# *Brief Articles*

## Synthesis and Histone Deacetylase Inhibitory Activity of New Benzamide Derivatives

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Newly synthesized benzamide derivatives were evaluated for their inhibitory activity against histone deacetylase. The structure of these derivatives was unrelated to the known inhibitors, and $IC_{50}$ values of the active compounds were in the range of 2−50 $\mu$M. Structure−activity relationship on the benzanilide moiety showed that the 2′-substituent, an amino or hydroxy group, was indispensable for inhibitory activity. Although the electronic influence of the substituent in the anilide moiety showed only a small effect on inhibitory activity, the steric factor in the anilide moiety, especially at positions 3′and 4′, played an important role in interaction with the enzyme. Among these benzamide derivatives, MS-275 (**1**), which showed significant antitumor activity in vivo, has been selected for further investigation.

### Introduction

Acetylation of histone molecules is catalyzed by two enzymes: histone acetyltransferase and histone deacetylase, which are considered to play an important role in cell cycle control by acting as a transcriptional coactivator or a transcriptional corepressor.[1] Inhibitors of histone deacetylase such as sodium butyrate,[2] trichostatin A,[3] and trapoxin[4] (Chart 1) have been reported to induce differentiation of several cancer cell lines and suppress cell proliferation. Some of these inhibitors or their related compounds are considered to be effective agents for the treatment of diseases ascribed to unusual cell proliferation such as malignant tumors. However their in vivo antitumor efficiency seems to be limited due to their instability, low retention, or nonspecific toxicity.

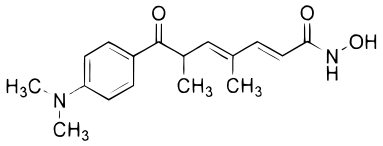
Recently we reported that a novel benzamide derivative, MS-275 (Chart 2), which had a structure unrelated to the known histone deacetylase inhibitors, inhibited histone deacetylase in vitro and in vivo and showed remarkable antitumor activity against several human tumors in vivo.[5]

In this paper we describe the synthesis and structural requirement for histone deacetylase inhibition of newly synthesized benzamide derivatives.

### Chemistry

The new benzamide derivatives **1**, **4a**−**e**, and **6a**−**h** (Table 1) were prepared as outlined in Scheme 1. Condensation of 3-(hydroxymethyl)pyridine and 4-(aminomethyl)benzoic acid using 1,1′-carbonyldiimidazole gave carboxylic acid **2** in 90% yield, which was converted into acyl chloride hydrochloride **3** by treatment of oxalyl chloride quantitatively.

**Chart 1.** Structure of Known Histone Deacetylase Inhibitors




Trichostatin A

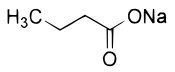
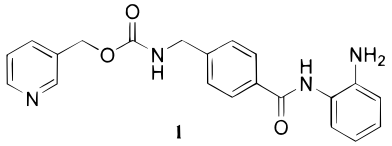
Sodium butyrate 


**Chart 2.** Structure of MS-275




**1**

Compounds **1** and **4a**−**e** were synthesized by method A: **3** was reacted with imidazole to form the acylimidazole intermediate, which was not isolated and was condensed with appropriately substituted aniline in the presence of trifluoroacetic acid to give **1** and **4a**−**e** in a 50−60% yield. This condensation reaction proceeded very slowly in the absence of acid.

Compounds **6a**−**h** were synthesized by method B: **3** was condensed with appropriately substituted 2-nitroaniline in pyridine to give **5** in a 40−50% yield, whose reduction with $SnCl_2$ gave **6** in a 50−60% yield.

