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ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 7830–7839
Bioorganic & Medicinal Chemistry

Molecular design of histone deacetylase inhibitors by aromatic ring shifting in chlamydocin framework

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Received 1 July 2007; revised 20 August 2007; accepted 23 August 2007
Available online 26 August 2007

Abstract—Chlamydocin, a cyclic tetrapeptide containing aminoisobutyric acid (Aib), L-phenylalanine (L-Phe), D-proline (D-Pro), and a unique amino acid L-2-amino-8-oxo-9,10-epoxydecanoic acid, inhibits the histone deacetylases (HDACs), a class of enzymes, which play important roles in regulation of gene expression. Sulfur containing amino acids can also inhibit potently, so zinc ligand, such as sulfhydryl group connected with a linker to the so-called capping group, corresponding to cyclic tetrapeptide framework in case of chlamydocin is supposed to interact with the surface of HDAC molecule. Various changes in amino acid residues in chlamydocin may afford specific inhibitors toward HDAC paralogs. To find out specific inhibitors, we focused on benzene ring of L-Phe in chlamydocin framework to shift to various parts of cyclic tetrapeptide. We prepared and introduced several aromatic amino acids into the cyclic tetrapeptides. By evaluating inhibitory activity of these macrocyclic peptides against HDACs, we could find potent inhibitors by shifting the aromatic ring to the Aib site.

1. Introduction

Common cancer therapy techniques, like chemotherapy, take the advantage of apoptosis to eliminate malignant cells within tumors. Reversible acetylation and deacetylation of the ε-amino groups of lysine residues on core histone tails by histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzymes play an important role in the epigenetic regulation of gene expression by altering the chromatin architecture and controlling the accessibility of transcriptional regulators to DNA and histones.[1–4]Modification of the level of histone acetylation and its consequences have received enormous interest, parallely the growing evidence supports their importance for basic cellular functions such as DNA replication, transcription, differentiation, and apoptosis. Acetylation of lysine residues in N-terminal tails of core histones reduces the interaction with DNA resulting in more open chromatin structure. In addition, acetylated histone tails are specifically recognized and bound by bromodomain containing proteins such as components of the basal transcription machinery or histone acetyltransferases.[5] These activator complexes, containing HAT activity, have been shown to induce activation of transcription. Whereas deacetylation is associated with condensed chromatin structure resulting in the repression of gene transcription. Therefore, alteration of equilibrium in histone acetylation leads to transcriptional deregulation. This deviation in equilibrium of histone acetylation either due to HAT mutation or abnormal recruitment of HDACs has been linked to a number of malignant diseases.[6–9] Inhibition of HDAC enzyme activity proved to reverse and induce re-expression of differentiation inducing genes. Therefore, the search for potent inhibitors possessing adequate selectivity among the HDAC paralogs demands a considerable effort to develop therapeutics for the treatment of epigenetic diseases.

Several structurally unrelated natural and synthetic compounds have been reported so far as HDAC inhibitors (Fig. 1). Among them trichostatin A (TSA, **1**),[10] depsipeptide FK228[11–13] and the cyclic tetrapeptide family including trapoxin (TPX),[14] chlamydocin,[15] TAN-1746,[16] FR-235222,[17] 9,10-desepoxy-9-hydroxychlamydocin,[18] HC-toxins,[19–23] Cyl-1, Cyl-2,[24–26] WF-3161,[27] apicidin,[28–31] and FR-225497[32] are examples of

Keywords: Histone deacetylase; Chlamydocin; Cyclic tetrapeptide; Inhibitor.
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0968-0896/$ - see front matter © 2007 Published by Elsevier Ltd.
doi:10.1016/j.bmc.2007.08.041

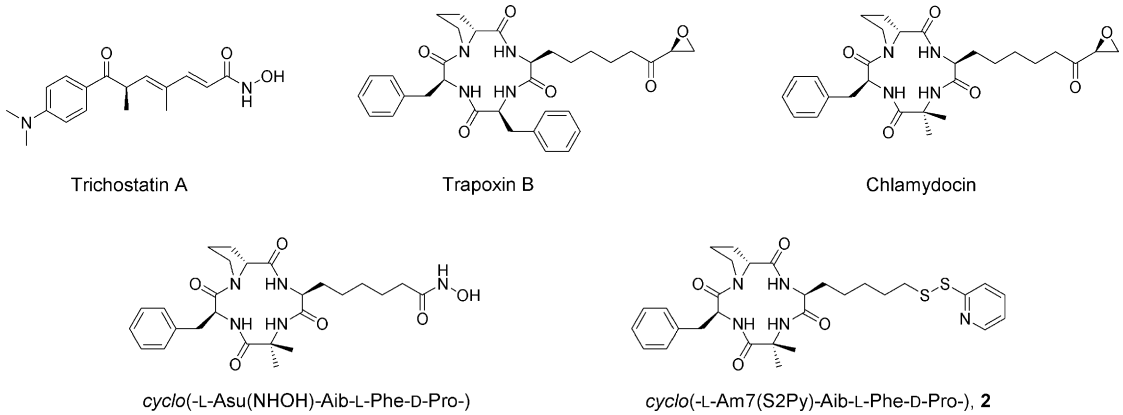


Figure 1. Structure of natural and synthetic HDAC inhibitors.

naturally occurring HDAC inhibitors. Inhibitors like suberoylanilide hydroxamic acid (SAHA),[33] straight chain TSA and SAHA analogs,[34–36] scriptaid[37], and the benzamide MS-275[38,39] have also been designed and synthesized.

In 1999 Finnin et al. defined that the aromatic dimethyl aminophenyl part in TSA plays the role of cap group.[40] They also proposed that, natural compounds trapoxin B and HC-toxins having a cyclic tetrapeptide structure with hydrophobic groups, such as L-Phe, serve as cap group. Larger size of cap group may give extensive contacts at the rim of enzyme pocket. There are evidences which support that the cyclic tetrapeptide framework has a significant structural role of specific hydrophobic interaction with the surface of HDAC enzymes.[41] Also in a recent report concerning to the SAR of apicidins[29] it is shown, the tryptophan residue in the cyclic tetrapeptide is the key constituent for HDACs inhibitory activity. The cyclic tetrapeptide chlamydocin, originally isolated from fungus *Diheterospora chlamydosphoria* is containing Aib, L-Phe, D-Pro, and L-Aoe, which reacts and inhibits histone deacetylases.[42] Recently we have reported chlamydocin hydroxamic acid analogs,[43] and sulfur containing chlamydocin analog (**2**),[44] in which the thiol function is protected as disulfide hybrid (Fig. 1).

Hydrophobicity in capping group of HDAC inhibitors is crucial in their activity. With the exception of HC-toxins, chlamydocin and other natural or synthetic cyclic tetrapeptide based inhibitors invariably contain an aromatic ring in their macrocyclic cap group. Various changes of amino acid residues in chlamydocin structure may afford specific inhibitors toward HDAC paralogs. In this work, to find out specific inhibitors, we focused on the benzene ring of L-Phe in chlamydocin framework. In a recent report[45] on docking study of TSA and trapoxin B into the homology model of HDAC1, it is revealed that aryl group of TSA corresponds to the cyclic tetrapeptide framework, not to the benzene ring of L-Phe.

