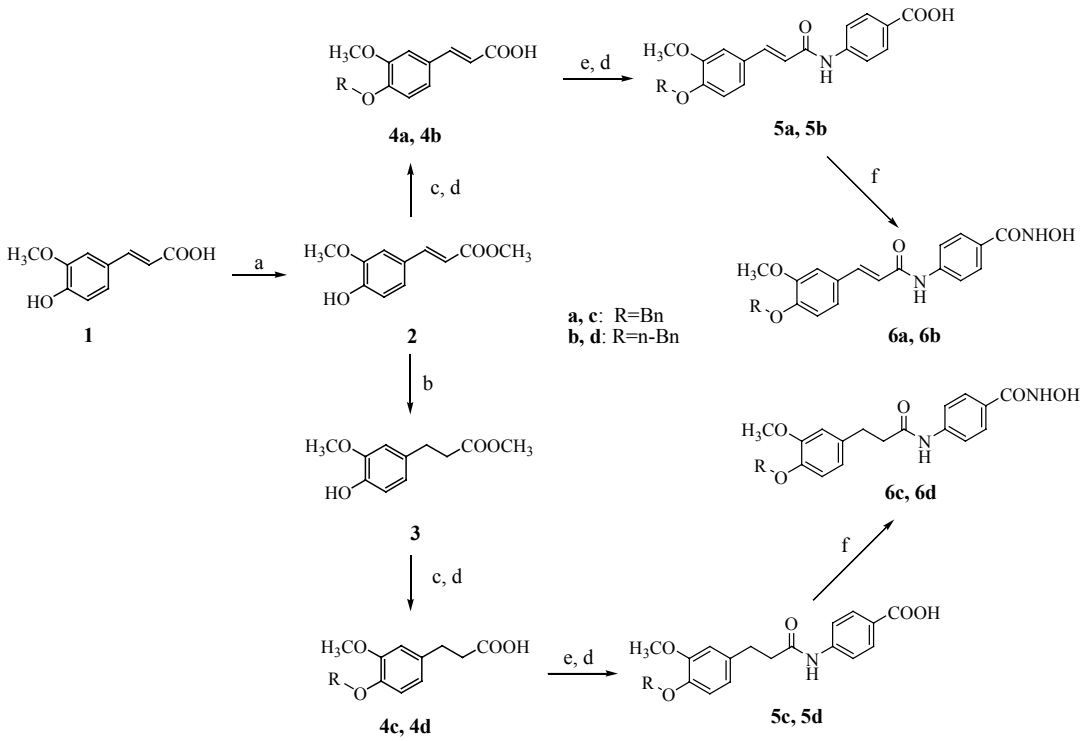
Esterification of ferulic acid **1** with *p*-methyl phenylsulfonic acid (PTS) in methanol yielded methyl ferulic acid **2**, which was reacted with BnBr in DMF by Williamson etherification and then hydrolyzed to provide compound **4a**. Compound **4a** was reacted with $SOCl_2$ to produce acylation of methyl 4-aminobenzoate, and then hydrolysis was performed to yield compound **5a**. Compound **5a** was reacted with ClCOOBu-*i* to provide a mixed anhydride and then reacted with NH_2OH to yield compound **6a**. Compound **6b** was prepared from compound **2** in the same way as compound **6a** using the *n*-bu substituted 4'-OH of compound **2**. In addition, hydrogenation of compound **2** yielded **3**, which provided compound **6c** and **6d** in the same way as compounds **6a** and **6b**.

3. Results and Discussion

In vitro bioactivity evaluation of compounds **6a-6d** was performed by HDAC activity assays using a HDAC colorimetric activity assay kit (AK501, Biomol Research Laboratories) and MTT assays of human colonic cancer cells (HCT116) because HDACs are highly expressed in this cell type.

3.1. *Procedures for HDAC activity assay*

The source of HDACs was HeLa nuclear extracts including HDAC1 and HDAC2 (the major contributors to HDAC activity in HeLa nuclear extracts), and the substrate was a type of [^{3}H]acetylated histone peptide. HDAC1 and HDAC2 are known to both be nucleus proteins in charge of the deacetylation of histones (*10*). Assays we performed according to kit instructions. The compound samples and the control drug were diluted to various concentrations: 20 μg/mL, 4 μg/mL, 0.8 μg/mL, and 0.16 μg/mL. On the 96-well plate, HDACs (5 μL/well) were incubated at 37°C with 10 μL of various concentrations of samples and 25 μL of substrate. After reacting for 30 min, Color de Lys Developer (50 μL/well) was added. Then, after 15 min the ultraviolet absorption of the wells was measured on a microtiter-plate reader at 405 nm. The % inhibition was calculated from the ultraviolet



Scheme 1. Synthetic scheme, reagents and conditions: (a) PTS, CH_3OH, 80°C; (b) H_2, 10% Pd-C, CH_3OH; (c) RBr, KOH, DMF, r.t.; (d) 2 mol/L NaOH, EtOH, 75°C; (e) $SOCl_2$, THF, methyl 4-aminobenzoate; (f) ClCOOBu-*i*, Et_3N, NH_2OH•HCl, THF, 0°C.

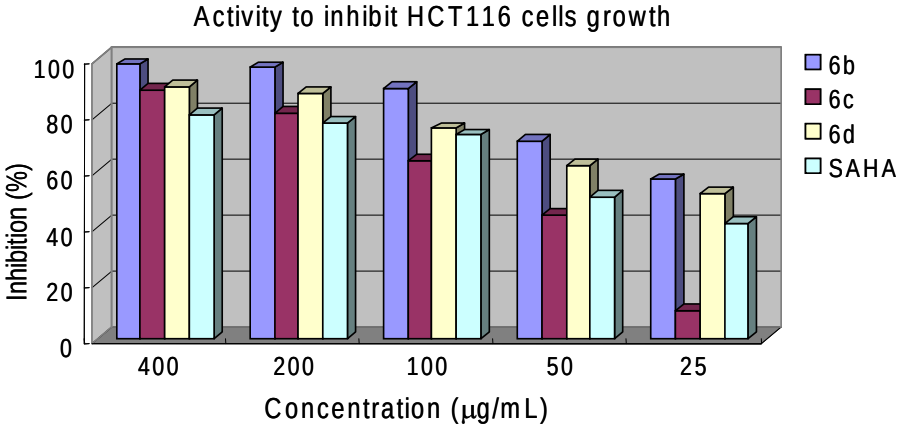


Figure 3. Activity to inhibit HCT116 cells growth of compounds **6b-6d** compared to SAHA.

absorption readings of inhibited wells relative to those of control wells. Finally, the IC_{50} values were determined using a regression analysis of the concentration/inhibition data.

3.2. *Procedures for the MTT assay*

The cells, being maintained in McCoy's 5a medium with 10% fetal bovine serum, were plated in 96-well plates (50 μL/well) at the density of 2.0×10^5/mL. After 4 h, compounds of various concentrations (400 μg/mL, 200 μg/mL, 100 μg/mL, 500 μg/mL, and 25 μg/mL) were dosed, and the cells were cultured for 2 days. Then 0.5% MTT (10 μL/well) were added to each well. After an additional 4 h of incubation, OD_{570} and OD_{630} were measured as a reference, and the IC_{50} values were calculated according to a regression analysis of the concentration/inhibition data.

3.3. *Inhibitory activity of compounds **6a~6d***

Results indicating the activity of compounds **6a-6d** are shown in Table 1. These compounds all had satisfactory

Table 1. Inhibitory activities of compounds **6a-6d** against HDACs and tumor cells

Compounds	HDACs IC_{50} (μM)	HCT 116 IC_{50} (μM)
6a	12.7	>1000
6b	16.4	175.6
6c	1.8	208.8
6d	4.0	193.6
SAHA	1.3	245.6

activity (IC_{50} = 1-17 μM) at inhibiting HDACs. In general, **HPPB** derivatives **6c** and **6d** were more potent than their cinnamamide analogues **6a** and **6b**. With the exception of **6a**, compounds exhibited a higher level of activity at inhibiting HCT116 cell growth than SAHA (Figure 3). Compound **6b** (IC_{50} = 16.4 μM) showed less potency than other compounds, but among these compounds it exhibited the highest level of activity at inhibiting HCT116 cell growth. The **HPPB** derivatives **6c** and **6d** both showed exciting bioactivity *in vitro* and could be used as leading compounds to guide further study on **HPPB** derivatives in the future.

4. Conclusion

A series of novel *N*-hydroxybenzamide HDAC inhibitors was designed and synthesized based on the structural features of SAHA. Preliminary biological evaluation *in vitro* found that most of these inhibitors showed satisfactory activity at inhibiting HDACs and HCT116 cell growth. These compounds could be used as leading compounds in the future.

Acknowledgments

The authors wish to thank Xuejian Wang for his work on the MTT assays of these compounds. This work was supported by the National "863" Foundation of China (Grant No. 2007AA02Z314).

References

1. Saverio M, Pier GP. Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. Nature 2006; 6:38-51.
2. Dong HK, Minjung K, Ho JK. Histone deacetylase in carcinogenesis and its inhibitors as anti-cancer agents. J Biochem Mol Biol 2003; 36:110-119.
3. Marks PA, Rifkind RA, Richon VM, Breslow R, Miller T, Kelly WK. Histone deacetylases and cancer: causes and therapies. Nat Rev Cancer 2001; 1:194-202.
4. Meinke PT, Liberator P. Histone deacetylase: a target for antiproliferative and antiprotozoal agents. Curr Med Chem 2001; 8:211-235.
5. Marielle P, Marina P, Monica B, Daniela F. Histone deacetylase inhibitors: from bench to clinic. J Med Chem 2008; 51:1505-1529.
6. Manfred J, Gerald B, Doris K, Hans S, Clarissa G, Peter L. Amide analogues of Trichostatin A as inhibitors of histone deacetylase and inducers of terminal cell differentiation. J Med Chem 1999; 42:4669-4679.
7. Stacy WR, Lidia CS, Peter A, Kenneth WB, Wendy DC, Michael AG, Kobporn LH, Manfred J, Paul K, Nancy T, Heather W. Inhibitors of human histone deacetylase: synthesis and enzyme and cellular activity of straight chain hydroxamates. J Med Chem 2002; 45:753-757.
8. Soon HW, Sylvie F, Elie AK, Giliane B, Arkadii V, Naomy B, Oscar M, Silvana L,Martin Al, Marielle F, Zuomei L, Jeffrey M. Besterman, Daniel D. Structurally simple Trichostatin A-like straight chain hydroxamates as potent histone deacetylase inhibitors. J Med Chem 2002; 45:2877-2885.
9. Qiang L, Da SW, Chang SC, Yuan DH, Ching SC. Structure-based optimization of phenylbutyrate-derived histone deacetylase inhibitors. J Med Chem 2005; 48:5530-5535.
10. Annemieke J M, Albert H, Huib NC, Stephan K. Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem Soc 2003; 370:737-749.

(*Received July 23, 2008; Revised August 1, 2008; Accepted August 11, 2008*)

Supplementary data

Synthesis of compound **6c**, general procedures, analytical data of the compounds **6a-6d**.

*(E)-Methyl 3-(4-hydroxy-3-methoxyphenyl)acrylate (**2**)*

Ferulic acid (30.0 g, 0.15 mol) was dissolved in dry MeOH (300 mL), then PTS (5.0 g, 0.03 mol) was added. The solution was heated to 80°C to be in reflux for 6 h, and then it was concentrated under vacuum. The residue was added by water (100 mL) and then extracted by EtOAc (100 mL) for 3 times. The organic layer was merged and washed by water and then dried with $MgSO_4$. The solution was evaporated to give a yellow crystal product (31.0 g, yield: 96.3%). mp 53-55°C, ESI-MS m/z: 209.4 $(M+H)^+$.