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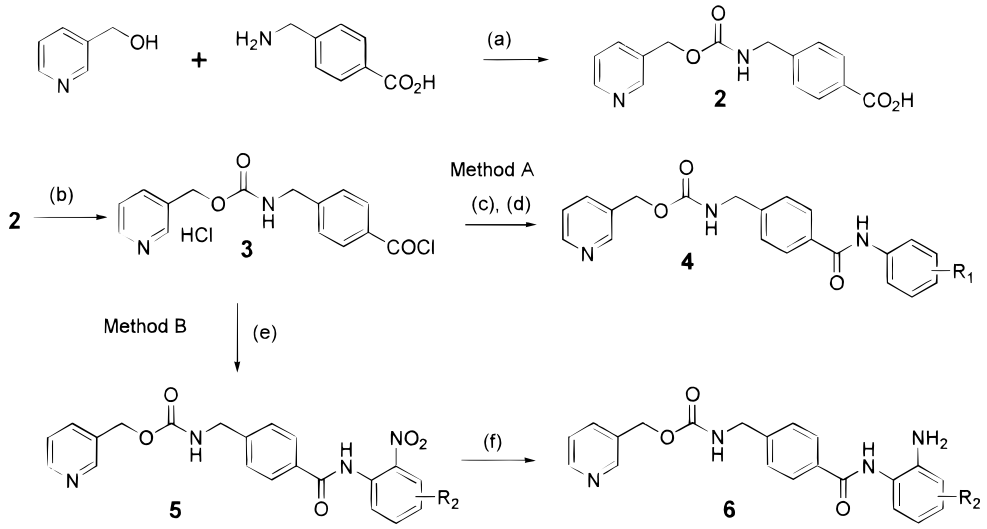
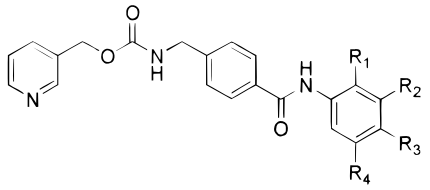
**Scheme 1.** General Synthetic Procedure for the Preparation of Benzamide Derivatives[a]




[a] Reagents: (a) CDI, DBU, $Et_3N$, THF; (b) oxalyl chloride, toluene; (c) imidazole, THF; (d) substituted aniline, TFA, THF; (e) substituted 2-nitroaniline, pyridine; (f) $SnCl_2$ dihydrate, $NH_4OAc$, MeOH.

**Table 1.** Inhibition of Histone Deacetylase with **1**, **4a**−**e**, and **6a**−**h**[a]




| compd | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $IC_{50}$[b] ($\mu$M) |
|---|---|---|---|---|---|
| **1** | $NH_2$ | H | H | H | 4.8 |
| **4a** | H | H | H | H | >100 |
| **4b** | H | $NH_2$ | H | H | >100 |
| **4c** | H | H | $NH_2$ | H | >100 |
| **4d** | NHAc | H | H | H | >100 |
| **4e** | OH | H | H | H | 2.2 |
| **6a** | $NH_2$ | $CH_3$ | H | H | >100 |
| **6b** | $NH_2$ | H | $CH_3$ | H | >100 |
| **6c** | $NH_2$ | H | $OCH_3$ | H | 44 |
| **6d** | $NH_2$ | H | Cl | H | 40 |
| **6e** | $NH_2$ | H | H | $CH_3$ | 2.8 |
| **6f** | $NH_2$ | H | H | $OCH_3$ | 4.6 |
| **6g** | $NH_2$ | H | H | Cl | 7.7 |
| **6h** | $NH_2$ | H | H | F | 6.0 |
| sodium butyrate | | | | | 140 |
| trichostatin A | | | | | 0.0046 |

[a] Expressed as the average of at least three determinations. [b] Concentration required to inhibit by 50%.