The cyclic tetrapeptides designed in the present study are given in Figure 2. We here tried to figure out the role of aromatic ring and explore its appropriate position in the chlamydocin macrocycle by targeting the aromatic ring of L-Phe residue to shift in different positions. We shifted the aromatic ring of L-Phe, keeping L-alanine (L-Ala) at L-Phe position in chlamydocin, to the Aib position by incorporating 2-aminoindan-2-carboxylic acid (A2in) (**3**) and DL-1-aminoindan-1-carboxylic acid (DL-A1in) (**4** and **5**) to make rigid aromatic ring. We then shifted to one of the methyl groups of Aib to make DL-2-methyl phenylalanine (DL-2MePhe) (**6** and **7**). Further, to shift the aromatic ring to the imino acid position, we placed commercially available D-1,2,3,4-tetrahydro-3-isoquinoline carboxylic acid (D-Tic) (**8**). To shift the aromatic ring from cyclic tetrapeptide, we varied the distance of aromatic ring of L-Phe from cyclic framework by incorporating several aromatic amino acids like L-phenylglycine (L-Phg) (**9**) with no methylene group from α-carbon, L-2-amino-4-phenylbutanoic acid (L-Ph4) (**10**) and L-2-amino-5-phenylpentanoic acid (L-Ph5) (**11**) with two and three methylene groups, respectively, at L-Phe position. To make more flexible aromatic ring, we incorporated *O*-benzyl-L-serine (L-Ser(Bzl)) (**12**). As reference compounds, we designed cyclic tetrapeptides containing no aromatic ring by incorporating L-serine (L-Ser) (**13**) and L-Ala (**14**) (not shown in Fig. 2) at L-Phe position. We designed and synthesized different cyclic tetrapeptides by introducing different unusual amino acids. We herein describe the account on the synthesis of chlamydocin analogs and a description of the interesting biological results including circular dichroism studies.

2. Results and discussion

2.1. Chemistry

Our aim was to synthesize potent inhibitors of HDACs by shifting the benzene ring of L-Phe to Aib position and proline position of the cyclic tetrapeptide and also varied the distance of benzene ring of L-Phe from cyclic framework. Synthesis of cyclic tetrapeptide was carried out according to the general Scheme 1 by the conventional solution phase method, starting from the Z-D-imino acid *tert*-butyl ester. After the removal of Z-protection by catalytic hydrogenation, free amine

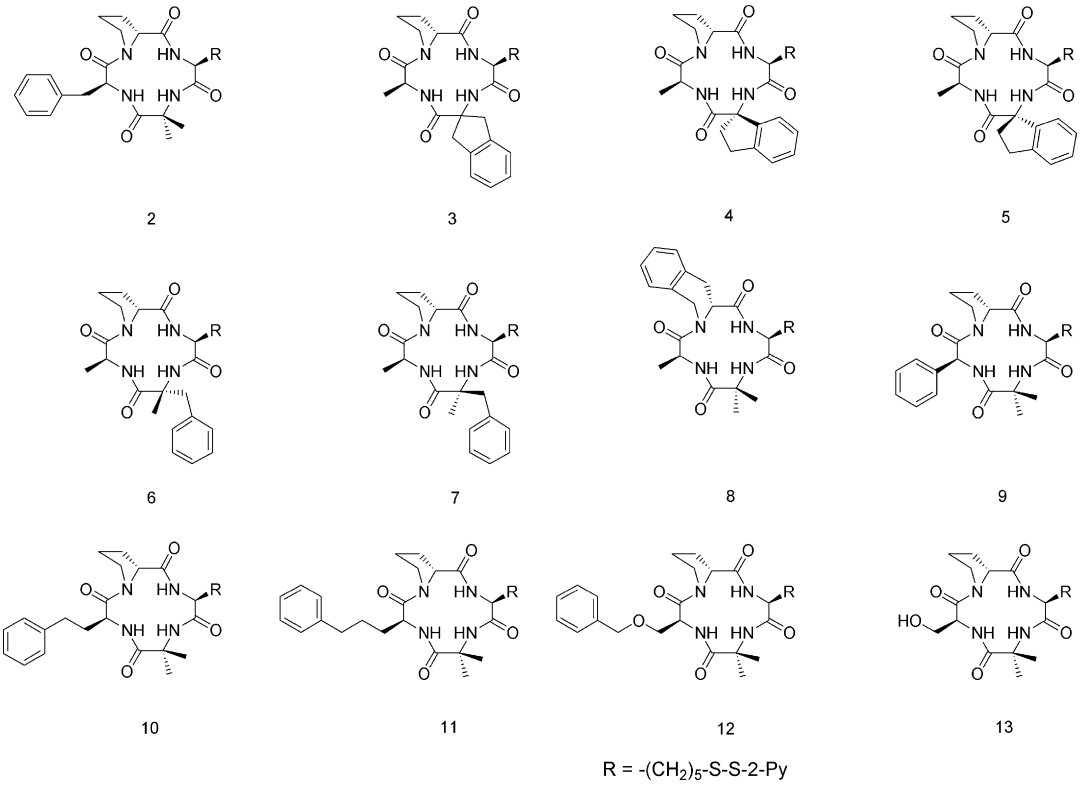
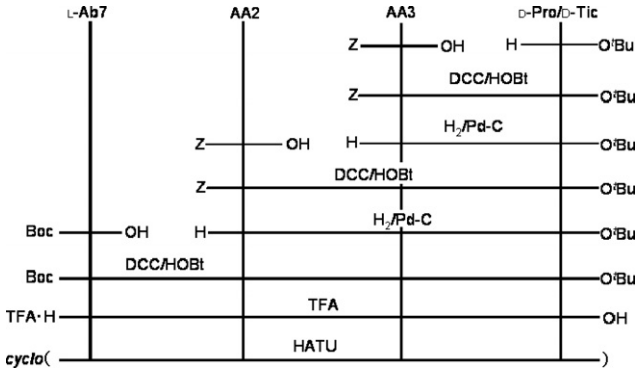


Figure 2. Structure of cyclic tetrapeptides containing different aromatic amino acids.



Scheme 1. Synthesis of cyclic tetrapeptides.

was extracted and used for condensation with another z-amino acid using DCC/HOBt. Boc-L-2-amino-7-bromo-heptanoic acid (Ab7)[46] was condensed to appropriate tripeptide free amine to prepare the linear tetrapeptide. After the removal of both side protections by treating with trifluoroacetic acid, cyclization reaction was carried out with the aid of HATU in DMF (2 mM) with minimum amount of DIEA (2.5 equiv). The yield of cyclic tetrapeptides was 50–60% after purification by silica gel chromatography. The cyclic tetrapeptide containing Ab7 was reacted with potassium thioacetate (Wako, Japan) to convert the bromide into thioacetate ester. Further treatment with methylamine in the presence of 2,2′-dipyridyl disulfide gave the desired cyclic tetrapeptides.

Initially, we introduced several aromatic Aib analogs such as A2in, DL-A1in, and DL-2MePhe at AA2 position and L-Ala at AA3 position as shown in Scheme 1, to know the effect of aromatic ring at Aib position. The cyclic tetrapeptides were synthesized according to the general method as described above to give the following compounds, *cyclo*(-L-Am7(S2Py)-A2in-L-Ala-D-Pro-) (**3**), *cyclo*(-L-Am7(S2Py)-D-A1in-L-Ala-D-Pro-) (**4**), *cyclo*(-L-Am7(S2Py)-L-A1in-L-Ala-D-Pro-) (**5**), *cyclo*(-L-Am7(S2Py)-D-2MePhe-L-Ala-D-Pro-) (**6**), and *cyclo*(-L-Am7(S2Py)-L-2MePhe-L-Ala-D-Pro-) (**7**). Compounds **4/5** and **6/7** were obtained as diastereomeric mixtures, which were successfully separated by HPLC. Detailed HPLC conditions are given in the experimental section. Next we changed the D-Pro residue of chlamydocin by D-Tic, to know the effect of aromatic ring at imino acid position. Using the general method as said above, we synthesized the following compound *cyclo*(-L-Am7(S2Py)-Aib-L-Ala-D-Tic-) (**8**).