*Methyl 3-(4-hydroxy-3-methoxyphenyl)propanoate (**3**)*

Compound **2** (25.0 g, 0.12 mol) was dissolved in MeOH (240 mL), then 10% Pd-C (0.5 g) was added. The solution was stirred at room temperature and filled with H_2 in one atm and for 12 h. The reaction mixture was filtered off Pd-C, and the filtrate was evaporated to get a diaphanous oil product (25.0 g, yield: 99.0%). ESI-MS m/z: 211.3 $(M+H)^+$, 228.4 $(M+NH_4)^+$, 233.3 $(M+Na)^+$.

*3-(4-Butoxy-3-methoxyphenyl)propanoic acid (**4c**)*

Compound **3** (2.1 g, 0.01 mol) was dissolved in dry DMF (20 mL), then KOH powder (0.62 g, 0.012 mol) was added. The solution was stirred for 1h at room temperature, then *n*-BuBr (1.7 g, 0.01 mol) was dropped into reaction mixture. After stirred for another 5 h, the reaction mixture was diluted by 100 mL water. Then the solution was extracted by ether (50 mL) for 3 times. The organic layer was merged and washed by water and then dried with $MgSO_4$. The solution was evaporated to give a yellow solid product which was dissolved in EtOH (50 mL), then 2 mol/L NaOH (10 mL) was added. The solution was stirred at 75°C for 3 h, and then concentrated under vacuum. The residue was added by 1 mol/L HCl (50 mL), then the solution was

filtered to get the sediment which was washed by water for several times. Recrystallisation of the sediment with acetone to get white crystal product 1.3 g. Yield: 45.5%, mp 90-91°C, ESI m/z $(M+H)^+$ 287.4, $(M+NH_4)^+$ 304.5, $(M+Na)^+$ 309.6, $(M+K)^+$ 325.5. ^{1}H NMR (DMSO-d_6, δ ppm, J Hz): 12.10 (s, 1H), 7.44-7.29 (m, 5H), 6.92-6.68 (m, 3H), 5.02 (s, 2H), 3.75 (s, 3H), 2.75 (t, 2H, 7.5 Hz), 2.50 (t, 2H, 7.5 Hz).

*4-(3-(4-(Benzyloxy)-3-methoxyphenyl) propanamido) benzoic acid (**5c**)*

Compound **4c** (1.0 g, 3.5 mmol) was dissolved in dry THF (50 mL), the solution was stirred under 0°C and then $SOCl_2$ (1.2 g, 10 mmol) was dropped into the solution. After 5 h, the reaction mixture was evaporated to get an oil product of acyl chloride which was dissolved in new dry THF (20 mL). The methyl 4-aminobenzoate (0.53 g, 3.5 mmol) was dissolved in dry THF (20 mL), the Et_3N (0.7 g, 7 mmol) was added into the solution, then the acyl chloride solution was dropped into solution. The reaction mixture was stirred at room temperature for 1 h evaporated under vacuum. The residue was added by water (100 mL) and extracted by EtOAc (50 mL) for 3 times. The organic layer was merged and washed by water and then dried with $MgSO_4$. The solution was evaporated to give a yellow crude product which was dissolved in EtOH (25 mL), and then 2 mol/L NaOH (5 mL) was added. The reaction mixture was stirred at 75°C for 5 h and then evaporated under vacuum. The residue was added by 1 mol/L HCl (30 mL) and then filtered. The sediment was washed by water for several times and recrystallized with acetone to get white crystal product (1.1 g, yield: 78.6%). Mp: 165-168°C, ESI-MS m/z: 406.4 $(M+H)^+$.

*4-(3-(4-(Benzyloxy)-3-methoxyphenyl)propanamido)-N-hydroxybenzamide (**6c**)*

Compound **5c** (1.0 g, 2.5 mmol) was dissolved in dry THF (30 mL), then Et_3N (0.50 g, 5 mmol) was added. The solution was cooled to 0°C and then ClCOOBu-*i* (0.34 g, 2.5 mmol) was dropped into the solution. The reaction mixture was stirred under 0°C for 5 min and then NH_2OH/MeOH (2.5 mL, 2 mmol/mL) was dropped into the reaction mixture. After stirred for 5 h, the reaction mixture was filtered and the filtrate was evaporated under vacuum. The residue was added by 1 mol/L HCl (30 mL) and extracted by EtOAc (20 mL) for 3 times. The organic layer was merged and washed by water and then dried with $MgSO_4$. The solution was evaporated to give a crude product which was recrystallized with acetone to get a white crystal product 0.3 g, (yield: 28.6%). Mp: 193-195°C, ESI-MS m/z: 421.4 $(M+H)^+$, IR (KBr, σ cm^{-1}): 3266, 1674, 1513, and 1256, ^{1}H NMR (DMSO-d_6, δ ppm, J Hz): 10.09 (s, 1H), 10.12 (s, 1H), 8.94 (s, 1H), 7.71-7.62 (m, 4H), 7.44-7.29 (m, 5H), 6.92 (d, 1H, J = 8.1 Hz), 6.88 (s, 1H, J = 8.1 Hz), 6.73 (d, 1H, J = 8.1 Hz), 5.02 (s, 2H), 3.73 (s, 3H), 2.85 (t, 2H, J = 7.5 Hz), 2.62 (t, 2H, J = 7.5 Hz).

*4-(3-(4-Butoxy-3-methoxyphenyl)propanamido)-N-hydroxybenzamide (**6d**)*

Mp: 163-165°C, ESI-MS m/z: 387.4 $(M+H)^+$, IR (KBr, σ cm^{-1}): 3302, 1668, 1514, and 1255, ^{1}H NMR (DMSO-d_6, δ ppm, J Hz): 11.10 (s, 1H), 10.13 (s, 1H), 8.95 (s, 1H), 7.71-7.62 (m, 4H), 6.85-6.82 (m, 2H), 6.72 (d, 1H, J = 8.1 Hz), 3.88 (t, 2H, J = 6.6 Hz), 3.71 (s, 3H), 2.84 (t, 2H, J = 7.5 Hz), 2.62 (t, 2H, J = 7.5 Hz), 1.70-1.61 (m, 2H), 1.47-1.35 (m, 2H), 0.91 (t, 3H, J = 7.5 Hz).

*(E)-4-(3-(4-(Benzyloxy)-3-methoxyphenyl)acrylamido)-N-hydroxybenzamide (**6a**)*

Mp: 214-215°C, ESI-MS m/z: 419.4 $(M+H)^+$, IR (KBr, σ cm^{-1}): 3247, 1597, 1512, and 1258, ^{1}H NMR (DMSO-d_6, δ ppm, J Hz): 11.10 (s, 1H), 10.34 (s, 1H), 8.94 (s, 1H), 7.74 (s, 4H), 7.56 (d, 1H, J = 15.6 Hz), 7.47-7.34 (m, 5H), 7.26-7.09 (m, 3H), 6.72 (d, 1H, J = 15.6 Hz), 5.15 (s, 2H), 3.84 (s, 3H).

*(E)-4-(3-(4-Butoxy-3-methoxyphenyl)acrylamido)-N-hydroxybenzamide (**6b**)*

Mp: 230-233°C, ESI-MS m/z: 385.5 $(M+H)^+$, IR (KBr, σ cm^{-1}): 3181, 1648, 1596, 1511, and 1263, ^{1}H NMR (DMSO-d_6, δ ppm, J Hz): 11.10 (s, 1H), 10.33 (s, 1H), 8.94 (s, 1H), 7.74 (s, 1H), 7.55 (d, 2H, J = 15.6 Hz), 7.22-7.00 (m, 3H), 6.71 (d, 1H, J = 15.6 Hz), 4.01 (t, 2H, J = 6.6 Hz), 3.83 (s, 3H), 1.76-1.68 (m, 2H), 1.44 (q, 2H, J = 7.2 Hz), 0.94 (t, 3H, J = 7.2 Hz).