## Results and Discussion

The synthesized benzamide derivatives **1**, **4a**−**e**, and **6a**−**h** were evaluated for their abilities to inhibit partially purified histone deacetylase. The results are given as $IC_{50}$ values and are shown in Table 1. Compound **1** (MS-275) showed a 30-fold stronger inhibitory activity ($IC_{50}$: 4.8 $\mu$M) than sodium butyrate ($IC_{50}$: 140 $\mu$M), and 1000-fold weaker than trichostatin A ($IC_{50}$: 0.0046 $\mu$M). Removal of the 2′-amino group of benzanilide resulted in loss of inhibitory activity (**4a**). Among three regioisomers (**1**, **4b**,**c**), only 2′-substituted derivative **1** showed inhibitory activity. These results indicate that the 2′-amino group of **1** may be involved in the specific interaction with the enzyme. 2′-Hydroxy derivative **4e** showed almost equal activity to **1**, suggesting that the 2′-substituent of benzanilide might act as a hydrogen-bonding site or other electrostatic interaction site and be indispensable to the specific interaction with the enzyme.

The effect of the substituent at positions 3′, 4′, and 5′ was examined. 3′-Methyl group of **6a** caused loss of activity. Introduction of the 4′-substituent, both the electron-donating group (**6c**) and the electron-withdrawing group (**6d**), weakened activity similarly. On the other hand, 5′-substituted derivatives **6e**−**h** showed almost equal activity to **1**. These results suggest that steric hindrance may play an important role near the specific binding site (at positions 3′ and 4′). There was little steric limitation for the 5′-substituent, which may not interfere in specific binding with the enzyme. On the other hand, the electronic nature of the 4′- and 5′-substituents did not change inhibitory activity remarkably.

## Conclusion

In this paper, we reported the synthesis and structural requirement for histone deacetylase inhibition of newly synthesized benzamide derivatives. These are structurally unrelated to the known histone deacetylase inhibitors, and some showed a 30-fold stronger inhibitory activity than sodium butyrate. Structure−activity relationship on the benzanilide moiety showed that the 2′-substituent, which might act as a hydrogen-bonding or other electrostatic interaction site, was indispensable for inhibitory activity. Although the electronic influence of the substituent seemed to play a small role, it appeared that the steric factor in the anilide moiety, especially at positions 3′ and 4′, had an important role for interaction with the enzyme. Among these benzamide derivatives, MS-275 (**1**), which showed significant antitumor activity in vivo,[5] has been selected for further investigation.

## Experimental Section

**Chemistry.** Melting points were determined on a Büchi 535 melting point apparatus and were not corrected. Proton magnetic resonance spectra ($^1$H NMR) were obtained on a JEOL EX-270 (270 MHz) spectrometer using deuterated dimethyl sulfoxide (DMSO-$d_6$) as the solvent. Chemical shifts

are reported in parts per million (ppm) downfield from tetramethylsilane (TMS). Infrared spectra (IR) were recorded on a JASCO IR 7300 spectrometer. High-resolution mass spectra (FAB-HRMS) were obtained by M. C. Research Center Inc. (Yokohama, Japan) on a JEOL SX-102A spectrometer. Elemental analyses were determined on a Perkin-Elmer CHN2400 elemental analyzer.

**4-[*N*-(Pyridin-3-ylmethoxycarbonyl)aminomethyl]-benzoic Acid (2).** To a suspension of 1,1′-carbonyldiimidazole (25.6 g, 158 mmol) in THF (120 mL) was added 3-pyridinemethanol (17.3 g, 158 mmol) in THF (50 mL) at 10 °C, and the mixture stirred for 1 h at room temperature. The resulting solution was added to a suspension of 4-(aminomethyl)benzoic acid (22.6 g, 158 mmol), DBU (24.3 g, 158 mmol), and triethylamine (22.2 mL, 158 mmol) in THF (250 mL). After stirring for 5 h at room temperature, the mixture was evaporated to remove THF and then dissolved in water (300 mL). The solution was acidified with hydrochloric acid (pH 5) to precipitate a white solid which was collected by filtration, washed with water (300 mL) and methanol (50 mL), respectively, and dried to give **2** (41.1 g, 91% yield): mp 207−208 °C; $^1$H NMR (DMSO-$d_6$) $\delta$ 4.28 (d, 2H, $J$ = 5.9 Hz), 5.10 (s, 2H), 7.3−7.5 (m, 3H), 7.7−8.1 (m, 4H), 8.5−8.7 (m, 2H); IR (KBr) 3043, 1718, 1568, 1434, 1266, 1108, 1037, 984, 756 cm$^{-1}$.

**4-[*N*-(Pyridin-3-ylmethoxycarbonyl)aminomethyl]-benzoyl Chloride Hydrochloride (3).** To a suspension of **2** (40 g, 140 mmol) in toluene (2000 mL) were added DMF (0.8 mL) and oxalyl chloride (24 mL). After the mixture stirred for 4 h, a white solid was collected by filtration, washed with toluene (500 mL) and diisopropyl ether (500 mL), respectively, and dried to give the title compound (47.7 g, quantitatively), which was very hygroscopic and used with no further purification.

**Method A: *N*-(2-Aminophenyl)-4-[*N*-(pyridin-3-ylmethoxycarbonyl)aminomethyl]benzamide (1, MS-275).** To a solution of imidazole (0.63 g, 9.2 mmol) in THF (20 mL) was added **3** (1 g, 2.9 mmol), and the mixture stirred for 1 h at room temperature. After imidazole hydrochloride was removed by filtration, 1,2-phenylenediamine (2.52 g, 23.2 mmol) and trifluoroacetic acid (0.2 mL, 2.6 mmol) were added to the filtrate and stirred for 15 h. The reaction mixture was evaporated to remove THF and partitioned between ethyl acetate (500 mL) and water (400 mL). The organic layer was washed with water and dried and then purified by silica gel column chromatography (ethyl acetate) to give **1** (0.62 g, 56% yield): mp 159−160 °C; $^1$H NMR (DMSO-$d_6$) $\delta$ 4.28 (d, 2H, $J$ = 5.9 Hz), 4.86 (s, 2H), 5.10 (s, 2H), 6.60 (t, 1H, $J$ = 7.3 Hz), 6.78 (d, 1H, $J$ = 7 Hz), 6.97 (t, 1H, $J$ = 7 Hz), 7.17 (d, 1H, $J$ = 8 Hz), 7.3−7.5(m, 3H), 7.78 (d, 1H, $J$ = 8 Hz), 7.93 (d, 2H, $J$ = 8 Hz), 8.53 (d, 1H, $J$ = 3.7 Hz), 8.59 (s, 1H), 9.61 (s, 1H); IR (KBr) 3295, 1648, 1541, 1508, 1457, 1309, 1183, 742 cm$^{-1}$. Anal. ($C_{21}H_{20}N_4O_3$) C, H, N.

**Method B: *N*-(2-Amino-5-methylphenyl)-4-[*N*-(pyridin-3-ylmethoxycarbonyl)aminomethyl]benzamide (6e). (i) *N*-(5-Methyl-2-nitrophenyl)-4-[*N*-(pyridin-3-ylmethoxycarbonyl)aminomethyl]benzamide (5e).** To a solution of 5-methyl-2-nitroaniline (1 g, 6.5 mmol) in pyridine (15 mL) was added **3** (2 g, 5.9 mmol). After stirring for 1 h, the mixture was evaporated and partitioned between ethyl acetate and water. The organic layer was washed with water, dried over anhydrous sodium sulfate, and then evaporated. The residue was recrystallized from ethyl acetate to give **5e** (1.3 g, 54% yield): mp 138 °C; $^1$H NMR (DMSO-$d_6$) $\delta$ 2.43 (s, 3H), 4.30 (d, 2H, $J$ = 5.9 Hz), 5.10 (s, 2H), 7.20−7.24 (m, 1H), 7.39−7.45 (m, 3H), 7.74−7.81 (m, 2H), 7.90−8.00 (m, 4H), 8.53−8.60 (m, 2H), 10.69 (s, 1H); IR (KBr) 3293, 1685, 1603, 1586, 1542, 1497, 1334, 1261, 745 cm$^{-1}$.

**(ii) *N*-(2-Amino-5-methylphenyl)-4-[*N*-(pyridin-3-ylmethoxycarbonyl)aminomethyl]benzamide (6e).** Compound **5e** (0.6 g, 1.38 mmol), $SnCl_2$ dihydrate (1.84 g, 8.15 mmol), and ammonium acetate (1.1 g, 14.3 mmol) in methanol (40 mL) were refluxed for 30 min. The mixture was evaporated to reduce the volume (to 5 mL) and extracted with ethyl acetate (150 mL). The organic layer was washed with saturated sodium bicarbonate (100 mL), water (100 mL, three times), and saline (100 mL), then dried over magnesium sulfate, and evaporated. Recrystallization from ethanol gave the title compound (0.31 g, 57% yield): mp 167−168 °C; $^1$H NMR (DMSO-$d_6$) $\delta$ 2.17 (s, 3H), 4.27 (d, 2H, $J$ = 6 Hz), 4.68 (s, 2H), 5.10 (s, 2H), 6.68 (d, 1H, $J$ = 8 Hz), 6.79 (d, 1H, $J$ = 8 Hz), 7.00 (s, 1H), 7.3−7.5 (m, 3H), 7.79 (d, 1H, $J$ = 8 Hz), 7.9−8.0 (m, 3H), 8.53 (d, 1H, $J$ = 3 Hz), 8.59 (s, 1H), 9.59 (s, 1H); IR (KBr) 3289, 1742, 1637, 1530, 1492, 1257, 1134, 1049, 817, 711 cm$^{-1}$; FAB-HRMS $m/z$ calcd 391.1770, found 391.1759 (M + H)$^+$. Anal. ($C_{22}H_{22}N_4O_3$) H, N; C: calcd, 67.68; found, 67.15.

**Pharmacology. In Vitro Inhibition of Histone Deacetylase.** Histone deacetylase fraction was prepared as described by Yoshida et al.[3] Human leukemia K562 ($2.5 \times 10^8$) cells were disrupted in buffer-A (15 mM potassium phosphate buffer (pH 7.5) containing 5% glycerol and 0.2 mM EDTA) (15 mL). The nuclei were collected by centrifugation (35000*g*, 10 min) and resuspended with buffer-A (15 mL) containing 1 M $(NH_4)_2SO_4$. After sonication, the supernatant was collected by centrifugation, and ammonium sulfate was added to make the final concentration 3.5 M. After stirring for 1 h at 0 °C, the precipitate was collected by centrifugation, dissolved with buffer-A (4 mL), and dialyzed against buffer-A (2000 mL). The dialysate was loaded onto a Mono Q HR 5/5 column (Pharmacia) equilibrated with buffer-A and eluted with a linear gradient of 0−1 M NaCl in buffer-A (30 mL). A single peak of histone deacetylase activity was eluted around 0.4 M NaCl, and the fraction was stored at −80 °C until use.

Inhibition of histone deacetylase was estimated as described by Yoshida et al.[3] with slight modifications. $^3$H-Labeled histone was prepared by the method of Yoshida et al.:[3] K562 cells ($10^8$ cells) were incubated in growth medium (25 mL) containing 0.5 mCi/mL [$^3$H]sodium acetate (152.8 GBq/mmol; NEN) and 5 mM sodium butyrate at 37 °C.

Histone deacetylase inhibitory activity of test compound was measured as follows: the mixture (total volume 50 $\mu$L) containing the above histone deacetylase fraction (2 $\mu$L), $^3$H-labeled histone (100 $\mu$g/mL), and test compound (5 $\mu$L) was incubated for 10 min at 37 °C. [$^3$H]Acetic acid, which was liberated from $^3$H-labeled histone, was extracted with ethyl acetate, and radioactivity was measured by a liquid scintillation counter.

**Supporting Information Available:** Melting point, $^1$H NMR spectra, and IR spectra for compounds **4a**−**e** and **6a**−**d**,**f**−**h** and HRMS and full $^1$H NMR spectra for compounds **4d** and **6a**,**b**,**e**,**g**. This material is available free of charge via the Internet at http://pubs.acs.org.

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