To explore the effect of variation in distance between cyclic framework and benzene ring, we replaced the L-Phe of chlamydocin at AA3 position, by other unnatural amino acids, like L-Phg, L-Ph4 amd L-Ph5 and with Aib at AA2 position. The cyclic tetrapeptides were synthesized according to general method as described above to obtain following compounds, *cyclo*(-L-Am7(S2Py)-Aib-L-Phg-D-Pro-) (**9**), *cyclo*(-L-Am7(S2Py)-Aib-L-Ph4-D-Pro-), (**10**) and *cyclo*(-L-Am7(S2Py)-Aib-L-Ph5-D-Pro-) (**11**).

Further, we synthesized the cyclic tetrapeptides *cyclo*(-L-Am7(S2Py)-Aib-L-Ser(Bzl)-D-Pro-) (**12**) and *cyclo*(-L-Am7(S2Py)-Aib-L-Ser-D-Pro-) (**13**) by introducing L-Ser(Bzl) and L-Ser to know the effect of oxygen atom

in the spacer. Finally we also have synthesized the cyclic tetrapeptide without having aromatic ring, to obtain *cyclo*(-L-Am7(S2Py)-L-Aib-L-Ala-D-Pro-) (**14**) as reference compound.

All the synthesized compounds were characterized by ^{1}H NMR and high resolution FAB-MS. The purity of compounds was determined by HPLC analysis and all the synthesized cyclic tetrapeptides showed purity above 97%. The configuration of diastereomers **4/5** containing D-A1 in and L-A1in,[43] and **6/7** containing D-2MePhe and L-2MePhe was assigned by ^{1}H, COSY, NOESY spectra in $CDCl_3$ and also high resolution FAB-MS. Difference in chemical shift positions of diastereomers **4** and **5**, containing D-A1in and L-A1in was shown in Figure 3a and b, respectively. In Figure 3a, the β-proton of D-A1in of compound **4** is appearing at around 2 ppm as multiplets, and the γ-proton is appearing around 3 ppm. In case of compound **5** (Fig. 3b), these two groups of protons are appearing at the same position at around 3 ppm. In the NOESY experiments, we observed NOE of 2.72 and 2.22 Åbetween NH- and the β-protons, in compound **4**, which corresponds to its D-configuration. We could not observe any NOE in case of compound **5**, which indicates, it is of L-configuration.

2.2. Enzyme inhibition and biological activity

The synthesized cyclic tetrapeptides were assayed for HDAC inhibitory activity using HDAC1, HDAC4, and HDAC6 enzymes prepared by 293T cells.[13] These compounds containing S–S disulfide were initially assayed for enzyme inhibition; they exhibited less activity in cell free condition. But in the p21 promoter assay most of the compounds showed good activity. Maybe the disulfide bond is reduced in cell medium to give a sulfhydryl group, to regulate the activity of promoter gene by inhibiting HDAC enzymes. It is already reported[13] that, FK-228 containing an intramolecular disulfide bridge is a potent HDAC inhibitor in cellular condition and its activity was found less in the absence of dithioltritol (DTT) in cell free conditions. The DTT is known to reduce the disulfide bonds to sulfhydryl group, which strongly ligates the Zn^{+2} ion of HDAC enzyme. Considering this point, cell free enzyme inhibition assays were carried out, in presence of 0.1 mM of DTT. Detailed experimental procedure for the preparation and assay methods were performed according to the reported methods[47] which are reproduced in the experimental section. In addition, to know the inhibitory activity of these compounds in cell based condition, we also carried out p21 promoter assay according to the literature.[47]The results of HDAC inhibitory activity and the p21 promoter assay of the compounds are shown in Table 1.

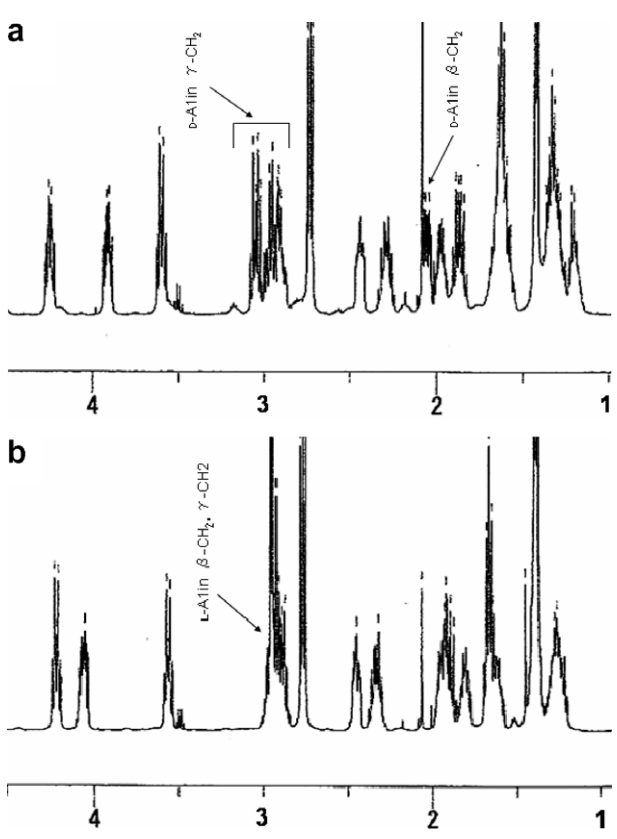


Figure 3. Selected region of ^{1}H NMR spectrum of compound **4** (a) compound **5** (b).

These compounds inhibited HDACs at nanomolar range. For comparison the inhibitory activity[48] of trichostatin A (**1**) is also shown. Compound **2**,[44] which has similar cyclic framework as chlamydocin showing good activity, is taken as standard for this study. In compound **3**, when Aib is replaced by A2in there was almost comparable in vitro activity with **2**. Among the diastereomers **4** and **5**, compound **4** with D-A1in found 10 times more active in vitro and in vivo than **5** with L-A1in. On the contrary among the diastereomers **6** and **7**, compound **7** with L-2MePhe was more active than compound **6** with D-2MePhe. These results indicate that, in compounds **4** and **7** there is a favorable interaction of benzene ring and the residues at the surface binding region of HDACs. Naturally occurring cyclic tetrapeptides rarely contain aromatic iminoacids. Therefore we replaced the D-Pro residue with D-Tic residue in compound **8**, to study the effect of benzene ring in at D-Pro position. It showed comparable activity with compound **2**.

Compound **9** with no methylene group on the α-carbon, showed less activity compared to **10** and **11** with two and three methylene groups, respectively, indicating that increasing the length between cyclic framework and benzene ring will give flexibility and favorable interaction to the surface binding region of HDACs. Compound **12** was found more active both in vitro and in vivo as compared to **13**. It further supports the fact that presence of an aromatic ring has a positive contribution to the activity of an inhibitor. The cyclic tetrapeptides **13** and **14** which lack an aromatic ring are still retaining the threshold amount of hydrophobicity to be active. The selectivity of HDAC1 over HDAC4 is actually insufficient as shown in Table 1, probably due to the similarity in surface binding regions of both the enzymes.

Table 1. HDAC inhibitory activity and p21 promoter assay data

Compound	No	IC_{50} (nM)			HDAC1/HDAC4[a]	p21 promoter assay EC_{1000} (nM)
		HDAC1	HDAC4	HDAC6		
Trichostatin A[48]	**1**	1.9	2.0	2.8	1.0	190
cyclo(L-Am7(S2Py)-Aib-L-Phe-D-Pro-)[44]	**2**	3.9	1.8	40	2.1	45
cyclo(L-Am7(S2Py)-A2in-L-Ala-D-Pro-)	**3**	2.0	1.6	8.8	1.2	140
cyclo(L-Am7(S2Py)-D-A1in-L-Ala-D-Pro-)	**4**	2.7	2.4	12	1.1	55
cyclo(L-Am7(S2Py)-L-A1in-L-Ala-D-Pro-)	**5**	36	25	33	1.4	2000
cyclo(L-Am7(S2Py)-D-2MePhe-L-Ala-D-Pro-)	**6**	170	70	71	2.4	25600
cyclo(L-Am7(S2Py)-L-2MePhe-L-Ala-D-Pro-)	**7**	3.7	2.2	56	1.6	550
cyclo(L-Am7(S2Py)-Aib-L-Ala-D-Tic-)	**8**	4.7	4.2	29	1.1	200
cyclo(L-Am7(S2Py)-Aib-L-Phg-D-Pro-)	**9**	88	56	86	1.5	7400
cyclo(L-Am7(S2Py)-Aib-L-Ph4-D-Pro-)	**10**	6.2	3.2	36	1.9	320
cyclo(L-Am7(S2Py)-Aib-L-Ph5-D-Pro-)	**11**	5.3	2.7	43	1.9	110
cyclo(L-Am7(S2Py)-Aib-L-Ser(Bzl)-D-Pro-)	**12**	3.2	1.6	24	2.0	72
cyclo(L-Am7(S2Py)-Aib-L-Ser-D-Pro-)	**13**	6.4	4.0	26	1.6	580
cyclo(L-Am7(S2Py)-Aib-L-Ala-D-Pro-)	**14**	9.4	5.2	43	1.8	750

[a] Selectivity of the enzymes.

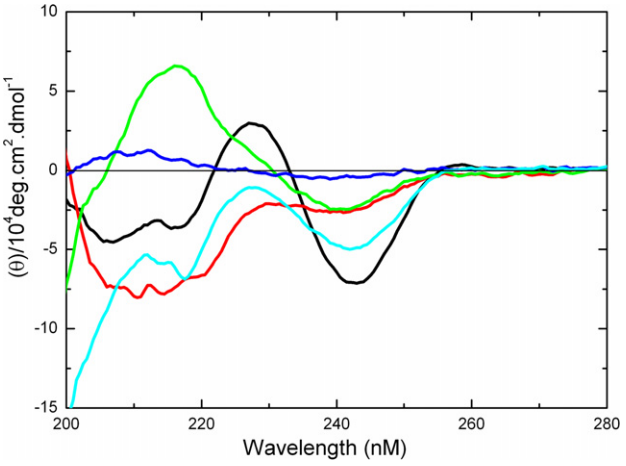


Figure 4. CD spectra of cyclic tetrapeptides — **2**,— **4**, — **5**, — **6**, — **7** in methanol.

2.3. Conformational change by aromatic ring shifting

The interesting HDAC activity data of diastereomers **4**/**5** and **6**/**7** forced us to study their conformations. We have carried out circular dichroism (CD) spectral studies of these diastereomers (Fig. 4). The CD spectrum of reference compound **2**is similar to chlamydocin[49] up to 260 nm. Compounds **4** and **7** showed negative ellipticity at 220 nm regions, whereas compounds **5** and **6** showed positive ellipticity. This explains the fact that, compounds **4**, **7**, and **5**, **6** have similar conformations. Further **4** and **7** with negative ellipticity are better inhibitors of HDAC paralogs. We are also carrying out conformational analysis of these diastereomers by NMR calculation methods. These results will be published elsewhere.

3. Conclusion

In order to develop HDAC inhibitors with good selectivity and potency, we attempted to shift the aromatic ring of L-Phe in chlamydocin scaffold to Aib and D-imino acid positions. We also varied the spacer length at L-Phe position. Thus, 11 cyclic tetrapeptides containing various aromatic amino acids have been successfully synthesized as the SS-hybrids. Upon p21 promoter assay we could find out the cyclic peptide **4** with aromatic ring rigidified tightly at Aib position as potent inhibitor as mother compound **2**. In addition, the data showed that the aromatic ring at L-Phe position is allowed flexible. Conformational changes were observed in the diastereomers due to the aromatic ring shift to the Aib site. The conformations in which the aromatic group and the zinc ligating spacer are directed to the same side are found to be more active. This fact leads us to conclude that there is some kind of hydrophobic interaction of aromatic ring with the surface binding region of HDAC enzymes. The cyclic tetrapeptides **13** and **14** which are devoid of aromatic rings are still retaining their minimal hydrophobicity to interact with the surface binding region of HDACs, thus inhibiting potently. Among the synthesized cyclic peptides, **4** and **12** were found promising, although they showed insignificant selectivity between HDAC1 and HDAC4.

4. Experimental

4.1. General

Unless otherwise noted, all solvents and reagents were of 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) 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 (4.6×50 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 to B over 15 min with a flow rate of 2 ml/min, with

detection at 220 nm. FAB-mass spectra and high resolution mass spectra (HR-MS) 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 millions (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 dicyclohexyl-carbodiimide (DCC), 2-(1*H*-benzatriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), or *O*-(7-azabenzotriazoyl-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HATU).

4.1.1. Synthesis of cyclo (-L-Am7(S2Py)-A2in-L-Ala-D-Pro-) (3). To a cooled solution of H-D-Pro-O^{t}Bu (3.5 g, 20 mmol), Z-L-Ala-OH (4.9 g, 22 mmol) and HOBt·H_2O (3 g, 20 mmol) in dimethylformamide (DMF) (120 mL) was added DCC (4.9 g, 24 mmol). The mixture was stirred at room temperature for 8 h. 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 Z-L-Ala-D-Pro-O^{t}Bu (6.5 g, 90%) as a colorless oil. The protected dipeptide (1.09 g, 3 mmol) was dissolved in acetic acid (10 mL). Pd–C (150 mg) was added and the mixture was stirred under hydrogen atmosphere for 10 h. The reaction was monitored by TLC and HPLC. After completion of the reaction Pd–C was filtered off and the acetic acid was evaporated. The residue was dissolved in ethyl acetate and the organic phase was washed with 2M Na_2CO_3 solution and dried over anhydrous Na_2CO_3. Evaporation of ethyl acetate gave H-L-Ala-D-Pro-O^{t}Bu (685 mg, 95%). To a cooled solution of H-L-Ala-D-Pro-O^{t}Bu (685 mg, 2.8 mmol) and Z-A2in-OH (1.04 g, 3.6 mmol) in DMF (6 mL) were successively added HBTU (1.27 g, 3.6 mmol), HOBt·H_2O (429 mg, 2.8 mmol), and DIEA (0.75 ml, 4.2 mmol). The product Z-A2in-L-Ala-D-Pro-O^{t}Bu (785 mg, 75%) was obtained in the same manner as described earlier as white foam. The tripeptide Z-A2in-L-Ala-D-Pro-O^{t}Bu (1.1 g, 2.1 mmol) was subjected to catalytic hydrogenation with Pd–C (100 mg) in acetic acid (8 mL). The free amine was taken into ethyl acetate (25 mL) the aid of 2 M Na_2CO_3 solution (10 mL). After dried over anhydrous Na_2CO_3 ethyl acetate solution was evaporated to remain H-A2in-L-Ala-D-Pro-O^{t}Bu (785 mg, 90%). The N-terminal free tripeptide H-A2in-L-Ala-D-Pro-O^{t}Bu (785 mg, 1.95 mmol) was coupled with Boc-L-Ab7-OH (758 mg, 2.3 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 v/v) to yield Boc-L-Ab7-A2in-L-Ala-D-Pro-O^{t}Bu (900 mg, 68%) as a white foam. Boc-L-Ab7-A2in-L-Ala-D-Pro-O^{t}Bu (745 mg, 1 mmol) was dissolved in TFA (3 mL) and left standing on ice for 3 h. After evaporation of TFA, the residue was solidified by trituration with ether to yield TFA·H-L-Ab7-A2in-L-Ala-D-Pro-OH (640 mg, 96%). HPLC, rt 5.53 min. To a volume of DMF (100 mL) TFA·H-L-Ab7-A2in-L-Ala-D-Pro-OH (640 mg, 0.96 mmol), HATU (548 mg, 1.44 mmol), and DIEA (0.42 mL, 2.4 mmol) were added in five aliquots with 30 min time interval, 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 was successively washed with 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 v/v) to yield *cyclo*(-L-Ab7-A2in-L-Ala-D-Pro-) (359 mg, 69%) as a white foam after drying in vacuo. HPLC, rt 7.41 min., HR-FAB MS, $[M+H]^+$ 533.1745 for $C_{25}H_{34}N_4O_4{}^{79}Br$ (calcd 533.1685) and 535.1732 for $C_{25}H_{34}N_4O_4{}^{81}Br$ (calcd 535.1664). The cyclic tetrapeptide *cyclo*(-L-Ab7-A2in-L-Ala-D-Pro-) (240 mg, 0.45 mmol) was dissolved in DMF (2 mL), potassium thioacetate (77 mg, 0.67 mmol) was added and stirred for 4–5 h. The solvent was evaporated and the residue was dissolved in ethyl acetate and washed with 10% citric acid and brine. The ethyl acetate solution was dried over anhydrous $MgSO_4$ and evaporated to thioester *cyclo*(-L-Am7(acetyl)-A2in-L-Ala-D-Pro-) (212 mg, 89%). HPLC, rt 7.20 min. To a solution of *cyclo*(-L-Am7(acetyl)-A2in-L-Ala-D-Pro-) (200 mg, 0.4 mmol) in DMF (2 mL), under argon atmosphere, 2,2′-dipyridyldisulfide (176 mg, 0.8 mmol) and 40% solution of CH_3NH_2/MeOH (0.22 mL, 2 mmol) were added and kept stirring for 5 h at room temperature. After evaporation of DMF residue was dissolved in ethyl acetate and washed with 10% citric acid, 4% sodium bicarbonate, brine, and finally dried over $MgSO_4$. After evaporation of ethyl acetate product was purified by silica gel chromatography using mixture of chloroform and methanol (99:1 v/v) to yield *cyclo*(-L-Am7(S2Py)-A2in-L-Ala-D-Pro-) as white foam (145 mg, 61.2%), HPLC, rt 7.78 min, HR-FAB MS, $[M+H]^+$ 596.2401 for $C_{30}H_{38}O_4N_5S_2$ (calcd 596.2287).^1H NMR (500 MHz, CDCl3): δ_H 1.23 (m, 1H), 1.31 (m, 1H), 1.39 (m, 2H), 1.41 (d, J = 7 Hz, 3H), 1.62 (m, 3H), 1.67 (m, 1H), 1.84 (m, 1H), 1.92 (m, 1H), 2.26 (m, 1H), 2.4 (m, 1H), 2.74 (t, 2H), 2.88 (s, 2H), 2.96 (s, 2H), 3.54 (ddd, J = 10, 10, 8.5 Hz, 1H), 4.02 (m, 1H), 4.19 (ddd, J = 10.0, 9.25, 8.5 Hz, 1H), 4.75 (dd, J = 2.0, 2.0 Hz, 1H), 6.39 (s, 1H), 7.07 (dd, J = 13.5, 10.0 Hz, 1H), 7.13 (d, J = 10.5 Hz, 1H), 7.17 (m, 2H), 7.21 (m, 2H), 7.34 (d, J = 8 Hz, 1H), 7.52 (d, J = 10 Hz, 1H).

4.1.2. Synthesis of *cyclo*(-L-Am7(S2Py)-D-A1in-L-Ala-D-Pro-) (4) and *cyclo*(-L-Am7(S2Py)-L-A1in-L-Ala-D-Pro-) (5). These compounds were synthesized using DL-A1in instead of Aib according the method described earlier, to yield *cyclo*(-L-Am7(S2Py)-DL-A1in-L-Ala-D-Pro-). The two distereoisomers were separated by preparative HPLC [Column: YMC pack, ODS-A (250 × 10 mm),

condition: Isocratic condition from solvent A to B over 20 min with 220 nm detection, Sol. A; 100% H_2O with 0.1% TFA and Sol. B; 100% acetonitrile with 0.1% TFA] to yield *cyclo*(-L-Am7(S2Py)-D-A1in-L-Ala-D-Pro-) (60 mg, 23%) HPLC, rt 5.9 min. HR-FAB MS, $[M+H]^+$ 596.2352 for $C_{30}H_{38}O_4N_5S_2$ (calcd 596.2287). ^1H NMR (500 MHz, CDCl3): δ_H 1.20 (m, 1H), 1.30 (m, 1H), 1.33 (m, 2H), 1.43 (d, J = 7 Hz, 3H), 1.62 (m, 4H), 1.87 (m, 1H), 1.97 (m, 1H), 2.05 (m, 2H), 2.29 (m, 1H), 2.45 (m, 1H), 2.74 (t, 2H), 2.93 (m, 1H), 2.93 (m, 1H), 3.04 (m, 1H), 3.59 (ddd, J = 10.0, 10.25, 8 Hz, 1H), 3.92 (m, 1H), 4.25 (ddd, J = 9.52, 9.52, 7.5 Hz, 1H), 4.82 (dd, J = 1.5, 1.5 Hz, 1H), 5.18 (m, 1H), 6.46 (s, 1H), 7.09 (d, J = 10.0 Hz, 1H), 7.22 (m, 1H), 7.25 (m, 4H), 7.64 (d, J = 10.0 Hz, 1H), 7.82 (m, 1H), 7.87 (d, J = 7.5 Hz, 1H), 8.51 (d, J = 4.5 Hz, 1H), and *cyclo*(-L-Am7(S2Py)-L-A1in-L-Ala-D-Pro-) (70 mg, 33%). HPLC, rt 6.38 min. HR-FAB MS, $[M+H]^+$ 596.2367 for $C_{30}H_{38}O_4N_5S_2$ (calcd 596.2287). ^1H NMR (500 MHz, CDCl3): δ_H 1.26 (m, 2H), 1.38 (d, J = 7 Hz, 3H), 1.62 (m, 1H), 1.67 (m, 2H), 1.80 (m, 1H), 1.92 (m, 2H), 2.33 (m, 1H), 2.45 (m, 1H), 2.76 (t, 2H), 2.90 (m, 1H), 2.95 (m, 2H) 3.56 (ddd, J = 10.0, 10.5, 7.5 Hz, 1H) 4.05 (m, 1H), 4.22 (ddd, J = 10.25, 10.0, 7.6 Hz, 1H), 4.77 (dd, J = 2.5, 2.0 Hz, 1H), 5.03 (m, 1H), 6.57 (s, 1H), 7.12 (d, 1H), 7.18 (dd, J = 13.5, 10.0 Hz, 1H), 7.33 (m, 2H), 7.36 (m, 2H), 7.49 (d, J = 10.5 Hz, 1H), 7.55 (m, 2H), 7.82 (d, J = 8 Hz, 1H), 8.53 (d, J = 4.5 Hz, 1H).

4.1.3. Synthesis of *cyclo* (-L-Am7(S2Py)-D-2-MePhe-L-Ala-D-Pro-) (6) and *cyclo* (-L-Am7(S2Py)-L-2-MePhe-L-Ala-D-Pro-) (7). These compounds were synthesized using DL-2MePhe instead of Aib according to the procedure described for **3**, to yield *cyclo*(-L-Am7(S2Py)-DL-2MePhe-L-Ala-D-Pro-). The two distereoisomers were separated by preparative HPLC [Column: YMC pack, ODS-A (250 × 10 mm), condition: Isocratic condition from solvent A to B over 20 min with 220 nm detection, Sol. A; 100% H_2O with 0.1% TFA and Sol. B; 100% acetonitrile with 0.1% TFA] to yield *cyclo*(-L-Am7(S2Py)-D-2MePhe-L-Ala-D-Pro-) (40 mg, 22%) as a white foam. HPLC, rt 6.52 min. HR-FAB MS, $[M+H]^+$ 598.256 for $C_{30}H_{40}O_4N_5S_2$ (calcd 598.2443). ^1H NMR (500 MHz, CDCl3): δ_H 1.23 (m, 2H), 1.27 (m, 1H), 1.38 (d, J = 6.5 Hz, 3H), 1.46 (m, 1H), 1.56 (m, 3H), 1.67 (m, 1H), 1.71(s, 3H), 1.81 (m, 1H), 1.92 (m, 1H),2.18 (m, 1H), 2.33 (m, 1H), 2.76 (t, 2H), 2.98 (d, J = 9 Hz, 1H), 3.12 (d, J = 14 Hz, 1H), 3.53 (ddd, J = 9.75, 7.5, 9.75 Hz, 1H) 3.87 (m, 1H), 4.14 (m, 1H), 4.71 (dd, J = 2.5, 2 Hz, 1H), 5.0 (m, 1H), 6.12 (s, 1H), 7.02 (d, J = 10 Hz), 7.28 (m, 1H), 7.28 (m, 4H), 7.81 (m, 1H), 7.88 (d, J = 8 Hz, 1H), 8.57 (d, J = 5 Hz, 1H), and *cyclo*(-L-Am7(S2Py)-L-2MePhe-L-Ala-D-Pro-) (50 mg, 28%) as a white foam. HPLC, rt 6.78 min. HR-FAB MS, $[M+H]^+$ 598.2510 for $C_{30}H_{40}O_4N_5S_2$ (calcd 598.2443). ^1H NMR (500 MHz, CDCl3): δ_H 1.20 (s, 3H), 1.3 (m, 2H), 1.42 (d, J = 7 Hz, 3H), 1.45 (m, 2H), 1.61 (m, 1H), 1.72 (m, 2H), 1.85 (m, 2H), 1.91 (m, 1H), 2.22 (m, 1H), 2.37, 1H), 2.82 (t, 2H), 3.22 (d, J = 13.5 Hz, 1H), 3.57 (ddd, J = 10.0, 10.5, 7.5 Hz, 1H), 3.78 (d, J = 13.5 Hz, 1H) 3.89 (m,1H), 4.18 (dd, J = 8.0, 8.0 Hz, 1H), 4.77 (dd, J = 2.5, 2.0 Hz, 1H), 5.10 (m, 1H), 5.95 (s, 1H), 7.14 (m, 2H), 7.24 (m, 4H), 7.75 (d, J = 10 Hz, 1H), 7.85 (m, 1H), 7.92 (d, J = 7.5 Hz, 1H), 8.60 (d, J = 5 Hz, 1H).

4.1.4. Synthesis of *cyclo* (-L-Am7(S2Py)-Aib-L-Ala-D-Tic-) (8). Starting from H-D-Tic-O^{t}Bu (2.95 g, 10 mmol), was obtained H-L-Ala-D-Tic-O^{t}Bu (1.4 g, 94%) similarly, as explained earlier. The free amine H-L-Ala-D-Tic-O^{t}Bu (1.4 g, 4.6 mmol) was coupled with Z-Aib-OH (1.14 g, 5 mmol) using DCC (1.13 g, 5.5 mmol) and HOBt·H_2O (704 mg, 4.6 mmol) in DMF (10 mL) to yield Z-Aib-L-Ala-D-Tic-O^{t}Bu (1.6 g, 70%). Further, similar reactions were carried out on Z-Aib-L-Ala-D-Tic-O^{t}Bu, according to procedure reported for **3** to yield finally *cyclo*(-L-Am7(S2Py)-Aib-L-Ala-D-Tic-) (80 mg 64%). HPLC, rt 6.92 min. HR-FAB MS, $[M+H]^+$ 584.2336 for $C_{29}H_{38}O_4N_5S_2$ (calcd 584.2287). ^1H NMR (500 MHz, CDCl3): δ_H 1.25 (m, 1H), 1.31 (m, 1H), 1.37 (s, 3H), 1.41 (m, 2H), 1.61 (m, 1H), 1.67 (m, 2H), 1.76 (m, 1H), 1.78 (s, 3H), 2.78 (t, 2H), 2.95 (dd, J = 8.0, 9.5 Hz, 1H), 3.42 (dd, J = 7.0, 7.0 Hz, 1H), 4.21 (ddd, J = 10.0, 10.0, 7.5 Hz, 1H), 4.30 (d, J = 15.5 Hz, 1H), 4.97 (d, J = 15.5 Hz, 1H), 5.13 (t, 1H), 5.32 (m, 1H), 6.13 (s, 1H), 6.72 (d, J = 10.0 Hz, 1H), 7.08 (dd, J = 13.5, 10.0 Hz, 1H), 7.14 (d, J = 7 Hz, 1H), 7.21 (m, 4H), 7.44 (d, J = 10 Hz, 1H), 7.65 (m, 1H), 7.71 (d, J = 8 Hz, 1H), 8.45 (d, J = 5 Hz, 1H).

4.1.5. Synthesis of *cyclo* (-L-Am7(S2Py)-Aib-L-Phg-D-Pro-) (9). The N-terminal free H-D-Pro-O^{t}Bu (1 g, 5.8 mmol), was coupled with Z-L-Phg-OH (1.82 g, 6.4 mmol) using DCC (1.32 g, 6.4 mmol) and HOBt·H_2O (980 mg, 6.4 mmol) in DMF (15 mL) to yield Z-L-Phg-D-Pro-O^{t}Bu (2.7 g, 99%). The dipeptide Z-L-Phg-D-Pro-O^{t}Bu (2.7 g, 6.2 mmol) was subjected to catalytic hydrogenation with Pd–C (300 mg) in acetic acid (15 mL). The free amine was taken into ethyl acetate (50 mL) the aid of 2M Na_2CO_3 solution (30 mL). After dried over anhydrous Na_2CO_3 ethyl acetate solution was evaporated to remain H-L-Phg-D-Pro-O^{t}Bu (1.65 g, 89%). The free amine H-L-Phg-D-Pro-O^{t}Bu (1.5 g, 4.8 mmol) was coupled with Z-Aib-OH (1.3 g, 5.5 mmol) in the same manner as described above to yield Z-Aib-L-Phg-D-Pro-O^{t}Bu (2.26 g, 94%). Further, similar reactions were carried out on Z-Aib-L-Phg-D-Pro-O^{t}Bu, according to procedure reported for **3** to obtain finally *cyclo*(-L-Am7(S2Py)-Aib-L-Phg-D-Pro-). Yield (229 mg, 45%), HPLC, rt 7.69 min. HR-FAB MS, $[M+H]^+$ 584.2347 for $C_{29}H_{37}O_4N_5S_2$ (calcd 584.2365). ^1H NMR (500 MHz, CDCl3): δ_H 1.29 (m, 1H), 1.40 (s, 3H), 1.42 (m, 1H), 1.60 (m, 1H), 1.69 (m, 2H), 1.79 (1H, m), 1.85 (S, 3H), 1.88 (m, 1H), 1.99 (m, 2H), 2.32 (m, 1H), 2.43 (m, 1H), 2.77 (t, 2H), 3.74 (ddd, J = 10.0, 10.0, 8.7 Hz, 1H), 4.04 (m, 1H), 4.22 (ddd, J = 11.7, 10.3, 7.0 Hz, 1H), 4.75 (dd, J = 2.5, 2.5 Hz, 1H), 6.05 (s, 1H), 6.15 (d, J = 10.5 Hz, 1H), 7.07 (m, 1H), 7.32 (m, 2H), 7.37 (m, 3H), 7.64 (m, 1H), 7.71 (d, J = 8 Hz, 1H), 8.04 (d, J = 10.5 Hz, 1H), 8.46 (d, J = 4.25 Hz, 1H).

4.1.6. Synthesis of *cyclo* (-L-Am7(S2Py)-Aib-L-Ph4-D-Pro-) (10). This compound was synthesized according to

the procedure reported for **9**. Yield (326 mg, 68%), HPLC, rt 7.88 min. HR-FAB MS, $[M+H]^+$ 612.2666 for $C_{31}H_{41}O_4N_5S_2$ (calcd 612.2678). ^1H NMR (500 MHz, CDCl3): δ_H 1.28 (m, 1H), 1.33 (s, 3H), 1.41 (m, 2H), 1.61 (m, 1H), 1.72 (s, 3H), 1.79 (m, 3H), 1.83 (m, 2H), 2.05 (m, 1H), 2.19 (m, 1H), 2.37 (m, 1H), 2.64 (m, 2H), 2.77 (t, 2H), 3.31 (ddd, J = 10.5, 10.5, 7 Hz, 1H), 3.74 (m, 1H), 4.17 (ddd, J = 10.5, 10.5, 7.5 Hz, 1H), 4.72 (d, 1H), 4.82 (ddd, J = 9.45, 9.75, 7.65 Hz, 1H), 5.93 (s, 1H), 7.07 (m, 1H), 7.18 (m, 3H), 7.27 (m, 3H), 7.63 (m, 1H), 7.73 (d, J = 8.5 Hz, 1H), 8.47 (d, J = 4.5 Hz, 1H).

4.1.7. Synthesis of *cyclo* (-L-Am7(S2Py)-Aib-L-Ph5-D-Pro-) (11). This compound was synthesized according to the procedure reported for **9**. Yield (100 mg, 63%), HPLC, rt 7.96 min. HR-FAB MS, $[M+H]^+$ 626.2798 for $C_{32}H_{43}O_4N_5S_2$ (calcd 626.2835) ^1H NMR (500 MHz, CDCl3): δ_H 1.28 (m, 1H), 1.33 (s, 3H), 1.41 (m, 1H), 1.64 (m, 1H), 1.70 (m, 3H), 1.76 (S, 3H), 1.82 (m, 3H), 1.89 (m, 2H), 2.23 (m, 1H), 2.36 (m, 1H), 2.65 (m, 1H), 2.77 (t, 2H), 3.47 (m, 1H), 3.89 (m, 1H), 4.17 (ddd, J = 10.5, 10.3, 7.7 Hz, 1H), 4.71 (dd, J = 2.0, 1.5 Hz, 1H), 4.85 (ddd, J = 8.75, 10, 4.4), 5.92 (s, 1H), 7.07 (m, 1H), 7.17 (m, 1H), 7.26 (m, 5H), 7.32 (d, J = 8 Hz, 1H)), 7.65 (m, 1H), 8.46 (d, J = 4.0 Hz, 1H).

4.1.8. Synthesis of *cyclo* (-L-Am7(S2Py)-Aib-L-Ser(Bzl)-D-Pro-) (12). This compound was synthesized according to the procedure reported for **9**. Yield (86 mg, 60%), HPLC, rt 7.61 min. HR-FAB MS, $[M+H]^+$ 628.2598 for $C_{30}H_{39}O_5N_5S_2$ (calcd 628.2627) ^1H NMR (500 MHz, CDCl3): δ_H 1.27 (m, 1H), 1.34 (s, 3H), 1.41 (m, 1H), 1.60 (m, 2H), 1.71 (m, 1H), 1.74 (S, 3H), 1.79 (m, 1H), 1.85 (m, 1H), 1.92 (m, 1H), 2.23 (m, 1H), 2.37 (m, 1H), 2.77 (t, 2H), 3.56 (m, 2H), 3.85 (m, 1H), 3.93 (t, 1H), 4.13 (ddd, J = 10.0, 10.0, 8.0 Hz, 1H), 4.55 (dd, J = 12, 12.5), 4.75 (dd, J = 2.0, 2.0 Hz, 1H), 5.12 (m, 1H), 5.98 (s, 1H), 7.07 (m, 1H), 7.32 (m, 5H), 7.65 (m, 1H), 7.71 (d, J = 10 Hz, 1H), 8.44 (d, J = 4.25 Hz, 1H).

4.1.9. Synthesis of *cyclo* (-L-Am7(S2Py)-Aib-L-Ser-D-Pro-) (13). This compound was synthesized according to the procedure reported for **9**. Yield (86 mg, 60%), HPLC, rt 4.98 min. HR-FAB MS, $[M+H]^+$ 538.2205 for $C_{24}H_{35}O_5N_5S_2$ (calcd 538.2158). ^1H NMR (500 MHz, CDCl3): δ_H 1.29 (m, 1H), 1.35 (s, 3H), 1.42 (m, 1H), 1.60 (m, 2H), 1.70 (m, 1H), 1.79 (S, 3H), 1.84 (m, 2H), 1.94 (m, 1H), 2.25 (m, 1H), 2.39 (m, 1H), 2.77 (t, 2H), 3.37 (dd, J = 3.0, 3.35 Hz, 1H, 3.58 (ddd, J = 10.3, 10.3, 7.5 Hz, 1H), 3.87 (m, 2H), 3.95 (m, 2H), 4.19 (ddd, J = 10.0, 10.0, 7.3 Hz, 1H), 4.76 (d, J = 6.5 Hz, 1H), 4.95 (m, 1H), 6.05 (s, 1H), 6.92 (d, J = 6.5 Hz, 1H), 7.08 (m, 1H), 7.65 (m, 1H), 7.72 (d, J = 8.25 Hz, 1H), 7.78 (d, J = 10.0 Hz, 1H), 8.46 (d, J = 3.5 Hz, 1H).

4.1.10. Synthesis of *cyclo* (-L-Am7(S2Py)-Aib-L-Ala-D-Pro-) (14). This compound was synthesized according to the procedure reported for **3**. Yield (50 mg, 30%), HPLC, rt 5.98 min. HR-FAB MS, $[M+H]^+$ 584.2336 for $C_{29}H_{38}O_4N_5S_2$ (calcd 584.2287). ^1H NMR (500 MHz, CDCl3): δ_H 1.27 (m, 2H), 1.34 (s, 3H), 1.36 (d, J = Hz, 3H), 1.42 (m, 2H), 1.60 (m, 1H), 1.71 (m, 1H), 1.77 (s, 3H), 1.81 (m, 1H), 1.91 (m, 1H), 2.24 (m, 1H), 2.38 (m, 1H), 2.78 (t, 2H), 3.53 (ddd, J = 16.0, 16.2, 8.9 Hz, 1H), 3.93 (m, 1H), 4.18 (ddd, J = 10.2, 10.5, 7.6 Hz, 1H), 4.73 (dd, J = 2.5, 2.5 Hz, 1H), 5.02 (m, 1H), 5.96 (s, 1H), 7.05 (d, J = 10 Hz, 1H), 7.09 (m, 4H), 7.41 (d, J = 10 Hz, 1H), 7.66 (m, 1H), 7.72 (d, J = 8.5 Hz, 1H), 8.46 (d, J = 14.5 Hz, 1H).

4.2. Circular dichroism measurement

CD spectra were recorded on a JASCO J-820 spectropolarimeter (Tokyo, Japan) using a quartz cell of 1 mm light path length at room temperature. Peptide solution (0.1 mM) was dissolved in methanol and CD spectra were recorded in terms of molar ellipticity, $[\theta]_M$ ($deg \times cm^2 \times dmol^{-1}$).

4.2.1. HDACs preparation and enzyme activity assay. In a 100-mm dish, 293T cells ($1–2 \times 10^6$) 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 tripsin (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.

4.2.2. The p21 promoter assay. A luciferase reporter plasmid (pGW-FL) was constructed by cloning the 2.4 kb genomic fragment containing the transcription start site into HindIII and SmaI 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 (pBK-CMV, 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 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 10-fold higher than the basal level are presented as the 1000% effective concentration 1000% (EC_{1000}). The human wild-type p21 promoter luciferase fusion plasmid, WWP-Luc, was a kind gift from Dr. B. Vogelstein.

Acknowledgment

This study was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO).

References and notes

1. Hassig, C. A.; Schrieber, S. L. *Curr. Opin. Chem. Biol.* **1997**, *1*, 300–308.
2. Kouzarides, T. *Curr. Opin. Gen. Dev.* **1999**, *9*, 40–48.
3. Grozinger, C. M.; Schrieber, S. L. *Chem. Biol.* **2002**, *9*, 3–16.
4. Yoshida, M.; Matsuayama, A.; Komatsu, Y.; Nishino, N. *Curr. Med. Chem.* **2003**, *10*, 2351–2358.
5. Yang, X. J. *Bioessays* **2004**, *26*, 1076–1081.
6. Urnov, F. D.; Wolfeffe *Emerging Ther. Targets* **2000**, *4*, 665–685.
7. Mahlknecht, U.; Hoelzer, D. *Mol. Med.* **2000**, *6*, 623–644.
8. Timmerman, S. et al. *Cell. Mol. Life Sci.* **2001**, *58*, 728–736.
9. Crsee, W. D.; Seto, E. J. *Cell. Physiol.* **2000**, *184*, 1–16.
10. Yoshida, M.; Kijima, M.; Akita, M.; Beppu, T. *J. Biol. Chem.* **1990**, *265*, 17174–17179.
11. Ueda, H.; Nakajima, H.; Hori, Y.; Fujita, T.; Nishimura, M.; Goto, T.; Okuhara, M. *J. Antibiot.* **1994**, *47*, 301–310.
12. Ueda, H.; Manda, T.; Matsumoto, S.; Mukumoto, S.; Nishigaki, F.; Kawamura, I.; Shimomura, K. *J. Antibiot.* **1994**, *47*, 315–323.
13. Furumai, R.; Matsuyama, A.; Kobashi, M.; Lee, K.-H.; Nishiyama, M.; Nakajima, H.; Tanaka, A.; Komatsu, Y.; Nishino, N.; Yoshida, M.; Horinouchi, S. *Cancer Res.* **2002**, *62*, 4916–4921.
14. Kijima, M.; Yoshida, M.; Suguta, K.; Horinouchi, S.; Beppu, T. *J. Biol. Chem.* **1993**, *268*, 22429–22435.
15. Closse, A.; Huguenin, R. *Helv. Chim. Acta* **1974**, *57*, 545–553.
16. Yoshimura, K.; Tsubotani, S.; Okazaki, K. *JP 7196686*, 1995.
17. Xie, W.; Zou, B.; Pei, D.; Ma, D. *Org. Lett.* **2005**, *7*, 2775–2777.
18. Gupta, S.; Peiser, G.; Nakajima, T.; Hwang, Y.-S. *Tetrahedron Lett.* **1994**, *35*, 6009–6012.
19. Liesch, J. M.; Sweeley, C. C.; Staffeld, G. D.; Anderson, M. S.; Weber, D. J.; Scheffer, R. P. *Tetrahedron* **1982**, *38*, 45–48.
20. Pope, M. R.; Ciuffetti, L. M.; Knoche, H. W.; McCrery, D.; Daly, J. M.; Dunkle, L. D. *Biochemistry* **1983**, *22*, 3502–3506.
21. Gross, M. L.; McCrery, D.; Crow, F.; Tomer, K. B.; Pope, M. R.; Ciuffetti, L. M.; Knoche, H. W.; Daly, J. M.; Dunkle, L. D. *Tetrahedron Lett.* **1982**, *23*, 5381–5384.
22. Tanis, S. P.; Horenstein, B. A.; Scheffer, R. P.; Rasmussen, J. B. *Heterocycles* **1986**, *24*, 3423–3431.
23. Kim, S.-D.; Knoche, H. W.; Dunkle, L. D.; McCrery, D. A.; Tomer, K. B. *Tetrahedron Lett.* **1985**, *26*, 969–972.
24. Hirota, A.; Suzuki, A.; Suzuki, H.; Tamura, S. *Agric. Biol. Chem.* **1973**, *37*, 643–647.
25. Hirota, A.; Suzuki, A.; Aizawa, K.; Tamura, S. *Agric. Biol. Chem.* **1973**, *37*, 955–956.
26. Hirota, A.; Suzuki, A.; Tamura, S. *Agric. Biol. Chem.* **1973**, *37*, 1185–1189.
27. Umehara, K.; Nakahara, K.; Kiyoto, S.; Iwami, M.; Okamoto, M.; Tanaka, H.; Kohsaka, M.; Aoki, H.; Imanaka, H. *J. Antiobiot.* **1983**, *36*, 478–483.
28. Darkin-Rattray, S. J.; Gurnett, A. M.; Myers, R. W.; Dulski, P. M.; Crumley, T. M.; Allocco, J. J.; Cannova, C.; Meinke, P. T.; Colletti, S. L.; Bednarek, M. A.; Singh, S. B.; Goetz, M. A.; Dombrowski, A. W.; Polishook, J. D.; Schmatz, D. M. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 13143–13147.
29. Singh, S. B.; Zink, D. L.; Liesch, J. M.; Mosley, R. T.; Dombrowski, A. W.; Bills, G. F.; Darkin-Rattray, S. J.; Schmatz, D. M.; Goetz, M. A. *J. Org. Chem.* **2002**, *67*, 815–825.
30. Han, J. W.; Ahn, S. H.; Park, S. H.; Wang, S. Y.; Bae, G. U.; Seo, D. W.; Known, H. K.; Hong, S.; Lee, Y. W.; Lee, H. W. *Cancer Res.* **2000**, *60*, 6068–6074.
31. Meinke, P. T.; Colletti, S. L.; Ayer, M. B.; Darkin-Rattray, S. J.; Myers, R. W.; Schmatz, D. M.; Wyvratt, M. J.; Fisher, M. H. *Tetrahedron Lett.* **2000**, *41*, 7831–7835.
32. Abe, F.; Hino, M.; Mori, H.; Yoshimura, S.; Takase, S. WO200008048-A2, **2000**.
33. Richon, V. M.; Emiliani, S.; Verdin, E.; Webb, Y.; Breslow, R.; Rifkind, R. A.; Marks, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 3003–3007.
34. Jung, M.; Brosch, G.; Kölle, D.; Scherf, H.; Gerhäuser, C.; Loidl, P. *J. Med. Chem.* **1999**, *42*, 4669–4679.
35. Remiszewski, S. W.; Sambucetti, L. C.; Atadja, P.; Bair, K. W.; Cornell, W. D.; Green, M. A.; Howell, K. L.; Jung, M.; Known, P.; Trogani, N.; Walker, H. *J. Med. Chem.* **2002**, *45*, 753–757.
36. Woo, S. H.; Frechette, S.; Khalil, E. A.; Bouchain, G.; Vaisburg, A.; Bernstein, N.; Moradei, O.; Leit, S.; Allan, M.; Fournel, M.; Trachy-Bourget, M.-C.; Li, Z.; Besterman, J. M.; Delorme, D. *J. Med. Chem.* **2002**, *45*, 2877–2885.
37. Su, G. H.; Sohn, T. A.; