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Bicyclic tetrapeptide histone deacetylase inhibitors with methoxymethyl ketone and boronic acid zinc-binding groups

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ABSTRACT

Histone deacetylase (HDAC) inhibitors are a class of potential therapeutics for the treatment of cancer. Bicyclic tetrapeptides equipped with methoxymethyl ketone and boronic acid as zinc-binding group were designed and synthesized. The inhibitory activities of these compounds were evaluated against HDAC enzymes. The cell-free and cell-based assay data showed that both potency and selectivity changed with the change in zinc-binding group. Boronic acid-based compound showed poor activity whereas methoxymethyl ketone-based compound displayed impressive activity in both cell-free and cell-based conditions.

1. Introduction

Histone deacetylase (HDAC) inhibitors, belonging to an emerging class of therapeutics with potential as anticancer drugs, not only cause growth arrest, differentiation, and apoptosis of tumor cells but also have shown promise as anti-parasitic, anti-neurodegenerative, anti-rheumatologic, and immunosuppressant agents [1,2]. As HDAC inhibitors display their biological effects across multiple pathways within the malignant cell, including extrinsic and intrinsic apoptosis, autophagy, inhibiting proliferation, migration, and tumor angiogenesis and effects in the immune response [1], design and synthesis of HDAC inhibitors has become an attracting field for research. The United States Food and Drug Administration (FDA) has approved two HDAC inhibitors, vorinostat and romidepsin, for the treatment of cutaneous T-cell lymphoma (CTCL) which are now available in the market [3]. This approval has added a new dimension in this research field.

So far, several structurally distinct HDAC inhibitors including hydroxamates, benzamides, short-chain fatty acids, electrophilic ketones, and macrocyclic peptides have been reported [2]. However, most of the reported HDAC inhibitors including trichostatin A (TSA) and SAHA are regarded as broad spectrum with wide range of side effects [4]. Therefore, research in this field is now going on to develop isoform selective HDAC inhibitors. The main focus of these researches is on the optimization of cap groups and/or zinc-binding groups, as the spacer groups have been optimized to six methylene units [5]. Komatsu and co-workers reported a series of cyclic tetrapeptides hydroxamic acid [6]. Our group reported a number of cyclic tetrapeptides HDAC inhibitors containing a variety of functional groups such as retrohydroxamate [7], SS-hybrid [8], trifluoromethyl, pentafluoroethyl ketones [9], carbonyl group [10], acryloyl chloride, and chloroacetic acid [11]. To increase the size of macrocyclic cap group, Nishino and co-workers synthesized bicyclic peptide disulfide hybrids which showed good activity in both cell-free and cell-based conditions in nanomolar range [12]. As a continuation, we reported bicyclic terapeptides hydroxamic acid as potent HDAC inhibitors in which the aliphatic loop length was optimized to eleven methylene units (compound **2**, Fig. 1) [13]. Comparative study of bicyclic tetrapeptide HDAC inhibitors containing disulfide hybrid and hydroxamic acid showed a significant change in both activity and selectivity as the zinc-binding group (ZBG) was changed from SS-hybrid to hydroxamic acid [12,13]. Inspired by these facts we designed two more bicyclic tetrapeptide HDAC inhibitors to explore the effect of other ZBG on the activity of bicyclic tetrapeptides. The designing was originated from our report of boronic acid and methoxymethyl ketone-based cyclic tetrapeptide HDAC inhibitors (compound **3** and **4**, Fig. 1) [11].

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In this paper, we describe the account on synthesis and a brief description of interesting biological results of methoxymethyl ketone and boronic acid-based bicyclic tetrapeptide HDAC inhibitors having optimized loop length (Fig. 2).

2. Results and discussion

2.1. Chemistry

Bicyclic tetrapeptide methoxymethyl ketone (**6**) was synthesized according to Scheme 1 by the conventional solution phase method. The synthesis was started by coupling H-D-Pro-O^{t}Bu (**7**) with Boc-L-2-amino-8-nonenoic acid (Boc-L-Ae9-OH) using DCC/HOBt to obtain protected dipeptide (**8**). Boc protection was selectively removed by 4 M HCl/dioxane, and the free amine was condensed with Boc-D-2-amino-7-octenoic acid (Boc-D-Ae8-OH) by the same DCC/HOBt method to obtain linear tripeptide (**9**). The linear tripeptide with fused side ring (**10**) was synthesized by ring-closing metathesis (RCM) between D-Ae8 and L-Ae9 using Grubb's first generation catalyst in dichloromethane (DCM), followed by catalytic hydrogenation in presence of Pd–C. After selective deprotection, Boc-L-Ae9-OH was incorporated to prepare the linear tetrapeptide (**11**). After removal of both side protections by treating with trifluoroacetic acid (TFA), cyclization reaction was carried out by the aid of HATU in DMF under high dilution conditions with minimum amount of DIEA (2.5 equiv) to yield bicyclic tetrapeptide with terminal alkene in the side chain (**12**). The side chain terminal alkene was modified to epoxide (**13**) by the aid of 3-Chloroperbenzoic acid (m-CPBA) in DCM. Opening of the epoxide ring by the use of NaOMe/MeOH and subsequent oxidation of alcoholic group ((**14**) to keto group using Dess–Martin periodinane (DMP) yielded the bicyclic tetrapeptide methoxymethyl ketone (**6**).

Bicyclic tetrapeptide boronic acid (**5**) was synthesized according to Scheme 2. In this case, the same linear tripeptide with fused ring (**10**) was used as starting material. After selective deprotection, Boc-L-2-amino-6-heptenoic acid (Boc-L-Ae7-OH) was incorporated to prepare the linear tetrapeptide (**15**). After removal of both the side protections by treating with trifluoroacetic acid, cyclization reaction was carried out by the aid of HATU in DMF (0.2 mM) with minimum amount of DIEA (2.5 equiv) to yield bicyclic tetrapeptide with terminal alkene in the side chain (**16**). Pinacole borane was incorporated to the side chain (**17**) by treating with pinacole borane in presence of $[Ir(cod)Cl]_2$ and bis(diphenylphosphino)methane (dppm) in DCM. Finally, pinacole protection was removed by treating with $NaIO_4$ and NH_4OAc to yield bicyclic tetrapeptide boronic acid (**5**).

All the synthesized compounds were characterized by ^{1}H NMR and high resolution FAB–MS. The purity of the compounds was determined by HPLC analysis all the synthesized cyclic/bicyclic tetrapeptides showed purity above 95%.

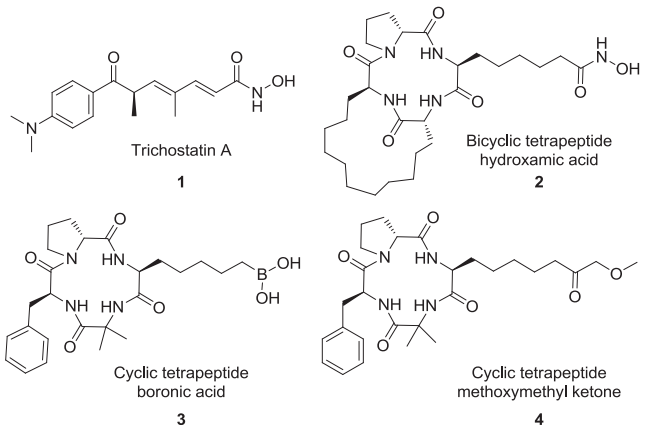


Fig. 1. Some reported HDAC inhibitors.

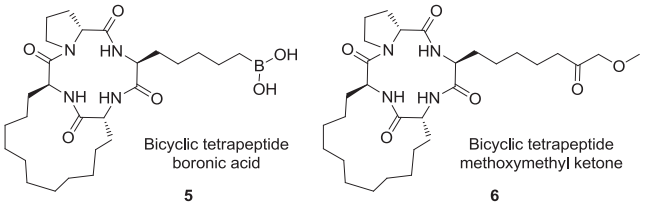


Fig. 2. Synthesized bicyclic tetrapeptide methoxymethyl ketone and boronic acid.

2.2. Enzyme inhibition and biological activity

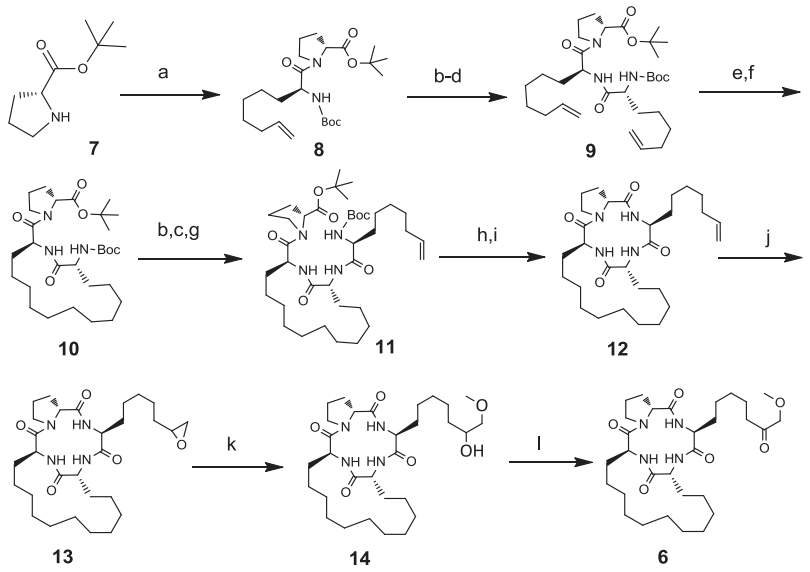
The synthesized bicyclic tetrapeptide boronic acid and methoxymethyl ketone were assayed for HDAC inhibitory activity using HDAC1, HDAC4, and HDAC6 enzymes prepared from 293T cells [14]. Additionally, to know the inhibitory activity of these compounds in cell-based condition; we carried out p21 promoter assay according to the literature [10]. The detail experimental procedures are described in the experimental section of this paper. The results of the HDAC inhibitory activity and the p21 promoter assay of the compounds are shown in Table 1.

To evaluate the potency and selectivity of synthesized compounds **5** and **6** as HDAC inhibitors, their activity on distinct isoforms (HDAC1, 4 and 6) was compared with that of compounds **2**, **3** and **4** (Table 1). Compounds **2**, **5** and **6** contain the same scaffold with different ZBG, hydroxamic acid, boronic acid and methoxymethyl ketone respectively. Among them, boronic acid showed poor activity with micromolar range in both cell-free and cell-based conditions. Suzuki and co-workers reported a series of boronic acid-based HDAC inhibitors, which also showed inhibitory activity in micromolar range [15]. Our reported cyclic tetrapeptide boronic acid (**3**) also exhibited HDAC inhibitory activity in micromolar range.

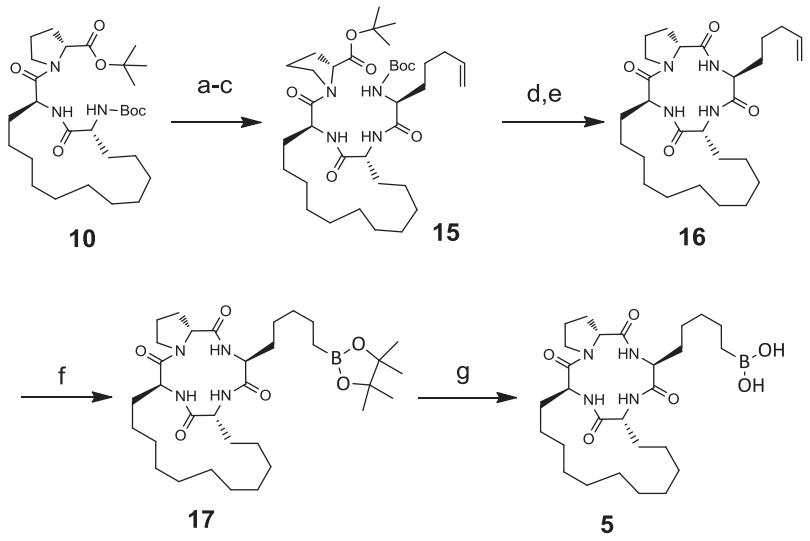
Therefore, boronic acid seems to be not promising as zinc-binding group. It may be due to the poor zinc-binding affinity for this group in the mechanism of HDAC inhibition. On the other hand, bicyclic tetrapeptide methoxymethyl ketone **6** displayed impressive inhibitory activity against HDAC1, 4, and 6. This reveals that the extent of zinc-binding ability of methoxymethyl ketone in the mechanism of HDAC inhibition is highly effective. It also showed better activity than monocyclic tetrapeptide methoxymethyl ketone, **4**. It implies that bicyclic tetrapeptide scaffold has better interaction with surface region of the HDAC enzymes. This observation supports the fact that complex cap-groups interact with HDAC enzyme's outer rim and demonstrate improved HDAC inhibition [2]. The nanomolar inhibitory activity of compound **6** is similar to our formerly reported bicyclic tetrapeptide hydroxamic acid **2**. Moreover, compound **6** showed much more selectivity towards HDAC 1 and 4 over HDAC 6 compared to that of compound **2**. As hydroxamic acid-based HDAC inhibitors suffer from some limitations [16], methoxymethyl ketone-based HDAC inhibitor might be an alternative in the field of HDAC inhibitors.

3. Conclusion

To explore the effect of zinc-binding groups on activity and selectivity, bicyclic tetrapeptide HDAC inhibitors bearing methoxymethyl ketone and boronic acid were synthesized and their inhibitory activity were evaluated. The cell-free and cell-based assay



Scheme 1. Synthesis of bicyclic tetrapeptide methoxymethyl ketone (**6**). Reagents and conditions: (a) Boc-L-Ae9-OH, DCC, HOBt, DMF, 12 h, 25 °C, 85%; (b) 4 M HCl/dioxane, 35 min, 25 °C, 77%; (c) saturated Na_2CO_3; (d) Boc-D-Ae8-OH, DCC, HOBt, DMF, 12 h, 25 °C, 85%; (e) Grubbs' first generation catalyst, DCM, 48 h, 25 °C, 74%; (f) AcOH, Pd–C, H_2, 12 h, 25 °C, 99%; (g) Boc-L-Ae9-OH, DCC, HOBt, DMF, 12 h, 25 °C, 75%; (h) TFA, 3 h, 25 °C, 100%; (i) HATU, DIEA, DMF, 4 h, 25 °C, 71%; (j) m-CPBA, DCM, 12 h, 25 °C, 74%; (k) NaOMe/MeOH, 12 h, 25 °C, 17%; and (l) DMP, DCM, 12 h, 25 °C, 65%.



Scheme 2. Synthesis of bicyclic tetrapeptide boronic acid (**5**). Reagents and conditions: (a) 4 M HCl/dioxane, 35 min, 25 °C, 75%; (b) saturated Na_2CO_3; (c) Boc-L-Ae7-OH, DCC, HOBt, DMF, 12 h, 25 °C, 75%; (d) TFA, 3 h, 25 °C, 98%; (e) HATU, DIEA, DMF, 4 h, 25 °C, 68%; (f) $[Ir(cod)Cl]_2$, Bis(diphenylphosphino)methane (dppm), Pinacolbarane, DCM, 48 h, 25 °C, 34%; and (g) NaIO4, NH_4OAc, Acetone, H_2O, 4 h, 25 °C, 20%.

Table 1
HDAC inhibitory activity and p21 promoter assay data for reported and reference compounds.

Compound	IC_{50} (nM)			p21 promoter assay, EC_{1000} (nM)
	HDAC1	HDAC4	HDAC6	
1 (TSA)	23	44	65	20
2	11	4.5	280	2.6
3	2100	150	21,000	3600
4	38	28	>100,000	43
5	12,000	9400	>100,000	10,000
6	27	26	>100,000	32

Values are means of at least three experiments.

studies showed that both potency and selectivity changed with the change in zinc-binding group. Boronic acid-based compound showed poor activity whereas methoxymethyl ketone-based compound displayed impressive activity in both cell-free and cell-based conditions. Therefore, methoxymethyl ketone-based bicyclic tetrapeptide HDAC inhibitors might be promising candidates in the field of HDAC inhibitor research.

4. Experimental

4.1. General

Unless otherwise noted, all solvents and reagents were reagent grade and used without purification. Flash chromatography was performed using silica gel 60 (230–400 mesh) eluting with solvents as indicated. All compounds were routinely checked by thin layer chromatography (TLC) and/or high performance liquid chromatography (HPLC). TLC was performed on aluminum-backed

silica gel plates (Merck DC-Alufolien Kieselgel 60 F_{254}) with spots visualized by UV light or charring. Analytical HPLC was performed on a Hitachi instrument equipped with a chromolith performance RP-18e column (4.6 × 100 mm, Merck). The mobile phases used were A: H_2O with 10% CH_3CN and 0.1% TFA, B: CH_3CN with 0.1% TFA using a solvent gradient of A–B over 15 min with a flow rate of 2 mL/min, with detection at 220 nm. FAB-mass spectra were measured on a JEOL JMS-SX 102A instrument. NMR spectra were recorded on a JEOL JNM A500 MHz spectrometer. Unless otherwise stated, all NMR spectra were measured in $CDCl_3$ solutions with reference to TMS. All ^1H shifts are given in parts per million (s = singlet; d = doublet; t = triplet; m = multiplet). Assignments of proton resonances were confirmed, when possible, by correlated spectroscopy. Amino acids were coupled using standard solution-phase chemistry with dicyclohexylcarbodiimide (DCC), O-(7-azabenzotriazoyl-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU).

4.2. Synthesis of bicyclic tetrapeptide methoxymethyl ketone (Mmk) (**6**)

To a cooled solution of H-D-Pro-O^{t}Bu (**7**) (1.71 g, 10 mmol), Boc-L-Ae9-OH (2.71 g, 10 mmol), and HOBt·H_2O (1.53 g, 10 mmol) in dimethylformamide (DMF) (20 mL), DCC (2.47 g, 12 mmol) was added. The mixture was stirred for 12 h at room temperature (25 °C). After completion of the reaction, DMF was evaporated and the residue was dissolved in ethyl acetate and successively washed with 10% citric acid, 4% sodium bicarbonate, and brine. The ethyl acetate solution was dried over anhydrous $MgSO_4$ and concentrated to remain an oily substance, which was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield Boc-L-Ae9-D-Pro-O^{t}Bu (**8**) (3.60 g, 85%) as an oil. The protected dipeptide (1.74 g, 4 mmol) was dissolved in 4 M HCl/dioxane (20 mL) and the mixture was kept at room temperature for 35 min. The reaction was monitored by TLC. After completion of the reaction HCl/dioxane was evaporated. The residue was dissolved in EtOAc, washed with saturated Na_2CO_3, and dried over anhydrous Na_2CO_3. EtOAc was evaporated to get H-L-Ae9-D-Pro-O^{t}Bu as a heavy oil (1.0 g, 77%). To a cooled solution of H-L-Ae9-D-Pro-O^{t}Bu (1.0 g, 3.1 mmol), Boc-D-Ae8-OH (956 mg, 3.7 mmol) and HOBt·H_2O (474 mg, 3.1 mmol) in DMF (8 mL), DCC (762 mg, 3.7 mmol) was added and stirred for 12 h at room temperature. The product Boc-D-Ae8-L-Ae9-D-Pro-O^{t}Bu (**9**) was obtained in the same manner as described earlier as a heavy oil (1.48 g, 85%), HPLC, retention time 8.84 min. To a solution of linear tripeptide Boc-D-Ae8-L-Ae9-D-Pro-O^{t}Bu (1.48 g, 2.64 mmol) in anhydrous and degassed DCM (325 mL), a solution of Grubbs first generation ruthenium catalyst (435 mg, 0.524 mmol) in anhydrous and degassed DCM (80 mL) was added. The reaction mixture was stirred at room temperature for 48 h. After the completion of reaction, DCM was evaporated and the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield linear tripeptide with fused cycle as a foam which on catalytic hydrogenation in presence of Pd–C (100 mg) in AcOH (10 mL) yield compound **10** (1.02 g, 73%), HPLC, retention time 7.92 min. The protected tripeptide (1.02 mg, 1.9 mmol) was dissolved in 4 M HCl/dioxane (8 mL) and the mixture was kept at room temperature for 35 min. The reaction was monitored by TLC. After completion of the reaction HCl/dioxane was evaporated. The residue was dissolved in EtOAc, washed with saturated Na_2CO_3, and dried over anhydrous Na_2CO_3. EtOAc was evaporated to get tripeptide free amine (620 mg, 75%). To a cooled solution of the free amine (310 mg, 0.71 mmol), Boc-L-Ae9-OH (212 mg, 0.78 mmol) and HOBt·H_2O (109 mg, 0.71 mmol) in DMF (2 mL), DCC (176 mg, 0.85 mmol) was added and stirred for 12 h at room temperature. The product linear tetrapeptide (**11**) (370 mg, 75%) was obtained in the same manner as described earlier as a foam. HPLC, retention time 11.43 min. The protected tetrapeptide (370 mg, 0.53 mmol) was dissolved in TFA (3 mL) at 0 °C and kept for 3 h. After evaporation of TFA, the residue was solidified using ether and petroleum ether to yield TFA salt of the linear tetrapeptide (335 mg, 100%). To DMF solvent (500 mL), the TFA salt (335 mg, 0.53 mmol), HATU (304 mg, 0.80 mmol), and DIEA (0.23 mL, 1.33 mmol) were added in separate five portions in every 30 min with stirring, for the cyclization reaction. After completion of the reaction, DMF was evaporated under vacuo, the residue was dissolved in ethyl acetate and washed with citric acid (10%) solution, sodium bicarbonate (4%) solution, and brine, successively. It was then dried over anhydrous $MgSO_4$ and filtered. After evaporation of ethyl acetate, the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield the bicyclic tetrapeptide **12** (195 mg, 71%). HPLC, retention time 9.14 min. Compound **12** (190 mg, 0.37 mmol) was dissolved in anhydrous DCM (4 mL) in ice bath and 3-Chloroperbenzoic acid (m-Chloroperbenzoic acid, m-CPBA) (128 mg) was added over 30 min. The mixture was stirred for 12 h. After the completion of the reaction, the reaction mixture was diluted to 30 mL by the addition of DCM. The resultant solution was washed with 4% $NaHCO_3$ and brine. The DCM solution was dried over anhydrous $MgSO_4$, concentrated and was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield bicyclic tetrapeptide epoxide (**13**) (145 mg, 74%) as a foam. HPLC retention time 6.50 min. To the epoxide (140 mg, 0.26 mmol) 0.5 M NaOMe/MeOH (3 mL) was added in ice bath, and was stirred at room temperature for 12 h. The reaction was quenched by the addition of acetic acid and water, and then methanol was evaporated. The residue was dissolved in ethyl acetate, and was washed successively with 10% citric acid, 4% $NaHCO_3$, and brine. It was then dried over anhydrous $MgSO_4$ and filtered. After evaporation of ethyl acetate, the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield compound **14** (25 mg, 17%). During the reaction peptide ring opening resulted in low yield. The methoxy alcohol (25 mg, 0.044 mmol) was dissolved in DCM (2 mL) and Dess–Martin periodinane (DMP) (25 mg, 0.057 mmol) was added. The reaction mixture was stirred for 12 h at room temperature under argon atmosphere. The reaction was quenched by the addition of saturated $NaHCO_3$ solution (7 mL) containing $Na_2S_2O_3·5H_2O$ (56 mg, 0.228 mmol), and was stirred vigorously to get two clear layers. The DCM layer was separated and was washed with brine followed by drying over $MgSO_4$. DCM was evaporated and the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield the bicyclic tetrapeptide methoxymethyl ketone (Mmk) (**6**) (16 mg, 65%) as a crystalline solid. HPLC, retention time 7.15 min; ^1H NMR (500 MHz, CDCl3) δH 1.14–1.17 (m, 2H), 1.20–1.36 (m, 18H), 1.41–1.47 (m, 4H), 1.54–1.63 (m, 4H), 1.78–1.86 (m, 2H), 1.90–2.06 (m, 4H), 2.24–2.33 (m, 1H), 3.42 (t, *J* = 7.4 Hz, 2H), 3.41 (s, 3H), 3.51 (dd, *J* = 17.7, 7.6 Hz, 1H), 3.99 (s, 2H), 4.12 (ddd, *J* = 9.9, 8.8, 4.7 Hz, 1H), 4.24 (dd, *J* = 17.9, 7.5 Hz, 1H), 4.55 (t, *J* = 10.8 Hz, 1H), 4.72 (d, *J* = 7.9 Hz, 1H), 4.86 (t, *J* = 10.1 Hz, 1H), 6.12 (d, *J* = 10.1 Hz, 1H), 6.23 (d, *J* = 10.4 Hz, 1H), 7.07 (d, *J* = 10.1 Hz, 1H); FAB–MS $[M + H]^+$ 563.3812 for $C_{30}H_{51}N_4O_6$ (calcd 563.3809).

4.3. Synthesis of bicyclic tetrapeptide boronic acid (**5**)

The protected tripeptide **10** (1.02 g, 1.90 mmol) was selectively deprotected using the method described above to obtain tripeptide free amine (620 mg, 75%). The tripeptide free amine (305 mg, 0.7 mmol) was coupled with Boc-L-Ae7-OH (204 mg, 0.84 mmol)

according to the method described earlier and the fully protected crude linear tetrapeptide was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield linear tetrapeptide **15** (350 mg, 75%, HPLC, rt 10.65 min) as a foam. Compound **15** (340 mg, 0.51 mmol) was dissolved in TFA (3 mL) at 0 °C and kept at room temperature for 3 h. After evaporation of TFA, the residue was crystalized by using the mixture of ether:-pet-ether (1:4) to yield TFA salt of linear tetrapeptide (302 mg, 98%, HPLC, retention time 6.66 min). To a volume of Sp. DMF (500 mL), TFA salt of linear tetrapeptide (302 mg, 0.5 mmol), HATU (285 mg, 0.75 mmol) and DIEA (218 μL, 1.25 mmol) were added in five aliquots in 30 min intervals while the solution was stirred vigorously. After the final addition, the reaction mixture was allowed to stir for an additional hour. Completion of the cyclization reaction was monitored by HPLC, and then DMF was evaporated under reduced pressure. The crude cyclic tetrapeptide was dissolved in ethyl acetate and the solution was washed successively by 10% citric acid, 4% sodium bicarbonate, and brine. Finally, the ethyl acetate solution was dried over anhydrous $MgSO_4$ and filtered. After evaporation of ethyl acetate, the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield bicyclic tetrapeptide **16** (166 mg, 68%, HPLC, retention time 7.85 min) as a foam after drying in vacuo.

To a solution of $[Ir(cod)Cl]_2$ (23 mg, 0.034 mmol) and bis(diphenylphosphino)methane (dppm) (27 mg, 0.068 mmol) in anhydrous and degassed DCM (4.0 mL), compound **12** (166 mg, 0.34 mmol) and pinacolborane (HBpin) (80 μL, 0.51 mmol) were added. The mixture was flashed with Ar for 5 min, and was stirred for 48 h at room temperature. The reaction mixture was concentrated by evaporating DCM and purified by silica gel column chromatography using a mixture of chloroform and methanol (99:1) to yield compound **17** (72 mg, 34%, HPLC, retention time 9.22 min) as a crystalline solid. To a solution of compound **17** (72 mg, 0.12 mmol) in acetone (2 mL) and H_2O (1 mL), $NaIO_4$ (77 mg, 0.36 mmol) and NH_4OAc (28 mg, 0.36 mmol), were added and the suspension was stirred at room temperature for 4 h. Then acetone was evaporated and the reaction mixture was poured into water and extracted with ethyl acetate. Finally, the ethyl acetate solution was dried over anhydrous $MgSO_4$ and filtered. After evaporation of ethyl acetate, the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (98:2) to yield bicyclic tetrapeptide boronic acid (**5**) (13 mg, 20%) as a crystalline solid. HPLC, retention time 6.49 min; ^1H NMR (500 MHz, $CDCl_3$) δ_H 1.12–1.18 (m, 2H), 1.24–1.33 (m, 18H), 1.37–1.47 (m, 6H), 1.53–1.57 (m, 4H), 1.77–1.86 (m, 2H), 1.90–2.06 (m, 4H), 2.24–2.33 (m, 1H), 3.52 (dd, J = 17.7, 7.6 Hz, 1H), 3.63 (t, J = 6.4 Hz, 1H), 4.12 (m, 1H), 4.27 (dd, J = 17.7, 7.8 Hz, 1H), 4.55 (t, J = 9.7 Hz, 1H), 4.72 (d, J = 7.6 Hz, 1H), 4.86 (t, J = 10.7 Hz, 1H), 6.19 (d, J = 10.1 Hz, 1H), 6.27 (d, J = 10.4 Hz, 1H), 7.10 (d, J = 10.4 Hz, 1H); FAB–MS $[M + Glycerol - 2H_2O - H]^-$ 589 for $C_{30}H_{50}BN_4O_7$ (calcd 589).

4.4. Preparation of HDACs and assay for enzyme activity

293T cells (1–2 × 10^6) were grown in a 100-mm dish for 24 h and transiently transfected with 10 μg each of the vector pcDNA3-HDAC1 for human HDAC1, pcDNA3-HDAC4 for human HDAC4, or pcDNA3-mHDA2/HDAC6 for mouse HDAC6, using the LipofectAMINE2000 reagent (Invitrogen). After successive cultivation in DMEM for 24 h, the cells were washed with PBS and lysed by sonication in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 120 mM NaCl, 5 mM EDTA, and 0.5% NP40. The soluble fraction collected by microcentrifugation was precleared by incubation with protein A/G plus agarose beads (Santa Cruz Biotechnologies, Inc.). After the cleared supernatant had been incubated for 1 h at 4 °C with 4 μg of an anti-FLAG M2 antibody (Sigma–Aldrich Inc.) for HDAC1, HDAC4, and HDAC6, the agarose beads were washed three times with lysis buffer and once with histone deacetylase buffer consisting of 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 10% glycerol. The bound proteins were released from the immune complex by incubation for 1 h at 4 °C with 40 μg of the FLAG peptide (Sigma–Aldrich Inc.) in histone deacetylase buffer (200 μL). The supernatant was collected by centrifugation. For the enzyme assay, 10 μL of the enzyme fraction was added to 1 μL of fluorescent substrate (2 mM Ac-KGLGK(Ac)-MCA) and 9 μL of histone deacetylase buffer, and the mixture was incubated at 37 °C for 30 min. The reaction was stopped by the addition of 30 μL of trypsin (20 mg/ml) and incubated at 37 °C for 15 min. The released aminomethyl coumarin (AMC) was measured using a fluorescence plate reader. The 50% inhibitory concentrations (IC_{50}) were determined as means with SD calculated from at least three independent dose response curves.

4.5. The p21 promoter assay

The human wild-type p21 promoter luciferase fusion plasmid, WWP-Luc, was a kind gift from Dr. B. Vogelstein. A luciferase reporter plasmid (pGW-FL) was constructed by cloning the 2.4 kb genomic fragment containing the transcription start site into *Hin*dIII and *Sma*I sites of the pGL3-Basic plasmid (Promega Co., Madison, WI). Mv1Lu (mink lung epithelial cell line) cells were transfected with the pGW-FL and a phagemid expressing neomycin/kanamycin resistance gene (pBKCMV, Stratagene, La Jolla, CA) with the Lipofectamine reagent (Life Technology, Rockville, MD, USA). After the transfected cells had been selected by 400 μg/mL Geneticin (G418, Life Technology), colonies formed were isolated. One of the clones was selected and named MFLL-9. MFLL-9 expressed a low level of luciferase, whose activity was enhanced by TSA in a dose-dependent manner. MFLL-9 cells (1 × 105) cultured in a 96-well multi-well plate for 6 h were incubated for 18 h in the medium containing various concentrations of drugs. The luciferase activity of each cell lysate was measured with a Luc-Lite luciferase Reporter Gene Assay Kit (Packard Instrument Co., Meriden, CT) and recorded with a Luminescencer-JNR luminometer (ATTO, Tokyo, Japan). Data were normalized to the protein concentration in cell lysates. Concentrations at which a drug induces the luciferase activity 10-fold higher than the basal level are presented as the 1000% effective concentration (EC_{1000}).

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