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ORIGINAL ARTICLE

Design and synthesis of mono and bicyclic tetrapeptides thioester as potent inhibitor of histone deacetylases

Md. Ashraful Hoque · Md. Shahidul Islam · Md. Nurul Islam · Tamaki Kato · Norikazu Nishino · Akihiro Ito · Minoru Yoshida

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Abstract Inhibitors of histone deacetylases (HDACs) are a promising class of anticancer agents that have an effect on gene regulation. The naturally occurring cyclic depsipeptide FK228 containing disulfide and Largazole possessing thioester functionalities act as pro-drugs and share the same HDAC inhibition mechanism in cell. Inspired from these facts, we have reported bicyclic tetrapeptide disulfide HDAC inhibitors resembling FK228 with potent activity and enhanced selectivity. In the present study, we report the design and synthesis of several mono and bicyclic tetrapeptide thioester HDAC inhibitors that share the inhibition mechanism similar to Largazole. Most of the compounds showed HDAC1 and HDAC4 inhibition and p21 promoting activity in nanomolar ranges. Among these the monocyclic peptides **1**, **2** and bicyclic peptide, **4** are notable demanding more advanced research to be promising anticancer drug candidates.

Md. A. Hoque
Department of Chemistry, Faculty of Science, Universiti Putra Malaysia, 43400 Serdang, Malaysia

Md. A. Hoque
Department of Biochemistry and Molecular Biology, Faculty of Science, University of Rajshahi, Rajshahi 6205, Bangladesh

Md. A. Hoque (✉) · Md. S. Islam · Md. N. Islam · T. Kato · N. Nishino
Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology, Wakamatsu, Kitakyushu 808-0196, Japan
e-mail: ashraf_bio2003@yahoo.com

Md. S. Islam · Md. N. Islam
Department of Chemistry, Faculty of Science, University of Rajshahi, Rajshahi 6205, Bangladesh

A. Ito · M. Yoshida
RIKEN, Saitama 351-0198, Japan

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Introduction

It is now well established that epigenetic changes play a crucial role in carcinogenesis. Histone deacetylases (HDACs) play a prominent role in epigenetic changes by histone deacetylation and subsequent gene transcriptions that are involved in cell cycle regulation, proliferation, differentiation, and apoptosis (Hassig and Schreiber 1997; Grozinger and Schreiber 2002; Yoshida et al. 2003). Due to over-expression of HDACs in carcinogenesis (Kouzarides 1999; Archer and Hodin 1999; Cress and Seto 2000; Mahlknecht et al. 2000), the design and synthesis of novel HDAC inhibitors is a promising field for cancer research.

In fact, there are a variety of HDAC inhibitors in clinical trials, and suberoylanilide hydroxamic acid (SAHA) as Vorinostat (Mann et al. 2007) and depsipeptide, FK228 as ISTODAX® (Zain et al. 2010) are now in the market (Fig. 1). Even though the hydroxamates are most powerful HDAC inhibitors, the short half-life and low oral bioavailability due to unfavorable pharmacokinetic behavior resulting from glucuronidation, sulfation (Mulder and Meerman 1983), and metabolic hydrolysis (Michaelides et al. 2001; Frey et al. 2002), limit their use as practical drugs. Moreover, most of the hydroxamates, such as trichostatin A (TSA) and SAHA are regarded as broad spectrum HDAC inhibitors with wide range of side effects (Suzuki et al. 2006). Also, it has been reported that gastrointestinal side effects, including change in taste, nausea, vomiting, and anorexia are commonly seen following romidepsin (FK228) treatment (Dickinson and Prince 2012). Therefore, the current challenge in this field is to design and synthesis of selective

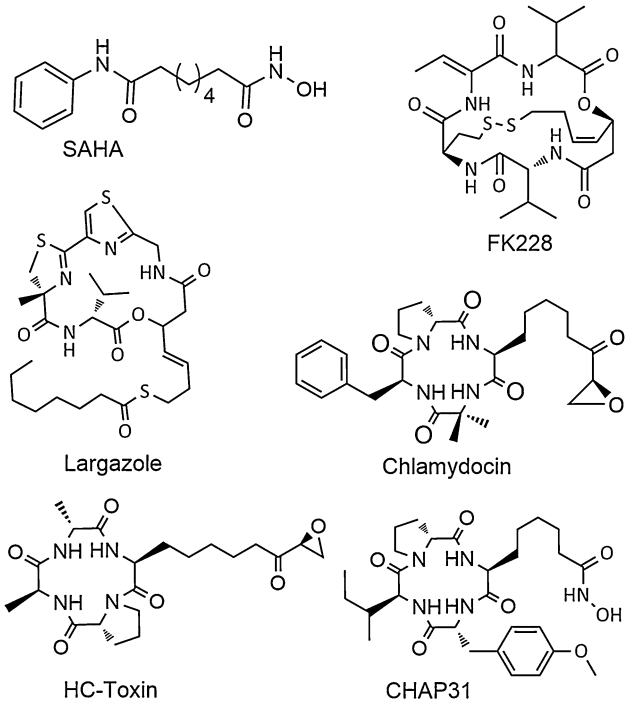


Fig. 1 Natural and conjugated cyclic peptide HDAC inhibitors

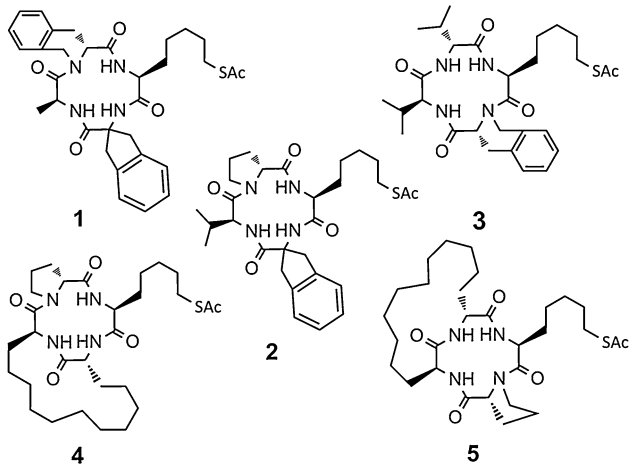


Fig. 2 The designed mono and bicyclic tetrapeptide thioesters

HDAC inhibitors which target cancer cells without affecting normal cells to reduce side effects.

