Synthesis and Biological Evaluation of 3-(4-Substituted-phenyl)-*N*-hydroxy-2-propenamides, a New Class of Histone Deacetylase Inhibitors

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Inhibitors of histone deacetylases (HDACs) have been shown to induce differentiation and/or apoptosis of human tumor cells. Novel 3-(4-substituted-phenyl)-*N*-hydroxy-2-propenamides have been prepared as a new class of HDAC inhibitors and evaluated for their antiproliferative activity and HDAC inhibitory activity. Incorporation of a 1,4-phenylene carboxamide linker, shown by **5**, and a 4-(dimethylamino)phenyl or 4-(pyrrolidin-1-yl)phenyl group as a cap substructure generated highly potent hydroxamic acid-based HDAC inhibitors **5a** and **5b**.

Introduction

Acetylation and deacetylation of histones of the core proteins of nucleosomes in chromatin play an important role in the regulation of gene expression.[1−3] The level of acetylation of histones is established and maintained by two classes of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs), which have been identified as transcriptional coactivators and transcriptional corepressors, respectively.[4,5] There is increasing evidence that aberrant histone acetylation has been linked to various malignant diseases.[6−8]

HDACs are zinc hydrolases that modulate deacetylation of the ϵ-*N*-acetyl groups of lysine residues in the *N*-terminal tails of core histones. Natural products such as trichostatin A (TSA) (**1**, Chart 1),[9] trapoxin B,[10] and depsipeptide[11] strongly inhibit HDACs, induce differentiation of cancer cells, and suppress cell proliferation. Depsipeptide is now under clinical evaluation; however, TSA and trapoxin B have not been developed clinically owing to their instability in vivo and toxicity.[12] A number of the hydroxamic acid-based synthetic inhibitors, such as suberoylanilide hydroxamic acid (SAHA) (**2**),[13−15] oxamflatin (**3**)[16] and others,[17−22] and the anilide-based synthetic inhibitors, such as MS-275 (**4**)[23,24] and its analogues,[25] have also been reported to induce differentiation of cancer cells and inhibit cell proliferation. Among them, SAHA[14,15] and MS-275[23] show potent in vivo antitumor effects in tumor-bearing animals and are now under clinical evaluation. Crystal structures of a bacterial HDAC homologue (HDAC-like protein, HDLP) bound to TSA and SAHA reveal that the hydroxamic acid functionality penetrates a narrow, hydrophobic channel and chelates a buried zinc ion.[26] HDAC inhibitors typically possess a metal-binding functionality, a cap substructure that interacts with amino acids at the entrance rim of the *N*-acetyllysine binding channel, and a linker connecting the cap and the metal-binding functionality.[26] A careful analysis of the structure−activity relationships (SAR) on the hydroxamic acid- and the anilide-based HDAC inhibitors suggests that the 1,4-phenylene carboxamide linker shown as the target compounds **5** would be the most optimized one.[13,16−22,24,25] In addition to this assumption, we further refined the cap substructure with 4-substituted phenyl or pyridyl groups. Since TSA has a dimethylamino group at the 4-position of the phenyl ring, we were particularly interested in introducing an acyclic or a cyclic tertiary amine at the same position. Thus, dimethylamino, pyrrolidinyl, and 4-substituted-piperazinyl groups were chosen for this purpose. In general, hydroxamates have poor bioavailability;[27,28] therefore, we adopted a pyridyl ring as a cap substructure, which would allow better physicochemical properties due to its both lipophilic and hydrophilic character, as shown in MS-275.

Results and Discussion

Chemical Synthesis. The hydroxamic acids **5a**−**d** were prepared as shown in Scheme 1. Coupling of the carboxylic acids **6a**−**c** with an amino alcohol **7**[29] by using either bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BOP−Cl) and Et_3N in CH_2Cl_2 (for **8a**) or 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole (HOBT) in the presence of DMAP in pyridine (for **8b** and **8c**) gave the amides **8a**−**c**. Swern oxidation of the hydroxy group of **8a**−**c** with $(COCl)_2$ and DMSO in THF followed by Wittig reaction of the resulting aldehydes with (carbethoxymethylene)-triphenylphosphorane in CH_3CN afforded the 2-propenoic acid esters **9a**−**c**. Coupling of picolinic acid (**6d**)

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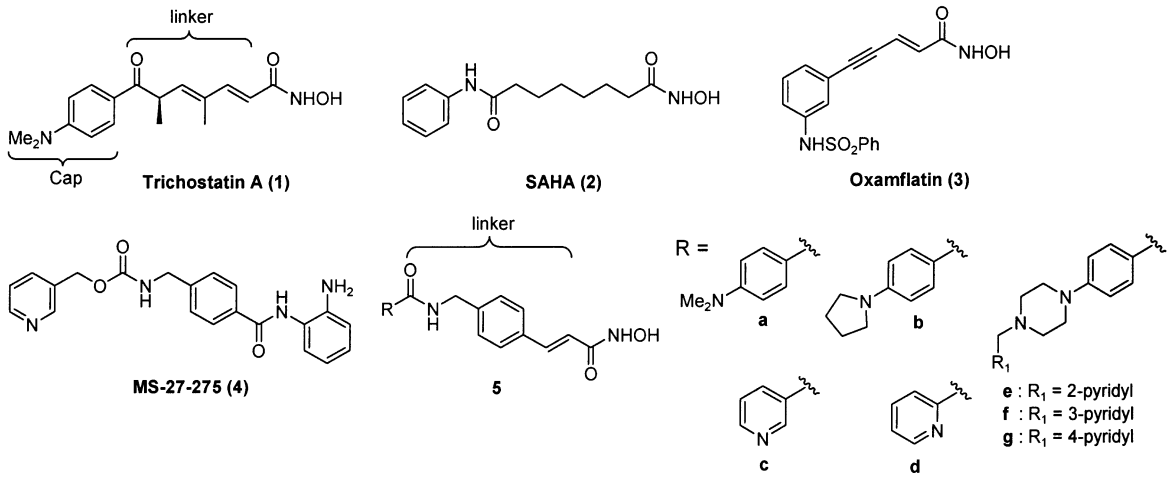
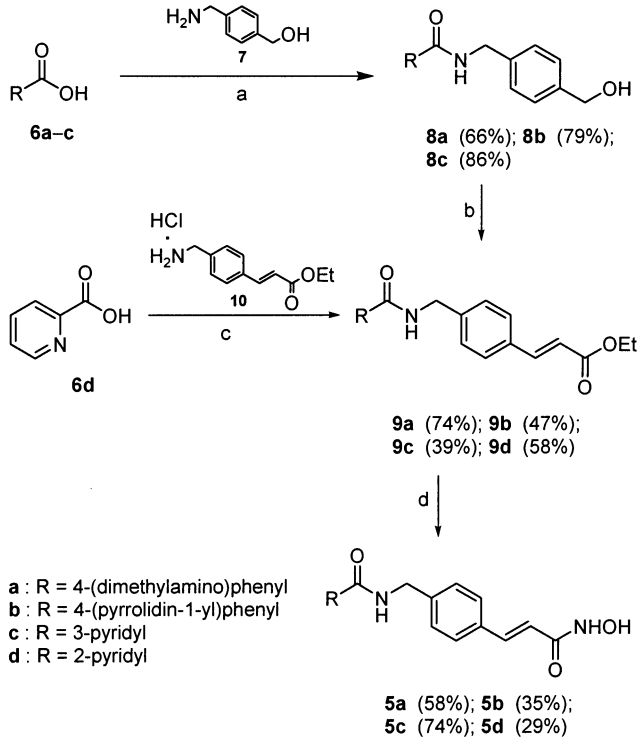
Chart 1

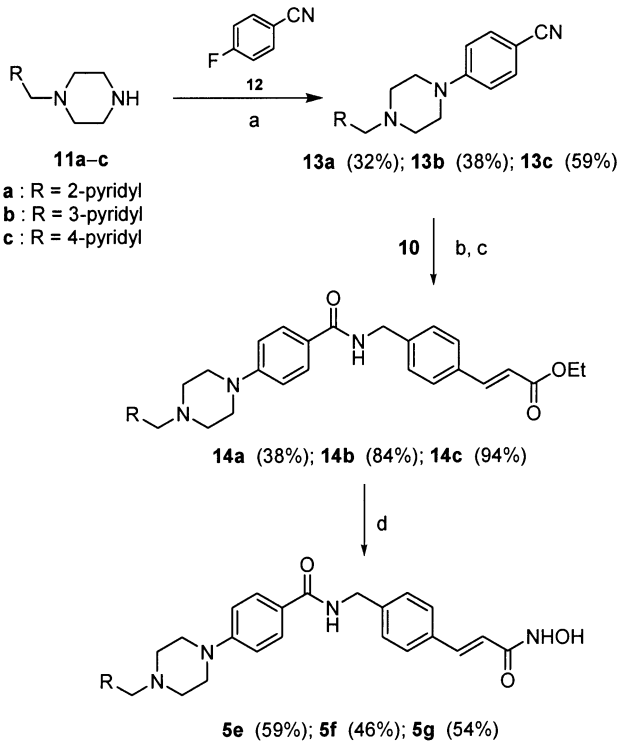


Scheme 1[a]



[a] (a) BOP−Cl, Et_3N, CH_2Cl_2, rt, overnight (for **8a**), or EDC, HOBT, DMAP, pyridine, rt, overnight (for **8b** and **8c**); (b) (i) $(COCl)_2$, DMSO, THF, −78 °C, 1 h, then warmed to −35 °C, 10 min, then cooled to −78 °C, add Et_3N, 0 °C, 1 h; (ii) $Ph_3P{=}CHCO_2Et$, CH_3CN, 70 °C, 2 h; (c) EDC, DMAP, DMF, rt, 4 h; (d) NH_2OH, MeOH, rt, overnight.

Scheme 2[a]



[a] (a) anhyd K_2CO_3, DMF, 80 °C, overnight; (b) conc HCl, 80 °C, overnight; (c) EDC, HOBT, DMAP, pyridine, rt, overnight; (d) NH_2OH, MeOH, rt, overnight.

with an amino ester **10**[30] under similar reaction conditions as for **8b** and **8c** produced the ester **9d**. Treatment of **9a**−**d** with NH_2OH in MeOH yielded the hydroxamic acids **5a**−**d**. The hydroxamic acids **5e**−**g** were synthesized in four steps, as shown in Scheme 2. Reaction of 1-(pyridylmethyl)piperazines **11a**−**c**[31] with 4-fluorobenzonitrile (**12**) in the presence of anhydrous K_2CO_3 in DMF provided the benzonitriles **13a**−**c**. Hydrolysis of **13a**−**c** with concentrated HCl followed by coupling with **10** under the same reaction conditions as for **8b** and **8c** afforded the esters **14a**−**c,** which were treated with NH_2OH in MeOH to give the hydroxamic acids **5e**−**g**.

Biological Evaluation. Antiproliferative activities of the new HDAC inhibitors **5a**−**g** against human lung cancer (A549), breast cancer (SK-BR-3), and stomach cancer (MKN45) cell lines were evaluated using the MTT assay[32,33] (spectrophotometric quantification of viable cells as measured by mitochondrion-catalyzed formation of a formazan from a tetrazolium salt) in comparison to TSA, oxamflatin, and MS-275. The results are given as IC_{50} values and are shown in Table 1. The 4-(dimethylamino)phenyl compound **5a** and the 4-(pyrrolidin-1-yl)phenyl compound **5b** showed better antiproliferative activity against all the three cancer cell

Table 1. Antiproliferative and HDAC Inhibitory Activities of Compounds 5a−g, Oxamflatin, TSA, and MS-275

compd	IC_{50} (μM)[a,b] A549	SK-BR-3	MKN45	HDAC inhibn[c] (nM)[b]
5a	0.48 ± 0.13	0.16 ± 0.06	0.83 ± 0.28	172.02 ± 8.22
5b	0.35 ± 0.22	0.11 ± 0.01	0.80 ± 0.27	205.27 ± 8.46
5c	2.89 ± 0.31	1.28 ± 0.23	4.98 ± 0.44	370.97 ± 4.54
5d	2.23 ± 0.99	0.87 ± 0.04	3.64 ± 1.74	941.61 ± 7.98
5e	11.98 ± 3.97	3.19 ± 0.95	38.59 ± 15.85	
5f	1.65 ± 0.32	0.27 ± 0.08	2.94 ± 0.91	569.97 ± 4.13
5g	3.02 ± 0.95	1.72 ± 0.33	56.05 ± 16.51	
TSA (**1**)	0.08 ± 0.02	0.02 ± 0.01	0.10 ± 0.03	53.29 ± 3.68
oxamflatin (**3**)	0.84 ± 0.19	0.59 ± 0.04	1.07 ± 0.20	233.15 ± 4.86
MS-275 (**4**)	3.58 ± 0.50	0.87 ± 0.10	4.16 ± 1.74	2194.59 ± 6.54

[a] Antiproliferative activity against human cancer cell lines was evaluated using the MTT assay, and the concentration of compound causing 50% cell growth inhibition (IC_{50}) was determined. [b] Data represent the mean ± SD (n = 3). [c] HDAC activity was analyzed at various concentrations of compound by measuring a fluorescent HDAC substrate. Test compounds were diluted serially, and the SNU-16 cell nuclear extract was added. Substrate was added and the mixture was incubated for 10 min at 25 °C prior to being assayed for HDAC activity.

lines tested than the pyridyl compounds **5c** and **5d** and the 4-(4-pyridylmethylpiperazin-1-yl)phenyl compounds **5e**−**g**. Compound **5b** showed the most significant antiproliferative activity with IC_{50} values of 0.35 ± 0.22, 0.11 ± 0.01, and 0.80 ± 0.27 μM against A549, SK-BR-3, and MKN45 cancer cell lines, respectively. These values were 5−10-fold lower than those seen with MS-275 and even lower than those seen with oxamflatin, one of the most potent synthetic hydroxamic acid-based HDAC inhibitors known. Furthermore, compound **5b** was only 4−8-fold less potent than TSA. The inhibitory potency of compound **5a** [IC_{50} = 0.48 ± 0.13 (A549), 0.16 ± 0.06 (SK-BR-3), 0.83 ± 0.28 (MKN45) μM] was comparable to that of compound **5b**. Among the pyridylmethyl-substituted piperazine derivatives **5e**−**g**, the 3-pyridyl derivative **5f** was more potent as an antiproliferative agent than the 2-pyridyl (**5e**) and the 4-pyridyl (**5g**) derivatives.

An HDAC inhibition assay[34,35] was performed using a nuclear extract from SNU-16 (human gastric adenocarcinoma) cells (Table 1). Expectedly, compounds with better antiproliferative profiles (**5a** and **5b**) inhibited HDACs more than the less active antiproliferative agents (**5c**, **5d**, and **5f**). Compound **5a** was the most active derivative (IC_{50} = 172.02 ± 8.22 nM), showing 13- and 1.4-fold more HDAC inhibition than MS-275 (IC_{50} = 2194.59 ± 6.54 nM) and oxamflatin (IC_{50} = 233.15 ± 4.86 nM), respectively. Compound **5a** exhibited only a 3.2-fold lower potency than TSA (IC_{50} = 53.29 ± 3.68 nM). Compound **5b** (IC_{50} = 205.27 ± 8.46 nM) was slightly weaker as an HDAC inhibitor than compound **5a**.

Docking Study. To examine a possible binding mode of **5a** at the active site of HDAC, flexible docking was conducted using the FlexX program implanted in Sybyl 6.9 (Tripos Inc.).[36] The reference protein coordinate used for docking was taken from the X-ray structure (PDB entry 1c3r) of bacterial HDLP complexed with TSA.[26] Three-dimensional structures of **5a** and reference molecule (TSA) were prepared and then docked into the protein active site. A superposition of FlexX-docked TSA onto the crystallographic geometry yielded a root-mean-square (rms) deviation of 0.75 Å and revealed that FlexX performed well in reproducing the binding conformation of TSA (result not shown). To compare the binding conformation of **5a** identified by FlexX with the X-ray pose of TSA, the top-ranked conformer of **5a** was retrieved and overlayed over TSA in the active site of HDLP. As shown in Figure 1, their binding poses are very similar.

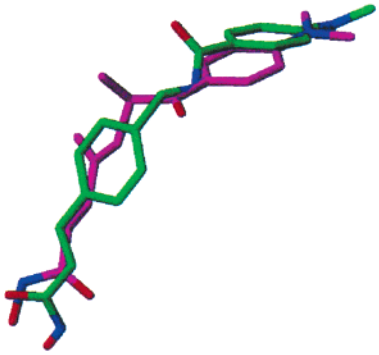


Figure 1. Compound **5a** (green) superimposed onto TSA (magenta) within the HDLP binding site. Oxygen atoms of the ligands are red and nitrogen atoms are blue.

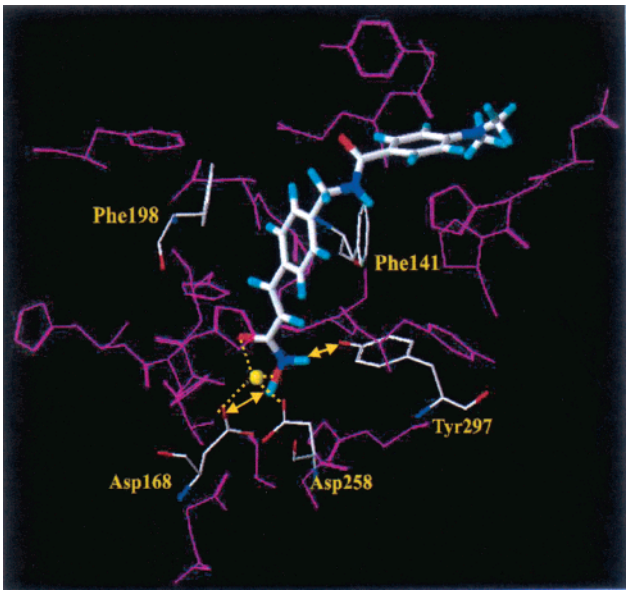


Figure 2. Binding pose of **5a** at the active site of HDLP, generated by FlexX. The amino acid residues within the HDLP binding site are represented in line form, and the zinc ion is shown as a yellow ball. Yellow dotted lines are coordination with Zn^{2+} (<2.7 Å), and arrows are hydrogen-bonding interactions (<2.5 Å).

The FlexX-docked pose of **5a** in the active site of HDLP is demonstrated in Figure 2. Compound **5a** snugly fits into the catalytic pocket of HDLP occupied

by TSA in the X-ray crystal structure, and the hydroxamic acid group anchors into the bottom of the pocket. In the X-ray structure of the TSA−HDLP complex, the hydroxamic acid group is coordinated to the zinc ion, which is critical for the HDAC catalysis, and forms hydrogen bonds with Asp168, His170, and Tyr297.[25] Similarly, the hydroxamic acid of **5a** is coordinated to zinc ion through its carbonyl (2.4 Å) and hydroxyl (2.7 Å) oxygen atoms. In addition, the hydroxyl and amino groups of hydroxamic acid form hydrogen bonds with Asp168 and Tyr297, respectively (Figure 2). The phenylene linker moiety of **5a**, which is equivalent to the methyl-substituted vinyl group of TSA, intercalates between Phe141 and Phe198 and possibly plays an important role in enhancing the affinity in the tube-like pocket of HDLP through the $\pi-\pi$ interaction.

The binding mode of **5a** generated by FlexX revealed that the structure of the molecule is compatible with the TSA-binding cavity of HDLP, and it would be related to the experimental result showing that **5a** has HDAC inhibitory activity comparable to that of TSA.

Conclusion

Incorporation of the 1,4-phenylene carboxamide linker shown in compounds **5a**−**g** and a 4-(dimethylamino)-phenyl or 4-(pyrrolidin-1-yl)phenyl group as a cap substructure generated the highly potent hydroxamic acid-based HDAC inhibitors **5a** and **5b**.

Compounds **5a** and **5b** have been selected as preclinical candidates, and work is underway to evaluate their effects on various cancer cells. Cancer cells sensitive to compound **5b** showed relatively higher levels of HDAC 1, suggesting that **5b** might selectively target the HDAC 1 isotype.[37] Compound **5b** has been found to induce cell cycle arrest at G1 and G2/M phases and to induce mitochondrial- and caspase-dependent apoptotic cell death.[37] These data and ongoing animal experiments will be published elsewhere in due course.

Experimental Section

Chemistry. ^{1}H NMR spectra were recorded on a Varian Unity 300 spectrometer. The chemical shifts are reported in parts per million (ppm) relative to internal tetramethylsilane in $CDCl_3$ or DMSO-d_6. Infrared spectra were recorded on a Magna 750 FTIR spectrophotometer. Fast-atom bombardment mass spectra (FAB-MS) were obtained on a VG Quattro mass spectrometer. Analytical thin-layer chromatography (TLC) was performed on Merck silica gel 60F-254 glass plates. Medium-pressure chromatography (MPLC) was performed using Merck silica gel 60 (230−400 mesh) with a VSP-2200 ceramic pump (Eyela). Elemental analyses were performed on a Carlo Erba 1106 elemental analyzer. Where indicated by the symbols of the elements, analyses were within ±0.4% of theoretical values.

4-(Dimethylamino)-*N*-(4-hydroxymethylbenzyl)benzamide (8a). To a mixture of 4-(dimethylamino)benzoic acid (**6a**) (915 mg, 5.54 mmol) and triethylamine (772 μL, 5.54 mmol) in CH_2Cl_2 (25 mL) at 0 °C was added BOP−Cl (1.50 g, 6.09 mmol), and the mixture was stirred at room temperature for 20 min. To the reaction mixture were added (4-aminomethylphenyl)methanol (**7**) (760 mg, 5.54 mmol) and triethylamine (1.54 mL, 11.08 mmol), and the mixture was stirred at room temperature overnight. To the reaction mixture was added a 50% aqueous $NaHCO_3$ solution (50 mL), and the mixture was extracted with 5% MeOH in $CHCl_3$ (100 mL × 1, 40 mL × 2). The organic layer was dried ($MgSO_4$) and evaporated under reduced pressure. The crude product was purified by MPLC on silica gel (2% MeOH in $CHCl_3$) to afford **8a** (1.04 g, 66%): ^{1}H NMR (DMSO-d_6) δ 2.97 (s, 6 H, 2 NCH_3), 4.41−4.47 (m, 4 H, NCH_2 and OCH_2), 5.10 (t, 1 H, J = 5.7 Hz, OH), 6.70 (m, 2 H, 2 Ar-H), 7.25 (apparent s, 4 H, 4 Ar-H), 7.76 (m, 2 H, 2 Ar-H), 8.63 (br t, 1 H, J = 6.0 Hz, NH); IR (neat) 3318 (NH and OH), 1622 (CO) cm^{-1}; MS (FAB) m/z 285 (MH^+). Anal. ($C_{17}H_{20}N_2O_2$) C, H, N.

***N*-(4-Hydroxymethylbenzyl)-4-pyrrolidin-1-ylbenzamide (8b).** To a mixture of 4-pyrrolidin-1-ylbenzoic acid (**6b**) (2.00 g, 10.5 mmol), **7** (4.30 g, 31.4 mmol), 1-hydroxybenzotriazole (1.70 g, 12.6 mmol), and DMAP (256 mg, 2.1 mmol) in pyridine (50 mL) was added EDC (3.00 g, 15.7 mmol), and the mixture was stirred at room temperature overnight. The reaction mixture was concentrated under reduced pressure, and an aqueous $NaHCO_3$ solution (50 mL) was added. The mixture was extracted with 5% MeOH in $CHCl_3$ (150 mL × 2), and the organic layer was dried ($MgSO_4$) and evaporated under reduced pressure. The crude product was purified by MPLC on silica gel (3% MeOH in $CHCl_3$) to afford **8b** (2.56 g, 79%): ^{1}H NMR (DMSO-d_6) δ 1.96 (m, 4 H, 2 CH_2), 3.28 (m, 4 H, 2 NCH_2), 4.41−4.47 (m, 4 H, NCH_2 and OCH_2), 5.10 (t, 1H, J = 5.7 Hz, OH), 6.53 (m, 2 H, 2 Ar-H), 7.25 (apparent s, 4 H, 4 Ar-H), 7.75 (m, 2 H, 2 Ar-H), 8.59 (br t, 1H, J = 6.0 Hz, NH); IR (neat) 3258 (NH and OH), 1615 (CO) cm^{-1}; MS (FAB) m/z 311 (MH^+). Anal. ($C_{19}H_{22}N_2O_2$) C, H, N.

***N*-(4-Hydroxymethylbenzyl)nicotinamide (8c)** was similarly obtained from **6c** as for **8b**: yield 86%; ^{1}H NMR (DMSO-d_6) δ 4.46−4.49 (m, 4 H, NCH_2 and OCH_2), 5.13 (br t, 1H, J = 5.6 Hz, OH), 7.28 (apparent s, 4 H, 4 Ar-H), 7.51 (dd, 1 H, J = 7.8 Hz, 4.8 Hz, Pyr-H), 8.22 (m, 1 H, Pyr-H), 8.71 (dd, 1 H, J = 4.8 Hz, 1.5 Hz, Pyr-H), 9.04 (d, 1 H, J = 2.1 Hz, Pyr-H), 9.21 (br t, 1 H, J = 5.7 Hz, NH); IR (neat) 3310 (NH and OH), 1631 (CO) cm^{-1}; MS (FAB) m/z 243 (MH^+). Anal. ($C_{14}H_{14}N_2O_2$) C, H, N.

3-{4-[(4-Dimethylaminobenzoylamino)methyl]phenyl}-acrylic Acid Ethyl Ester (9a). To a solution of oxalyl chloride (0.95 mL, 10.8 mmol) in THF (10 mL) at −78 °C was added a solution of DMSO (1.68 mL, 23.6 mmol) in THF (10 mL), and the reaction mixture was stirred for 30 min. A solution of **8a** (1.40 g, 4.8 mmol) in THF (80 mL) was added to it, and then the mixture was stirred for 1 h and warmed to −35 °C. After 20 min, the mixture was cooled to −78 °C, and triethylamine (3.43 mL, 24.6 mmol) was added. After stirring at 0 °C for 30 min, the mixture was diluted with water (100 mL), and then THF was evaporated under reduced pressure. The resulting residue was extracted with 5% MeOH in $CHCl_3$ (200 mL × 2), and the organic layer was dried ($MgSO_4$) and evaporated to dryness under reduced pressure to give a crude product which was crystallized from MeOH/$CHCl_3$/Et_2O to afford the corresponding aldehyde (1.10 g, 80%) as a white solid.

A solution of the aldehyde (800 mg, 2.83 mmol) above and $(Ph)_3P{=}CHCO_2Et$ (1.48 g, 4.25 mmol) in CH_3CN (40 mL) was stirred at 60 °C for 2 h. The reaction mixture was concentrated under reduced pressure, and the residue was purified by MPLC on silica gel (2% MeOH in $CHCl_3$) to afford **9a** (916 mg, 92%): ^{1}H NMR (DMSO-d_6) δ 1.25 (t, 3 H, J = 7.2 Hz, CH_2CH_3), 2.97 (s, 6 H, 2 NCH_3), 4.18 (q, 2 H, J = 7.2 Hz, CH_2-CH_3), 4.46 (d, 2 H, J = 6.0 Hz, NCH_2), 6.59 (d, 1 H, J = 15.9 Hz, CH), 6.71 (m, 2 H, 2 Ar-H), 7.33 (d, 2 H, J = 8.1 Hz, 2 Ar-H), 7.63 (d, 1 H, J = 15.9 Hz, CH), 7.67 (d, 2 H, J = 8.1 Hz, 2 Ar-H), 7.77 (m, 2 H, 2 Ar-H), 8.70 (br t, 1 H, J = 6.0 Hz, NH); IR (neat) 3247 (NH), 1704 (CO), 1634 (CO) cm^{-1}; MS (FAB) m/z 353 (MH^+). Anal. ($C_{21}H_{24}N_2O_3$) C, H, N.

3-{4-[(4-Pyrrolidin-1-ylbenzoylamino)methyl]phenyl}-acrylic acid ethyl ester (9b) was similarly obtained from **8b** as for **9a**: yield 47%; ^{1}H NMR (DMSO-d_6) δ 1.25 (t, 3 H, J = 7.2 Hz, CH_2CH_3), 1.96 (m, 4 H, 2 CH_2), 3.28 (m, 4 H, 2 NCH_2), 4.18 (q, 2 H, J = 7.2 Hz, CH_2CH_3), 4.46 (d, 2 H, J = 5.7 Hz, $NHCH_2$), 6.54 (m, 2 H, 2 Ar-H), 6.58 (d, 1 H, J = 16.5 Hz, CH), 7.33 (d, 2 H, J = 8.1 Hz, 2 Ar-H), 7.62 (d, 1 H, J = 16.5 Hz, CH), 7.67 (d, 2 H, J = 8.1 Hz, 2 Ar-H), 7.76 (m, 2 H, 2 Ar-H), 8.66 (br t, 1 H, J = 5.7 Hz, NH); IR (neat) 3256 (NH), 1707 (CO), 1629 (CO) cm^{-1}; MS (FAB) m/z 379 (MH^+). Anal. ($C_{23}H_{26}N_2O_3$) C, H, N.

3-{4-[(Nicotinoylamino)methyl]phenyl}acrylic acid ethyl ester (9c) was similarly obtained from **8c** as for **9a**: yield 39%; ^{1}H NMR (DMSO-d_6) δ 1.26 (t, 3 H, J = 7.2 Hz, CH_2CH_3), 4.19 (q, 2 H, J = 7.2 Hz, CH_2CH_3), 4.53 (d, 2 H, J = 6.0 Hz, NCH_2), 6.60 (d, 1 H, J = 15.9 Hz, CH), 7.38 (d, 2 H, J = 8.1 Hz, 2 Ar-H), 7.52 (m, 1 H, Pyr-H), 7.63 (d, 1 H, J = 15.9 Hz, CH), 7.69 (d, 2 H, J = 8.1 Hz, 2 Ar-H), 8.23 (m, 1 H, Pyr-H), 8.72 (m, 1 H, Pyr-H), 9.06 (m, 1 H, Pyr-H), 9.26 (br t, 1 H, J = 6.0 Hz, NH); IR (neat) 3279 (NH), 1703 (CO), 1632 (CO) cm^{-1}; MS (FAB) m/z 311 (MH^+). Anal. ($C_{18}H_{18}N_2O_3$) C, H, N.

3-{4-[(Picolinoylamino)methyl]phenyl}acrylic Acid Ethyl Ester (9d). To a mixture of picolinic acid (**6d**) (170 mg, 1.4 mmol), **10** (284 mg, 1.4 mmol), and DMAP (34 mg, 2.1 mmol) in DMF (10 mL) was added EDC (291 mg, 15.7 mmol), and the mixture was stirred at room temperature for 4 h. The reaction mixture was concentrated under reduced pressure, and an aqueous $NaHCO_3$ solution (20 mL) was added. The mixture was extracted with 5% MeOH in $CHCl_3$ (30 mL × 3), and the organic layer was dried (Na_2SO_4) and evaporated under reduced pressure. The crude product was purified by MPLC on silica gel (1% MeOH in $CHCl_3$) to afford **9d** (247 mg, 58%): ^{1}H NMR ($CDCl_3$) δ 1.34 (t, 3 H, J = 7.2 Hz, CH_2CH_3), 4.26 (q, 2 H, J = 7.2 Hz, CH_2CH_3), 4.69 (d, 2 H, J = 6.3 Hz, NCH_2), 6.42 (d, 1 H, J = 15.9 Hz, CH), 7.38 (d, 2 H, J = 8.1 Hz, 2 Ar-H), 7.44 (m, 1 H, Pyr-H), 7.50 (d, 2 H, J = 8.1 Hz, 2 Ar-H), 7.67 (d, 1 H, J = 15.9 Hz, CH), 7.87 (m, 1 H, Pyr-H), 8.24 (m, 1 H, Pyr-H), 8.42 (m, 1 H, NH), 8.54 (m, 1 H, Pyr-H); IR (neat) 3384 (NH), 1717 (CO), 1636 (CO) cm^{-1}; MS (FAB) m/z 311 (MH^+). Anal. ($C_{18}H_{18}N_2O_3$) C, H, N.

4-Dimethylamino-*N*-[4-(2-hydroxycarbamoylvinyl)benzyl]benzamide (5a). To a solution of 1.76 M NH_2OH in MeOH (0.97 mL) was added **9a** (100 mg, 0.28 mmol), and the mixture was stirred at room temperature overnight. The reaction mixture was neutralized by adding 1 N HCl aqueous solution (pH 7) at 0 °C. The solid precipitated was collected by filtration, washed with H_2O and Et_2O, dried under vacuum, and crystallized from MeOH/CH_2Cl_2 to afford **5a** (55 mg, 58%): ^{1}H NMR (DMSO-d_6) δ 2.97 (s, 6 H, 2 NCH_3), 4.45 (d, 2 H, J = 5.7 Hz, NCH_2), 6.42 (d, 1 H, J = 15.9 Hz, CH), 6.71 (m, 2 H, 2 Ar-H), 7.33 (d, 2 H, J = 8.1 Hz, 2 Ar-H), 7.43 (d, 1 H, J = 15.9 Hz, CH), 7.51 (d, 2 H, J = 8.1 Hz, 2 Ar-H), 7.77 (m, 2 H, 2 Ar-H), 8.68 (br t, 1 H, J = 5.7 Hz, CONH), 9.00 (br s, 1 H, N*H*OH), 10.71 (br s, 1 H, NHO*H*); IR (neat) 3178 (NH), 1633 (CO), 1608 (CO) cm^{-1}; MS (FAB) m/z 340 (MH^+). Anal. ($C_{19}H_{21}N_3O_3$) C, H, N.

***N*-[4-(2-Hydroxycarbamoylvinyl)benzyl]-4-pyrrolidin-1-ylbenzamide (5b)** was similarly obtained from **9b** as for **5a**: yield 35%; ^{1}H NMR (DMSO-d_6) δ 1.96 (m, 4 H, 2 CH_2), 3.28 (m, 4 H, 2 NCH_2), 4.45 (d, 2 H, J = 5.7 Hz, $NHCH_2$), 6.42 (d, 1 H, J = 15.9 Hz, CH), 6.54 (m, 2 H, 2 Ar-H), 7.32 (d, 2 H, J = 8.1 Hz, 2 Ar-H), 7.43 (d, 1 H, J = 15.9 Hz, CH), 7.50 (d, 2 H, J = 8.1 Hz, 2 Ar-H), 7.76 (m, 2 H, 2 Ar-H), 8.64 (br t, 1 H, J = 5.7 Hz, CONH), 9.00 (br s, 1 H, N*H*OH), 10.70 (br s, 1 H, NHO*H*); IR (neat) 3302 (NH), 1630 (CO), 1608 (CO) cm^{-1}; MS (FAB) m/z 366 (MH^+). Anal. ($C_{21}H_{23}N_3O_3$) C, H, N.

***N*-[4-(2-Hydroxycarbamoylvinyl)benzyl]nicotinamide (5c)** was similarly obtained from **9c** as for **5a**: yield 74%; ^{1}H NMR (DMSO-d_6) δ 4.51 (m, 2 H, NCH_2), 6.43 (d, 1 H, J = 15.9 Hz, CH), 7.37 (d, 2 H, J = 8.1 Hz, 2 Ar-H), 7.44 (d, 1 H, J = 15.9 Hz, CH), 7.52 (m, 1 H, Pyr-H), 7.53 (d, 2 H, J = 8.1 Hz, 2 Ar-H), 8.23 (m, 1 H, Pyr-H), 8.72 (m, 1 H, Pyr-H), 9.05 (m, 1 H, Pyr-H), 9.25 (m, 1 H, CONH); IR (neat) 3249 (NH), 1653 (CO), 1626 (CO) cm^{-1}; MS (FAB) m/z 298 (MH^+). Anal. ($C_{16}H_{15}N_3O_3$) C, H, N.

***N*-[4-(2-Hydroxycarbamoylvinyl)benzyl]picolinamide (5d)** was similarly obtained from **9d** as for **5a**: yield 29%; ^{1}H NMR (DMSO-d_6) δ 4.51 (m, 2 H, NCH_2), 6.45 (d, 1 H, J = 15.9 Hz, CH), 7.35 (d, 2 H, J = 8.1 Hz, 2 Ar-H), 7.42 (d, 1 H, J = 15.9 Hz, CH), 7.50 (d, 2 H, J = 8.1 Hz, 2 Ar-H), 7.59−7.64 (m, 1 H, Pyr-H), 7.97−8.06 (m, 2 H, 2 Pyr-H), 8.66 (m, 1 H, Pyr-H), 9.02 (br s, 1 H, N*H*OH), 9.36 (m, 1 H, CONH), 10.76 (br s, 1 H, NHO*H*); IR (neat) 3254 (NH), 1653 (CO), 1636 (CO) cm^{-1}; MS (FAB) m/z 298 (MH^+). Anal. ($C_{16}H_{15}N_3O_3$) C, H, N.

4-(4-Pyridin-2-ylmethylpiperazin-1-yl)benzonitrile (13a). A suspension of 1-(pyridin-2-ylmethyl)piperazine (**11a**) (638 mg, 3.60 mmol), 4-fluorobenzonitrile (436 mg, 3.60 mmol), and K_2CO_3 (746 mg, 5.40 mmol) in DMF (50 mL) was stirred at 80 °C overnight. The reaction mixture was cooled to room temperature and filtered, and the filtrate was washed with EtOAc (30 mL). The combined filtrate and washings were evaporated to dryness under reduced pressure. The crude residue was purified by MPLC on silica gel (3% MeOH in CH_2Cl_2) to afford **13a** (317 mg, 32%): ^{1}H NMR ($CDCl_3$) δ 2.66 (apparent t, 4 H, J = 5.1 Hz, 2 NCH_2), 3.36 (apparent t, 4 H, J = 5.1 Hz, 2 NCH_2), 3.72 (s, 2 H, NCH_2Pyr), 6.84 (m, 2 H, 2 Ar-H), 7.19 (m, 1 H, Pyr-H), 7.42 (m, 1 H, Pyr-H), 7.48 (m, 2 H, 2 Ar-H), 7.68 (m, 1 H, Pyr-H), 8.59 (m, 1 H, Pyr-H); IR (neat) 2216 (CN) cm^{-1}; MS (FAB) m/z 279 (MH^+). Anal. ($C_{17}H_{18}N_4$) C, H, N.

4-(4-Pyridin-3-ylmethylpiperazin-1-yl)benzonitrile (13b) was similarly obtained from **11b** as for **13a**: yield 38%; ^{1}H NMR ($CDCl_3$) δ 2.59 (apparent t, 4 H, J = 5.1 Hz, 2 NCH_2), 3.33 (apparent t, 4 H, J = 5.1 Hz, 2 NCH_2), 3.57 (s, 2 H, NCH_2Pyr), 6.85 (m, 2 H, 2 Ar-H), 7.27 (m, 1 H, Pyr-H), 7.48 (m, 2 H, 2 Ar-H), 7.69 (m, 1 H, Pyr-H), 8.53 (m, 1 H, Pyr-H), 8.57 (m, 1 H, Pyr-H); IR (neat) 2206 (CN) cm^{-1}; MS (FAB) m/z 279 (MH^+). Anal. ($C_{17}H_{18}N_4$) C, H, N.

4-(4-Pyridin-4-ylmethylpiperazin-1-yl)benzonitrile (13c) was similarly obtained from **11c** as for **13a**: yield 59%; ^{1}H NMR ($CDCl_3$) δ 2.59 (apparent t, 4 H, J = 5.1 Hz, 2 NCH_2), 3.35 (apparent t, 4 H, J = 5.1 Hz, 2 NCH_2), 3.56 (s, 2 H, NCH_2Pyr), 6.85 (m, 2 H, 2 Ar-H), 7.30 (m, 2 H, 2 Pyr-H), 7.49 (m, 2 H, 2 Ar-H), 8.56 (m, 2 H, 2 Pyr-H); IR (neat) 2213 (CN) cm^{-1}; MS (FAB) m/z 279 (MH^+). Anal. ($C_{17}H_{18}N_4$) C, H, N.

3-(4-{[4-(4-Pyridin-2-ylmethylpiperazin-1-yl)benzoylamino]methyl}phenyl)acrylic Acid Ethyl Ester (14a). A solution of **13a** (330 mg, 1.19 mmol) in concentrated HCl (20 mL) was stirred at 80 °C overnight. The reaction mixture was cooled to room temperature and then evaporated to dryness under reduced pressure. The residue was dissolved in saturated LiOH solution (pH 9) and then evaporated to dryness under reduced pressure. The residue was dissolved in 10% aqueous HCl solution (pH 2), evaporated, and dried under vacuum to give the corresponding acid, which was used in the next step without further purification.

To a mixture of the acid above, 3-(4-aminomethylphenyl)-acrylic acid ethyl ester hydrochloride (**10**) (305 mg, 1.26 mmol), 1-hydroxybenzotriazole (195 mg, 1.44 mmol), and DMAP (22 mg, 0.18 mmol) in pyridine (12 mL) was added EDC (276 mg, 1.44 mmol), and the mixture was stirred at room temperature for 20 h. The reaction mixture was concentrated under reduced pressure, and to it an aqueous $NaHCO_3$ solution (20 mL) was added. The mixture was extracted with 10% MeOH in $CHCl_3$ (30 mL × 3), and the organic layer was dried (Na_2SO_4) and evaporated under reduced pressure. The crude product was purified by MPLC on silica gel (5% MeOH in $CHCl_3$) to afford **14a** (220 mg, 38%): ^{1}H NMR (DMSO-d_6) δ 1.25 (t, 3 H, J = 7.2 Hz, CH_2CH_3), 2.57 (m, 4 H, 2 NCH_2), 3.28 (m, 4 H, 2 NCH_2), 3.66 (s, 2 H, NCH_2Pyr), 4.18 (q, 2 H, J = 7.2 Hz, CH_2CH_3), 4.46 (d, 2 H, J = 5.7 Hz, $NHCH_2$), 6.59 (d, 1 H, J = 16.2 Hz, CH), 6.96 (m, 2 H, 2 Ar-H), 7.26−7.34 (m, 3 H, Pyr-H and 2 Ar-H), 7.48 (m, 1 H, Pyr-H), 7.60−7.68 (m, 3 H, CH and 2 Ar-H), 7.75−7.81 (m, 3 H, 2 Ar-H and Pyr-H), 8.51 (m, 1 H, Pyr-H), 8.80 (br t, 1 H, J = 5.7 Hz, NH); IR (neat) 3350 (NH), 1702 (CO), 1637 (CO) cm^{-1}; MS (FAB) m/z 485 (MH^+). Anal. ($C_{29}H_{32}N_4O_3$) C, H, N.

3-(4-{[4-(4-Pyridin-3-ylmethylpiperazin-1-yl)benzoylamino]methyl}phenyl)acrylic acid ethyl ester (14b) was similarly obtained from **13b** as for **14a**: yield 84%; ^{1}H NMR ($CDCl_3$) δ 1.34 (t, 3 H, J = 7.2 Hz, CH_2CH_3), 2.60 (apparent t, 4 H, J = 5.1 Hz, 2 NCH_2), 3.30 (apparent t, 4 H, J = 5.1 Hz, 2 NCH_2), 3.57 (s, 2 H, NCH_2Pyr), 4.26 (q, 2 H, J = 7.2 Hz, CH_2CH_3), 4.64 (d, 2 H, J = 5.7 Hz, $NHCH_2$), 6.32 (br t, 1 H, J = 5.7 Hz, NH), 6.41 (d, 1 H, J = 15.9 Hz, CH), 6.88 (m, 2 H, 2 Ar-H), 7.27 (m, 1 H, Pyr-H), 7.36 (d, 2 H, J = 8.1 Hz, 2 Ar-H), 7.49 (d, 2 H, J = 8.1 Hz, 2 Ar-H), 7.64−7.72 (m, 4 H, CH, Pyr-H, and 2 Ar-H), 8.53 (m, 1 H, Pyr-H), 8.57 (m, 1 H, Pyr-

H); IR (neat) 3349 (NH), 1707 (CO), 1638 (CO) cm^{-1}; MS (FAB) *m*/*z* 485 (MH^+). Anal. ($C_{29}H_{32}N_4O_3$) C, H, N.

3-(4-{[4-(4-Pyridin-4-ylmethylpiperazin-1-yl)benzoylamino]methyl}phenyl)acrylic acid ethyl ester (14c) was similarly obtained from **13c** as for **14a**: yield 94%; ^1H NMR ($CDCl_3$) δ 1.33 (t, 3 H, J = 7.2 Hz, CH_2CH_3), 2.60 (apparent t, 4 H, J = 5.1 Hz, 2 NCH_2), 3.31 (apparent t, 4 H, J = 5.1 Hz, 2 NCH_2), 3.56 (s, 2 H, NCH_2Pyr), 4.26 (q, 2 H, J = 7.2 Hz, CH_2CH_3), 4.65 (d, 2 H, J = 5.7 Hz, $NHCH_2$), 6.36 (br t, 1 H, J = 5.7 Hz, NH), 6.41 (d, 1 H, J = 15.9 Hz, CH), 6.88 (m, 2 H, 2 Ar-H), 7.30 (d, 2 H, J = 6.0 Hz, 2 Pyr-H), 7.36 (d, 2 H, J = 8.1 Hz, 2 Ar-H), 7.49 (d, 2 H, J = 8.1 Hz, 2 Ar-H), 7.66 (d, 1 H, J = 15.9 Hz, CH), 7.72 (m, 2 H, 2 Ar-H), 8.56 (m, 2 H, 2 Pyr-H); IR (neat) 3356 (NH), 1701 (CO), 1634 (CO) cm^{-1}; MS (FAB) *m*/*z* 485 (MH^+). Anal. ($C_{29}H_{32}N_4O_3$) C, H, N.

***N*-[4-(2-Hydroxycarbamoylvinyl)benzyl]-4-(4-pyridin-2-ylmethylpiperazin-1-yl)benzamide (5e)** was similarly obtained from **14a** as for **5a**: yield 59%; ^1H NMR (DMSO-d_6) δ 2.59 (m, 4 H, 2 NCH_2), 3.28 (m, 4 H, 2 NCH_2), 3.68 (br s, 2 H, NCH_2Pyr), 4.45 (d, 2 H, J = 5.7 Hz, $NHCH_2$), 6.42 (d, 1 H, J = 15.9 Hz, CH), 6.96 (m, 2 H, 2 Ar-H), 7.26−7.34 (m, 3 H, 2 Ar-H and Pyr-H), 7.43 (d, 1 H, J = 15.9 Hz, CH), 7.47−7.52 (m, 3 H, 2 Ar-H and Pyr-H), 7.77−7.81 (m, 3 H, 2 Ar-H and Pyr-H), 8.51 (m, 1 H, Pyr-H), 8.78 (br t, 1 H, J = 5.7 Hz, CONH), 9.02 (br s, 1 H, N*H*OH), 10.73 (br s, 1 H, NHO*H*); IR (neat) 3270 (NH), 1631 (CO), 1608 (CO) cm^{-1}; MS (FAB) *m*/*z* 472 (MH^+). Anal. ($C_{27}H_{29}N_5O_3$) C, H, N.

***N*-[4-(2-Hydroxycarbamoylvinyl)benzyl]-4-(4-pyridin-3-ylmethylpiperazin-1-yl)benzamide (5f)** was similarly obtained from **14b** as for **5a**: yield 46%; ^1H NMR (DMSO-d_6) δ 2.56 (m, 4 H, 2 NCH_2), 3.28 (m, 4 H, 2 NCH_2), 3.62 (br s, 2 H, NCH_2Pyr), 4.45 (d, 2 H, J = 5.7 Hz, $NHCH_2$), 6.42 (d, 1 H, J = 15.9 Hz, CH), 6.96 (m, 2 H, 2 Ar-H), 7.31 (d, 2 H, J = 8.1 Hz, 2 Ar-H), 7.37−7.45 (m, 2 H, CH and Pyr-H), 7.50 (d, 2 H, J = 8.1 Hz, 2 Ar-H), 7.76−7.79 (m, 3 H, 2 Ar-H and Pyr-H), 8.50 (m, 1 H, Pyr-H), 8.54 (m, 1 H, Pyr-H), 8.78 (br t, 1 H, J = 5.7 Hz, CONH), 9.01 (br s, 1 H, N*H*OH), 10.73 (br s, 1 H, NHO*H*); IR(neat) 3278 (NH), 1631 (CO), 1607 (CO) cm^{-1}; MS (FAB) *m*/*z* 472 (MH^+). Anal. ($C_{27}H_{29}N_5O_3$) C, H, N.

***N*-[4-(2-Hydroxycarbamoylvinyl)benzyl]-4-(4-pyridin-4-ylmethylpiperazin-1-yl)benzamide (5g)** was similarly obtained from **14c** as for **5a**: yield 54%; ^1H NMR (DMSO-d_6) δ 2.52 (m, 4 H, 2 NCH_2), 3.28 (m, 4 H, 2 NCH_2), 3.57 (s, 2 H, NCH_2Pyr), 4.45 (d, 2 H, J = 5.7 Hz, $NHCH_2$), 6.42 (d, 1 H, J = 15.9 Hz, CH), 6.96 (m, 2 H, 2 Ar-H), 7.32 (d, 2 H, J = 8.1 Hz, 2 Ar-H), 7.36 (d, 2 H, J = 6.0 Hz, 2 Pyr-H), 7.42 (d, 1 H, J = 15.9 Hz, CH), 7.51 (d, 2 H, J = 8.1 Hz, 2 Ar-H), 7.78 (m, 2 H, 2 Ar-H), 8.53 (d, 2 H, J = 6.0 Hz, 2 Pyr-H), 8.78 (br t, 1 H, J = 5.7 Hz, CONH), 9.02 (br s, 1 H, N*H*OH), 10.73 (br s, 1 H, NHO*H*); IR (neat) 3335 (NH), 1635 (CO), 1609 (CO) cm^{-1}; MS (FAB) *m*/*z* 472 (MH^+). Anal. ($C_{27}H_{29}N_5O_3$) C, H, N.

Antiproliferative Activity. Three human cancer cell lines (lung cancer, A549; breast cancer, SK-BR-3; and stomach cancer, MKN45) were tested in the MTT assay. A549 and SK-BR-3 were obtained from the American Type Culture Collection (Bethesda, MD), and MKN45 was kindly provided by Dr. N. Saijo, National Cancer Center Hospital and Research Institute, Japan. These cell lines were grown in RPMI 1640 medium containing penicillin−streptomycin (100 units/mL) and 10% heat-inactivated fetal bovine serum at 37 °C in an atmosphere of 5% CO_2. Single-cell suspensions were prepared by trypsinization and pipet disaggregation. The number of cells for each cell line plated in 96-well microtiter plates was determined from the growth curve obtained in the MTT assay. Test compounds were diluted from stock solution in DMSO into fresh medium to a 10-fold concentration. Cells were inoculated into each well in 180 μL of medium, and eight different concentrations of 20 μL of test compounds were added to each well. The plates were then incubated for 4 days at 37 °C in an atmosphere of 5% CO_2. After 4 days of culture, 0.1 mg (20 μL of 5 mg/mL) of MTT was added to each well. The plates were then incubated at 37 °C for 4 h. After the plates were centrifuged at 1000 rpm for 10 min, the supernatant was aspirated. DMSO (150 μL) was added to each well to solubilize formazan crystals. The plates were read immediately at 550 nm on an Elisa reader (Dynatech, MR 5000). The IC_{50} was defined as the concentration of compounds that produced a 50% reduction in surviving cells and was calculated from the expansion of control cultures over 4 days (number of cells at day 4 minus the number of cells at day 0) by quantal probit analysis of pharmacologic calculations with a computer program.

HDAC Inhibition. SNU-16 (human gastric adenocarcinoma) cells were quickly cooled by placing the plates on ice and were then washed twice with ice-cold PBS. Collected cells were centrifuged and resuspended in five packed cell volumes of buffer A (20 mM Tris pH 7.6, 10 mM KCl, 0.2 mM EDTA, 20% glycerol, 1.5 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 0.4 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4, and 2 μg/mL each of leupeptin, pepstatin, and aprotinin). Nuclei were pelleted (2500*g*, 10 min) and resuspended in two packed cell volumes of buffer B (identical to buffer A except that the KCl level was increased to 0.42 M). Nuclear debris was removed by centrifugation (15 000*g*, 20 min). HDAC inhibition assay was performed using an HDAC Fluorescent Activity Assay Kit (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA) according to the manufacturer's recommendation. In brief, 5 μg of SNU-16 nuclear extract was added to the diluted HDAC inhibitor and then substrate was added. Samples were incubated for 10 min at 25 °C, then the reaction was stopped by addition of developer. Fluorescence was analyzed using LS 55 Luminescence spectrometer (Perkin-Elmer, Wellesley, MA).

Preparation of Three-Dimensional Database and Target Protein Structure. All docking experiments have been performed with Sybyl (Tripos Inc., version 6.9) on a SGI−Octane 2 with a single 475-MHz processor and 128MB main memory. The structures of TSA and **5a** were prepared in mol2 format using the sketcher module of Sybyl 6.9, and Gasteiger−Huckel charges were assigned to the ligand atoms. The minimization was run until it converged to a maximum derivative of 0.001 kcal mol^{-1} $Å^{-1}$, and the final coordinate was stored in database. The X-ray coordinate of HDLP−TSA complex (1c3r) was retrieved from the PDB,[26] and all crystallographic water molecules were removed. The active site was defined as all the amino acid residues and zinc ion enclosed within a 6.5 Å radius sphere centered by the bound ligand, TSA.

Flexible Docking. The docking and subsequent scoring were performed using the default parameters of the FlexX programs implanted in Sybyl 6.9.[36] For the docking of ligand into the target active site, the main settings are 1000 solutions per iteration during the incremental construction algorithm and a maximum protein−ligand atom−atom overlap of 2.5 $Å^3$. Final scores for all FlexX solutions (up to 1000) per compound were calculated by a standard scoring function and used for database ranking.

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