Recently, the US FDA approved sulfur-containing cyclic peptide, FK228 as anticancer drug for the treatment of cutaneous T cell lymphoma (CTCL) (Zain et al. 2010). As a consequence, the sulfur-containing group draws much more attention as zinc binding group for their attractive inhibitory and specificity profile. Therefore, researchers are now trying to find more thiol-based HDAC inhibitors. Our group reported sulfur-containing cyclic tetrapeptides (SCOPs) as homo- and heterodimer (Nishino et al. 2003), bicyclic tetrapeptide disulfide hybrids (Nishino et al. 2008). As a continuation, we reported bicyclic tetrapeptides with intramolecular disulfide bridge (Hoque et al. 2012) as pro-drug like FK228. Moreover, it has been reported that Largazole (Taori et al. 2008), a thioester containing natural depsipeptide, shares HDAC inhibition mechanism similar to FK228 after ester hydrolysis. Considering these facts, herein we reported the design and synthesis of mono and bicyclic tetrapeptide thioester HDAC inhibitors based on Chlamydocin, HC-toxin, and CHAP31 scaffolds (Fig. 2).

Interactions between HDAC enzyme and the cyclic tetrapeptide scaffold of inhibitors are of great importance to selective inhibitors design (Butler and Kozikowski 2008). To increase inhibitory activity and selectivity by allowing cap group to bind properly to the hydrophobic rim region of HDAC enzyme, at first, we introduced several hydrophobic amino acids in the cyclic tetrapeptide scaffold and designed and synthesized three HDAC inhibitors (**1**-**3**) (Fig. 2).

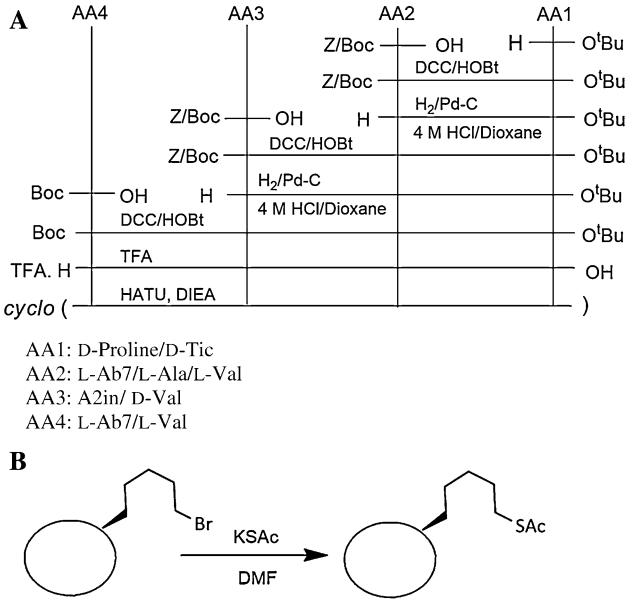
We designed chlamydocin-based cyclic tetrapeptide thioester, cyclo(-L-Am7(Ac)-A2in-L-Ala-D-Tic-) (**1**) by introducing D-1,1,2,2,-tertahydroisoquinoline-3-carboxylic acid (D-Tic), L-Ala, amino-2-indane carboxylic acid (A2in) and L-2-amino-7-bromoheptanoic acid (L-Ab7) in place of D-Pro, L-Phe, amino isobutyric acid (Aib) and 2-amino-8-oxo-9,10-epoxydecanoic acid (Aoe), respectively, of chlamydocin scaffold. Cyclo(-L-Am7(Ac)-A2in-L-Val-D-Pro-) (**2**) was designed by replacing D-Tic and L-Ala with D-Pro and L-Val, respectively, of compound **1** and HC-toxin-based compound cyclo(-L-Am7(Ac)-D-Tic-L-Val-D-Val-) (**3**) was designed by introducing more hydrophobic amino acids valine and Tic.

In the second step, the author designed and synthesized bicyclic tetrapeptides **4** and **5** (Fig. 2) to demonstrate the effect of aliphatic loop and its position on the biological activity of HDAC inhibitors. The aliphatic loop length was of eleven methylene groups as it was reported to be optimum (Islam et al. 2010). The sequence and configuration of amino acids in CHAP31 and HC-toxin were considered as the basis for designing the bicyclic peptides. L-2-amino-8-nonenoic acid (L-Ae9) and D-2-amino-7-octenoic acid (D-Ae8) were incorporated in the sequence, and the terminal alkenes were fused by the aid of Grubbs' first generation ruthenium (Ru) catalyst.

Results and discussion

Chemistry

Our aim was to synthesize several mono and bicyclic tetrapeptide thioester HDAC inhibitors resembling Largazole



Scheme 1 **a** Synthetic strategy of cyclic tetrapeptide scaffolds. **b** Side chain modification of cyclic tetrapeptides

with potent activity and enhanced selectivity. Synthesis was started from the preparation of the artificial building block amino acids. Boc-D/L-2-amino-7-octenoic acid (Boc-D/L-Ae8-OH), Boc-D/L-2-amino-8-nonenoic acid (Boc-D/L-Ae9-OH), Boc-L-2-amino-7-bromoheptanoic acid (Boc-L-Ab7), D-Tic and A2in were synthesized according to the reported procedure (Watanabe et al. 2004).

The designed compounds were obtained by synthesizing monocyclic tetrapeptides followed by side chain modification. Synthesis of cyclic tetrapeptides was carried out with tert-butyl-protected D-proline/D-Tic and coupled it with N-terminal protected L-amino acids (Boc-L-Ab7/Z-L-Ala/Z-L-Val) using DCC/HOBt to get protected dipeptides (Scheme 1a). After the removal of N-terminal protection by HCl-dioxane/catalytic hydrogenation, the free amines were extracted and used for condensation with other N-terninal protected amino acids (Z-A2in/Boc-D-Val) by the same DCC/HOBt method to obtain linear tripeptides.

The N-terminal of the tripeptides was further deprotected using catalytic hydrogenation/HCl-dioxane and coupled with Boc-protected L-Ab7/Boc-L-Val to yield linear tetrapeptides. After the removal of both side protections by treating with trifluoroacetic acid (TFA), the linear tetrapeptides were cyclized under high dilution conditions in dimethylformamide (DMF) using 2-(1H-7-Azobenzotriazol-1-yl)-tetramethyluronium hexafluorophosphate (HATU) and diisopropylethylamine (DIEA) to give cyclic tetrapeptides after purification by silica gel column chromatography.

The side chain of the cyclic tetrapeptides modified to a thioacetate group by treatment with potassium thioacetate to yield the desired compounds **1, 2** and **3** (Scheme 1b) after purification by silica gel column chromatography.

The synthesis of CHAP31-based bicyclic tetrapeptide thioester (**4**) was started by the conventional solution-phase method by coupling H-D-Pro-O^{t}Bu (**6**) with Boc-L-Ae9 using DCC/HOBt to obtain protected dipeptide (**7**). Boc protection was selectively removed by 4 M HCl/dioxane, and the free amine was condensed with Boc-D-Ae8 by the same DCC/HOBt method to obtain linear tripeptide (**8**). The linear tripeptide with fused side ring (**9**) was synthesized by ring-closing metathesis (RCM) between D-Ae8 and L-Ae9 using Grubbs' first generation catalyst in dichloromethane (DCM), followed by catalytic hydrogenation in presence of Pd-C. After selective deprotection, Boc-L-Ab7-OH was incorporated to prepare the linear tetrapeptide (**10**). 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 (**11**) which was treated with potassium thioacetate to obtain compound **4** (Scheme 2).

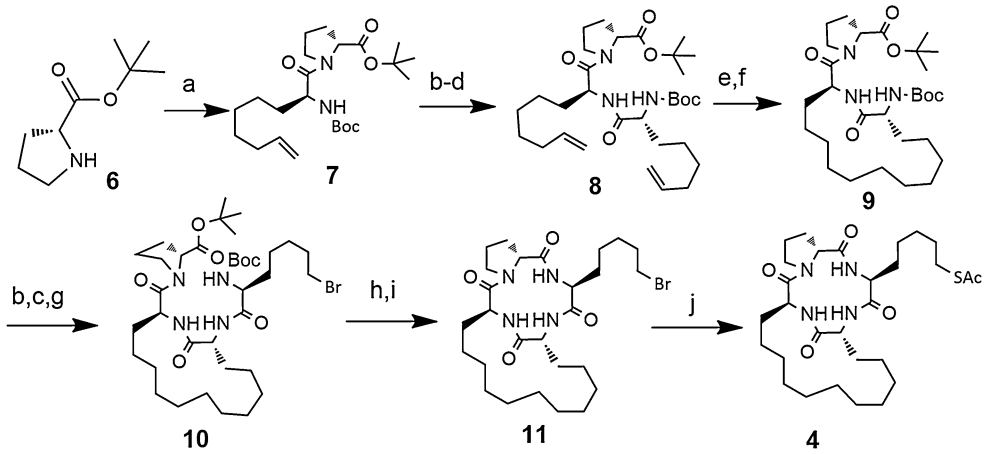
HC-toxin-based bicyclic tetrapeptide (**5**) was synthesized through fragment condensation of H-L-Ab7-D-Pro-O^{t}Bu (**13**) and Boc-fused side chain dipeptide–OH (**16**) by DCC/HOBt followed by cyclization and side chain modification to thioester (Scheme 3).

All the synthesized compounds were characterized by ^{1}H-NMR and high-resolution FAB-MS. The purity of compounds were determined by HPLC analysis and showed purity above 98 %.

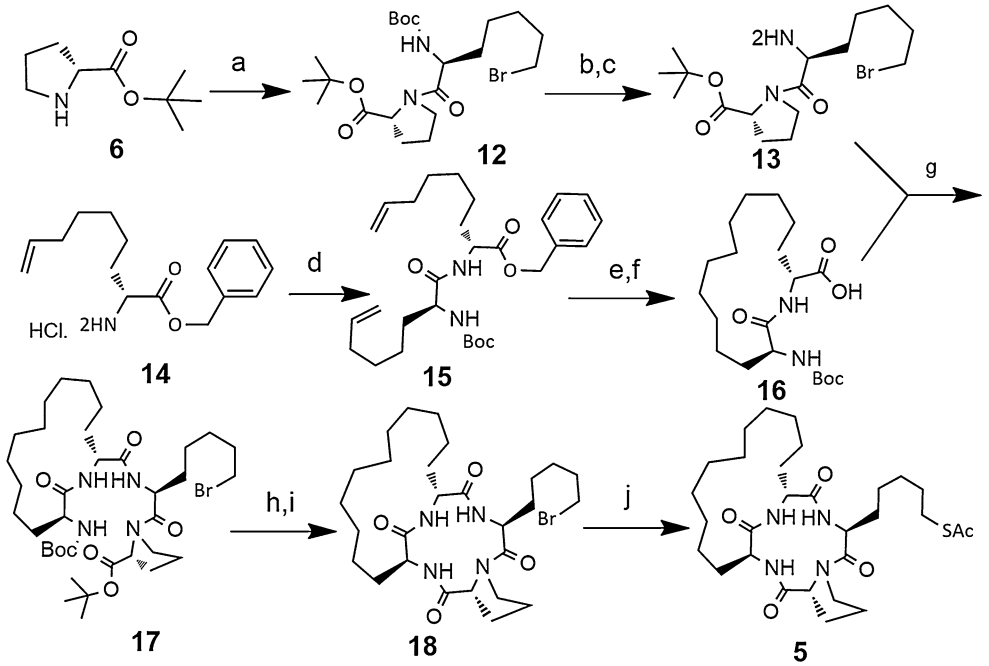
Enzyme inhibition and biological activity

The synthesized compounds were assayed for HDAC inhibitory activity using HDAC1, HDAC4, and HDAC6 enzymes prepared by 293T cells (Furumai et al. 2002) with the aid of 0.1 mM of dithiothreitol (DTT). In addition, to know the inhibitory activity of these compounds in cell-based condition; we carried out p21 promoter assay according to the literature (Bhuiyan et al. 2006). The detail experimental procedures for the preparation and assays of HDAC inhibitory activity and p21 promoter assay are described in the experimental section of this paper. Tricostatin A and FK228 were used as the reference compounds and the results are summarized in Table 1.

All the compounds were active in nanomolar range against HDAC1 and HDAC4. Compound **1** was less active than compound **2** in vitro conditions. The presence of aromatic ring at imino acid position has no effect on activity (Nishino et al. 2004) and hence the hydrophobicity in the third position of compounds **1** and **2** affect the activity. The



Scheme 2 Synthesis of CHAP31-based bicyclic tetrapeptide thioester (**4**). Reagents and conditions: (**a**) Boc-L-Ae9-OH, DCC, HOBt, DMF, 12 h, 80 %; (**b**) 4 M HCl/dioxane, 30 min, 70 %; (**c**) saturated Na_2CO_3; (**d**) Boc-D-Ae8-OH, DCC, HOBt, DMF, 12 h, 78 %; (**e**) Grubbs' first generation catalyst, DCM, 48 h, 83 %; (**f**) AcOH, Pd-C, H_2, 20 h, 90 %; (**g**) Boc-L-Ab7-OH, DCC, HOBt, DMF, 5 h on ice bath, 70 %; (**h**) TFA, 3 h, 91 %; (**i**) HATU, DIEA, DMF, 1 h, 61 %; (**j**) DMF, KSAc, 12 h, 80 %



Scheme 3 Synthesis of HC-toxin-based bicyclic tetrapeptide thioester (**5**). (**a**) Boc-L-Ab7-OH, DCC, HOBt, DMF, 5 h on ice bath; (**b**) 4 M HCl/dioxane, 30 min; (**c**) saturated Na_2CO_3; (**d**) Boc-L-Ae8-OH, DCC, HOBt, DMF, 12 h; (**e**) Grubbs' first generation catalyst, DCM, 48 h; (**f**) AcOH, Pd-C, H_2, 12 h; (**g**) DCC, HOBt, DMF, 5 h on ice bath; (**h**) TFA, 3 h; (**i**) HATU, DIEA, DMF, 1 h; (**j**) DMF, KSAc, 12 h

presence of less hydrophobic alanine at the third position in compound **1** is responsible for its lesser activity than compound **2** that contains comparatively more hydrophobic valine at the same position. It supports the fact that presence of relatively more hydrophobic amino acid residue in third position of cyclic tetrapeptide scaffold has a positive contribution to the activity of an inhibitor (Shivashimpi et al. 2007). Although HC-toxin-based compound **3** is potent against HDAC isoforms, it is less active compared to compounds **1** and **2**. However, compound **3** is more selective toward HDAC1 over HDAC6 compared to compound **1** and **2**. As the third position of compounds **2** and **3** is the same, the activity and selectivity were affected by the nature of amino acids at the second and fourth positions in

Table 1 HDAC inhibitory activity and p21 promoter assay data of synthesized and reference compounds

Compounds.	IC_{50}, nM (in vitro)			HDAC6/HDAC1[a]	p21 promoter assay EC_{1000} (nM)
	HDAC1	HDAC4	HDAC6		
TSA	23	34	65	3	20
FK228	1	nt	624	624	15.7
1	18 ± 1.30	50 ± 8.20	81 ± 4.30	5	2 ± 0.83
2	6.5 ± 0.63	8.5 ± 0.85	50 ± 6.40	8	20 ± 1.30
3	87 ± 3.40	73 ± 3.20	5,100 ± 320	59	4,200 ± 830
4	22 ± 1.30	8.9 ± 0.46	9,900 ± 2,300	450	7.4 ± 1.80
5	960 ± 54.0	850 ± 20.0	9,700 ± 120	10	540 ± 130

The (IC_{50}s) were determined as the mean ± SD of the concentrations calculated from at least three independent dose–response curves

nt Not tested

[a] Selectivity for HDAC1 toward HDAC6

the cyclic tetrapeptide scaffold. The enhanced potency of compound **1** over compounds **2** and **3** in cell-based condition proves the fact that the hydrophobicity is necessary for cell permeability.

CHAP31-based bicyclic compound **4** was more potent than HC-toxin-based compound **5** in both cell-free and cell-based conditions. These results imply that the loop size and its position is an important factor for improving activity and selectivity. Compound **4** is 73 fold more active than compound **5** and its activity and selectivity is higher than TSA and comparable with FK228 (Nishino et al. 2003). The loop position in compound **4** seems to be suitable for interacting with the surface of HDAC1 and HDAC4. The same selectivity and two fold in vivo activity of compound **4** over newly approved anticancer drug FK228 suggests its possibility to be a promising anticancer drug.

Experimental

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. 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 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. HR-FAB-mass spectra were measured on a JEOL JMS-SX 102A instrument. NMR spectra were recorded on a JEOL JNM A500 MHz spectrometer 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 acid coupling reactions were performed by standard solution-phase chemistry using *N*, *N*-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazol (HOBt). Peptide cyclization was mediated by 2-(1H-7-Azobenzotriazol-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate (HATU) as a coupling reagent.

Synthesis of cyclic tetrapeptides thioester (**1**-**3**)

Cyclo(-L-*thioacetate of 2-amino-7-mercaptoheptanoic acid, Am7(Ac)-amino-2-indane carboxylic acid, A2in-*L-*Ala-*D-*tetrahydroisoquinoline-carboxylic acid*, *Tic*-) (**1**) To a cold solution of Z-L-Ala-OH (2.68 g, 12 mmol) and H-D-Tic-O^{t}Bu (2.34 g, 10 mmol) in DMF (30 mL), HOBt·H_2O (1.53 g, 10 mmol) and DCC (2.50 g, 12 mmol) were added. The mixture was stirred overnight at room temperature. After completion of the reaction, DMF was removed by evaporation. The residue was dissolved in ethyl acetate (AcOEt) and filtered. Then it was washed with 10 % citric acid, 4 % $NaHCO_3$ and brine, respectively. The AcOEt solution was dried over anhydrous $MgSO_4$ and concentrated to remain an oily substance, which was purified by silica gel chromatography using 1 % methanol in chloroform (v/v) to yield Z-L-Ala-D-Tic-O^{t}Bu (3.950 g, 90 %) as oil. To a solution of protected dipeptide (1.97 g, 4.5 mmol) in acetic acid (20 mL), Pd-C (225 mg) was added and the mixture was stirred under hydrogen atmosphere overnight. The reaction was monitored by TLC and HPLC. After completion of the reaction, Pd-C was filtered off and the acetic acid was evaporated. Then the residue was dissolved in AcOEt and was washed with saturated Na_2CO_3 solution.

The AcOEt solution was dried over anhydrous Na_2CO_3 and concentrated to remain dipeptide free amine, H-L-Ala-D-Tic-O^{t}Bu (1.26 g, 92 %). While cooling on ice, HBTU (1.04 g, 4.98 mmol), HOBt·H_2O (625 mg, 4.15 mmol) were added to DMF (12 mL) containing Z-A2in-OH (1.55 g, 5 mmol) and H-L-Ala-D-Pro-O^{t}Bu (1.26 mg, 4.15 mmol). After stirring for 4 h, the product was isolated in the same manner as described earlier to obtain Z-A2in-L-Ala-D-Pro-O^{t}Bu (1.85 g, 3.1 mmol, 75 %) as oil. Z-A2in-L-Ala-D-Pro-O^{t}Bu was dissolved in acetic acid (12 mL) and subjected to catalytic hydrogenation in presence of the catalyst Pd-C (150 mg). After 10 h, the catalyst was filtered and reaction solution was removed by evaporation. The residue was dissolved in ethyl acetate (50 mL), washed with 2 M Na_2CO_3 solution (20 mL), ethyl acetate was evaporated to get H-A2in-L-Ala-D-Pro-O^{t}Bu (1.3 mg, 2.8 mmol, 90 %). To a cold solution of Boc-L-Ab7-OH (0.91 g, 2.8 mmol) and H-A2in-L-Ala-D-Tic-O^{t}Bu (1.30 g, 2.8 mmol) in DMF (7 mL), HOBt·H_2O (0.428 g, 2.8 mmol) and DCC (0.695 g, 3.36 mmol) were added. The mixture was stirred overnight at room temperature. After completion of the reaction, DMF was removed by evaporation. The residue was dissolved in AcOEt and filtered. Then it was washed with 10 % citric acid, 4 % $NaHCO_3$ and brine, respectively. The AcOEt solution was dried over anhydrous $MgSO_4$ and concentrated to remain an oily substance, which was purified by silica gel chromatography using 1 % methanol in chloroform (v/v) to yield Boc-L-Ab7-A2in-L-Ala-D-Tic-O^{t}Bu (1.29 g, 60 %) as foam. The protected tetrapeptide (1.29 g, 1.68 mmol) was dissolved in TFA (4 mL) on ice bath and kept for 3 h at room temperature. After evaporation of TFA, the residue was solidified using ether and petroleum ether to yield TFA salt of the linear tetrapeptide (1.19 g, 97 %). The linear tetrapeptide, H-L-Ab7-A2in-L-Ala-D-Tic-OH·TFA salt (1.19 g, 1.63 mmol), HATU (0.931 g, 2.44 mmol) and DIEA (0.71 mL, 4.1 mmol) were added in separate two portions to DMF (400 mL) in every 30 min with stirring. After completion of the cyclization reaction, the reaction was quenched by adding a small amount of acetic acid and DMF was evaporated and the residue was dissolved in AcOEt. Then solution was washed with 10 % citric acid, 4 % $NaHCO_3$ and brine, respectively. The AcOEt solution was dried over anhydrous $MgSO_4$ and concentrated to remain a foamy substance, which was purified by silica gel chromatography using 1 % methanol in chloroform (v/v) to yield cyclic tetrapeptide *cyclo*(-L-Ab7-A2in-L-Ala-D-Tic-) (0.370 g, 38 %, HPLC: retention time (r.t.) 8.22 min). To a solution of cyclic tetrapeptide (0.360 g, 0.60 mmol) in DMF (5 mL), KSAc (0.207 g, 1.8 mmol) was added and was stirred for 7 h at room temperature. DMF was evaporated and the residue was dissolved in AcOEt. Then it was washed with 10 % citric acid and brine, respectively. The AcOEt solution was dried over anhydrous $MgSO_4$ and concentrated to remain crude substance, which was purified by silica gel chromatography using 1 % methanol in chloroform (v/v) to yield *cyclo*(-L-Am7(Ac)-A2in-L-Ala-D-Tic-) (**1**) (0.275 g, 0.46 mmol, 77 %, HPLC: r.t. 8.40 min) as white powder. HR-FAB-MS, $[M + H]^+$ 591.2607 for $C_{32}H_{39}N_4O_5S$ (calcd 591.2641). ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.42 (d, $J = 10$ Hz, 1H), 7.13–7.25 (m, 6H), 6.81 (d, $J = 10$ Hz, 1H), 6.44(s, 1H), 5.25–5.33 (m, 1 H), 5.11 (t, $J = 8$ Hz, 1H), 4.98 (d, $J = 16$ Hz, 1H), 4.38 (d, $J = 15$ Hz, 1H), 4.19–4.26 (m, 1H), 4.01 (d, $J = 15$ Hz, 1H), 3.92–3.94 (d, $J = 17$ Hz, 1H), 3.62 (d, $J = 17$ Hz, 1H), 3.42–3.51 (m, 1H), 2.92–2.99 (m, 1H), 2.78–2.89 (m, 3H), 2.31 (s, 3H), 1.50–1.81 (m, 6H), 1.18–1.40 (m, 7H).

Cyclo(-L-Am7(Ac)-A2in-L-Val-D-Pro-) (**2**) This compound was synthesized as solid (0.372 g, 0.67 mmol, 71 %, HPLC: r.t. 8.25 min) in a similar manner as described in case of compound **1** starting from H-D-Pro-O^{t}Bu and using Z-L-Val-OH instead of Z-L-Ala-OH for the synthesis of dipeptide. HR-FAB-MS, $[M + H]^+$ 557.2753 for $C_{29}H_{41}N_4O_5S$ (calcd 557.2798). ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.34 (d, $J = 7.5$ Hz, 1H), 7.14–7.23 (m, 1H), 6.27 (s, 1H), 4.76 (d, $J = 8$ Hz, 1H), 4.44 (d, $J = 10$ Hz, 1H), 4.14–4.22 (m, 1H), 3.95–4.10 (m, 3H), 3.42–3.62 (m, 3H), 2.77–2.88 (m, 3H), 2.36–2.45 (m, 1H), 2.30 (s, 3H), 1.82–1.98 (m, 3H), 1.65–1.79 (m, 4H), 1.56 (m, 1H), 1.49–1.56 (m, 2H), 1.26–1.40 (m, 3H), 1.03–1.26 (m, 2H), 0.94 (d, $J = 7$ Hz, 3H), 0.89 (d, $J = 7$ Hz, 3H).

Cyclo(-L-Am7(Ac)-D-Tic-L-Val-D-Val-) (**3**) This compound was synthesized as heavy oil (0.044 g, 0.08 mmol, 64 %, HPLC: r.t. 7.14 min) from H-D-Tic-O^{t}Bu by adding Boc-L-Ab7-OH, Boc-D-Val-OH and Boc-L-Val-OH sequentially followed by cyclization and subsequent replacement of bromo group to thioacetate HR-FAB-MS, $[M + H]^+$ 559.2960 for $C_{29}H_{43}N_4O_5S$ (calcd 559.2954). ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.17–7.25 (m, 1H), 7.14 (d, $J = 7.5$ Hz, 1H), 6.90 (d, $J = 10$ Hz, 1H), 6.39 (d, $J = 10$ Hz, 1H), 6.14 (d, $J = 10$ Hz, 1H), 5.06–5.15 (m, 2H), 4.94 (d, $J = 15$ Hz, 1H), 4.49 (d, $J = 15$ Hz, 1H), 4.03 (t, $J = 10$ Hz, 1H), 3.90 (t, $J = 10$ Hz, 1H), 3.46 (dd, $J = 7$, 7 Hz, 1H), 2.96 (dd, $J = 7$, 7 Hz, 1H), 2.79 (t, $J = 7.5$ Hz, 2H), 2.30 (s, 3H), 2.08–2.21 (m, 2H), 1.81–1.90 (m, 1H), 1.79 (s, 1H), 1.62–1.71 (m, 1H), 1.47–1.54 (m, 2H), 1.30–1.38 (m, 2H), 1.13–1.30 (m, 2H), 0.87–0.98 (m, 13H).

Synthesis of cyclic tetrapeptides thioester (**4** and **5**)

Cyclo(-L-Am7(Ac)-D-Ae8(-)-L-Ae9(-)-D-Pro-) (**4**) To a cold solution of Boc-L-Ae9-OH (1.58 g, 5.83 mmol) and H-D-Pro-O^{t}Bu (**6**) (1.00 g, 5.83 mmol) in DMF (12 mL), HOBt·H_2O (0.892 g, 5.83 mmol) and DCC (1.45 g, 7.0 mmol) were added. The mixture was stirred overnight at room temperature. After completion of the reaction, DMF was removed by evaporation. The residue was

dissolved in ethyl acetate (AcOEt) and filtered. Then it was washed with 10 % citric acid, 4 % $NaHCO_3$ and brine, respectively. The AcOEt solution was dried over anhydrous $MgSO_4$ and concentrated to remain an oily substance, which was purified by silica gel chromatography using 1 % methanol in chloroform (v/v) to yield Boc-L-Ae9-D-Pro-O^{t}Bu (**7**) (1.98 g, 80 %, HPLC: r.t. 8.13 min). The protected dipeptide (1.98 g, 4.66 mmol) was dissolved in 4 M HCl/dioxane (12 mL) in ice bath and kept for 30 min at room temperature. After completion of the reaction, HCl/dioxane was removed by evaporation and the residue was dissolved in AcOEt. The solution was washed with saturated Na_2CO_3 solution and dried over anhydrous Na_2CO_3. The AcOEt solution was filtered and concentrated to remain free amine, H-L-Ae9-D-Pro-O^{t}Bu (1.06 g, 70 %, HPLC: r.t. 5.66 min) which was condensed with Boc-D-Ae8-OH (0.842 g, 3.26 mmol) following the same procedure described above and purified by silica gel chromatography using 1 % methanol in chloroform (v/v) to obtain Boc-D-Ae8-L-Ae9-D-Pro-O^{t}Bu (**8**) (1.44 g, 78 %, HPLC: r.t. 9.06 min) as a white foam. The tripeptide, Boc-D-Ae8-L-Ae9-D-Pro-O^{t}Bu (1.44 g, 2.54 mmol) was dissolved in anhydrous and degassed DCM (318 mL) and flashed with Ar. Then the Grubbs' first generation catalyst (418 mg, 0.51 mmol) dissolved in anhydrous and degassed DCM (65 ml) was added. The mixture was stirred at room temperature for 48 h. After completion of the reaction, DCM was evaporated and the residue was purified by silica gel chromatography using chloroform as solvent to yield Boc-D-Ae8(=)-L-Ae9(-)-D-Pro-O^{t}Bu (1.13 g, 83 %, HPLC: r.t. 8.93 min). Then the Boc-D-Ae8(=)-L-Ae9(-)-D-Pro-O^{t}Bu (1.13 g, 2.1 mmol) was dissolved in acetic acid (11 mL) and Pd-C (110 mg) was added. The mixture was stirred under hydrogen for 20 h. After completion of the reaction, Pd-C was filtered off and the acetic acid was evaporated. The residue was flashed with diethylether and pumped up to obtain Boc-D-Ae8(-)-L-Ae9(-)-L-Pro-O^{t}Bu (**9**) (1.02 g, 90 %, HPLC: r.t. 8.44 min), HR-FAB-MS, $[M + H]^+$ 538.3836 for $C_{29}H_{52}N_3O_6$ (calcd 538.3856).

The protected tripeptide (0.530 g, 0.98 mmol) was selectively deprotected using the method described above to obtain H-D-Ae8(-)-L-Ae9(-)-D-Pro-O^{t}Bu (0.230 g, 0.53 mmol, 55 %) and was condensed with Boc-L-Ab7-OH (0.172 g, 0.53 mmol) according to the method described earlier and the fully protected crude linear tetrapeptide was purified by silica gel chromatography using 1 % methanol in chloroform (v/v) to yield Boc-L-Ab7-D-Ae8(-)-L-Ae9(-)-D-Pro-O^{t}Bu (**10**) (0.350 g, 75 %, HPLC: r.t. 9.54 min) as a white foam. The protected tetrapeptide (0.300 g, 0.40 mmol) was dissolved in TFA (2 mL) in ice bath and kept for 3 h at room temperature. After evaporation of TFA, the residue was solidified using ether and petroleum ether to yield TFA salt of the linear tetrapeptide (0.255 g, 91 %, HPLC: r.t. 6.87 min). The TFA salt of linear tetrapeptide, TFA·H-L-Ab7-D-Ae8(-)-L-Ae9(-)-D-Pro-OH (0.255 g, 0.36 mmol,), HATU (0.205 g, 0.54 mmol), and DIEA (0.157 mL, 0.90 mmol) were added in separate two portions to DMF (170 mL) in every 30 min with stirring. After completion of the cyclization reaction, the reaction was quenched by adding a small amount of acetic acid and DMF was evaporated and the residue was dissolved in AcOEt. Then solution was washed with 10 % citric acid, 4 % $NaHCO_3$ and brine, respectively. The AcOEt solution was dried over anhydrous $MgSO_4$ and concentrated to remain a foamy substance which was purified by silica gel chromatography using 1 % methanol in chloroform (v/v) to yield cyclic tetrapeptide *cyclo*(-L-Ab7-D-Ae8(-)-L-Ae9(-)-D-Pro-) (**11**) (0.125 g, 61 % HPLC: r.t. 8.22 min) as a white foam.

To a solution of cyclic tetrapeptide (0.125 g, 0.22 mmol) in DMF (2 mL), KSAc (0.078 g, 0.68 mmol) was added and was stirred for 7 h at room temperature. DMF was evaporated and the residue was dissolved in AcOEt. Then it was washed with 10 % citric acid and brine, respectively. The AcOEt solution was dried over anhydrous $MgSO_4$ and concentrated to remain crude substance which was purified by silica gel chromatography using 1 % methanol in chloroform (v/v) to yield *cyclo*(-L-Am7(Ac)-D-Ae8(-)-L-Ae9(-)-D-Pro-) (**4**) (0.055 g, 44 %, HPLC: r.t. 7.85 min) as solid. HR-FAB-MS, $[M + H]^+$ 565.3401 for $C_{29}H_{49}N_4O_5S$ (calcd 565.3424). ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.06 (d, $J = 10$ Hz, 1H), 6.21 (d, $J = 10$ Hz, 1H), 6.07 (d, $J = 10$ Hz, 1H), 4.82–4.89 (m, 1H), 7.19 (dd, $J = 2$, 2 Hz, 1H), 4.52–4.59 (m, 1H), 4.21–4.28 (m, 1H), 4.08–4.15 (m, 1H), 3.47–3.55 (m, 1H), 2.84 (t, $J = 8$ Hz, 2H), 2.36–2.43 (m, 1H), 2.32 (s, 3H), 2.88–2.89 (m, 3H), 1.77–1.86 (m, 2H), 1.76 (s, 2H), 1.50–1.66(m, 5H), 1.18–1.50 (m, 20H),), 1.11–1.18 (m, 1H).

Synthesis of H-L-Ab7-D-Pro-O^{t}Bu (**13**). To a cold solution of Boc-L-Ab7-OH (1.88 g, 5.8 mmol) and H-D-Pro-O^{t}Bu (**6**) (0.922 g, 5.8 mmol) in DMF (12 mL), HOBt·H_2O (0.888 g, 5.8 mmol) and DCC (1.44 g, 7.0 mmol) were added. The mixture was stirred for 5 h at room temperature. After completion of the reaction, DMF was removed by evaporation. The residue was dissolved in AcOEt and filtered. Then it was washed with 10 % citric acid, 4 % $NaHCO_3$ and brine, respectively. The AcOEt solution was dried over anhydrous $MgSO_4$ and concentrated to remain an oily substance which was purified by silica gel chromatography using 1 % methanol in chloroform (v/v) to yield Boc-L-Ab7-D-Pro-O^{t}Bu (**12**) (1.96 g, 70 %) as oil. The protected dipeptide (1.35 g, 2.8 mmol) was dissolved in 4 M HCl/dioxane (7 mL) in ice bath and was kept at room temperature for 30 min. After completion of the reaction, HCl/dioxane was removed by evaporation and the residue was dissolved in AcOEt. The solution was washed with

saturated Na_2CO_3 and dried over anhydrous Na_2CO_3. The AcOEt solution was filtered and concentrated to remain free amine, H-L-Ab7-D-Pro-O^{t}Bu as syrup (**13**) (0.710 g, 67 %).

Synthesis of Boc-L-Ae8(-)-D-Ae9(-)-OH (**16**). To a cold solution of Boc-L-Ae8-OH (1.70 g, 6.58 mmol) and HCl·H-D-Ae9-OBzl (**14**) (1.96 g, 6.58 mmol) in DMF (13 mL), triethylamine (TEA) (0.915 mL, 6.58 mmol), HOBt·H_2O (0.1.00 g, 6.58 mmol) and DCC (1.63 g, 7.91 mmol) were added. The mixture was stirred overnight at room temperature. After completion of the reaction, DMF was removed by evaporation. The residue was dissolved in AcOEt and filtered. Then it was washed with 10 % citric acid, 4 % $NaHCO_3$, and brine, respectively. The AcOEt solution was dried over anhydrous $MgSO_4$ and concentrated to remain an oily substance which was purified by silica gel chromatography using 1 % methanol in chloroform (v/v) to yield Boc-L-Ae8-D-Ae9-OBzl (**15**) (2.90 g, 86 %) as crystalline solid. To a solution of linear dipeptide (2.05 g, 4.1 mmol) in anhydrous and degassed dichloromethane (500 mL), a solution of Grubbs' first generation ruthenium catalyst (0.680 g, 0.85 mmol) in anhydrous and degassed dichloromethane (100 mL) was added. The reaction mixture was stirred at room temperature for 48 h. After the completion of reaction, dichloromethane was evaporated and the residue was purified by silica gel chromatography using chloroform to get fused cyclic dipeptide which on catalytic hydrogenation in presence of Pd-C (0.150 g) in AcOH (15 mL) yielded compound Boc-L-Ae8(-)-D-Ae9(-)-OH (**16**) (1.14 g, 72 %) as solid. HR-FAB-MS, $[M + H]^+$ 385.2635 for $C_{20}H_{37}N_2O_5$ (calcd 385.2702).

Synthesis of cyclo(-L-Am7(Ac)-D-Pro-L-Ae8(-)-D-Ae9(-)-) (**5**) To a cold solution of Boc-L-Ae8(-)-D-Ae9(-)-OH (**16**) (0.723 g, 1.9 mmol) and H-L-Ab7-D-Pro-O^{t}Bu (**13**) (0.710 g, 1.9 mmol), HOBt·H_2O (0.291 g, 1.9 mmol) in DMF (4 mL) and DCC (0.472 g, 2.28 mmol) were added. The mixture was stirred for 5 h at room temperature. After completion of the reaction, the product linear tetrapeptide (**17**) (0.900 g, 65 %, HPLC: r.t. 9.41 min) was obtained in a similar manner as described earlier as foam. The protected tetrapeptide (0.520 g, 0.70 mmol) was dissolved in TFA (2 mL) on ice bath and kept for 3 h at room temperature. After evaporation of TFA, the residue was solidified using ether and petroleum ether to yield TFA salt of the linear tetrapeptide (0.440 g, 90 %, HPLC: r.t. 6.27 min). The TFA salt of linear tetrapeptide, TFA·H-L-Ae8(-)-D-Ae9(-)-L-Ab7-D-Pro-OH (0.440 g, 0.63 mmol,), HATU (0.360 g, 0.95 mmol), and DIEA (0.274 mL, 1.6 mmol) were added in separate two portions to DMF (150 mL) in every 30 min with stirring. After completion of the cyclization reaction, the reaction was quenched by adding a small amount of acetic acid and DMF was evaporated and the residue was dissolved in AcOEt. Then solution was washed with 10 %) citric acid, 4 % $NaHCO_3$, and brine, respectively. The AcOEt solution was dried over anhydrous $MgSO_4$ and concentrated to remain a foamy substance which was purified by silica gel chromatography using 1 % methanol in chloroform (v/v) to yield cyclic tetrapeptide *cyclo*(-L-Ab7-D-Pro-L-Ae8(-)-D-Ae9(-)-) (**18**) (0.260 g, 72 %, HPLC: r.t. 8.15 min) as a white foam.

To a solution of cyclic tetrapeptide (0.260 g, 0.45 mmol) in DMF (4 mL), KSAc (0.154 g, 1.35 mmol) was added and was stirred for 7 h at room temperature. DMF was evaporated and the residue was dissolved in AcOEt. Then it was washed with 10 % citric acid and brine, respectively. The AcOEt solution was dried over anhydrous $MgSO_4$ and concentrated to remain crude substance which was purified by silica gel chromatography using 1 % methanol in chloroform (v/v) to yield *cyclo*(-L-Am7(Ac)-D-Pro-L-Ae8(-)-D-Ae9(-)-) **(5)** (0.200 g, 78 %, HPLC: r.t. 8.08 min) as solid. HR-FAB-MS, $[M + H]^+$ 565.3455 for $C_{29}H_{49}N_4O_5S$ (calcd 565.3424). ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 7.09 (d, $J = 10$ Hz, 1H), 6.44 (d, $J = 10$ Hz, 1H), 6.01 (d, $J = 10$ Hz, 1H), 4.68–4.78 (m, 1H), 4.40–4.53 (m, 1H), 4.06 (d, $J = 7$ Hz, 1H), 3.90–3.98 (m, 1H), 3.43–3.54 (m, 2H), 2.84 (t, $J = 7$ Hz, 2H), 2.32 (s, 3H), 2.10–2.19 (m, 1H), 1.89–1.98 (m, 2H), 1.75–1.88 (m, 2H), 1.73 (s, 1H), 1.65–1.72 (m, 4H), 1.52–1.60 (m, 2H), 1.18–1.48 (m, 20H), 1.05–1.17 (m, 2H).

HDACs preparation and enzyme activity assay

In a 100-mm dish, 293T cells (1–2 × 10^6) were grown 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 amino methyl coumarin (AMC) was measured using a fluorescence plate reader. The 50 % inhibitory concentrations (IC_{50}) were determined as the means with SD calculated from at least three independent dose–response curves.

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 *Sm*aI sites of the pGL3-Basic plasmid (Promega Co., Madison, WI, USA). Mv1Lu (mink lung epithelial cell line) cells were transfected with the pGW-FL and a phagemid expressing neomycin/kanamycin resistance gene (pBK-CMV, Stratagene, La Jolla, CA, USA) 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, of which activity was enhanced by TSA in a dose-dependent manner. MFLL-9 cells (1 × 105) cultured in a 96-well multiwell 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 LucLite 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 tenfold higher than the basal level are presented as the 1,000 % effective concentration 1,000 % (EC_{1000}).

Conclusion

To develop novel HDAC inhibitors, we report the design and synthesis of some mono and bicyclic tetrapeptide thioester HDAC inhibitors with enhanced activity and selectivity. The improved potency of compounds **1**, **2** and **4** demands more advanced research for these compounds to be promising anticancer drug candidates.

Conflict of interest The authors declare that they have no conflict of interest.

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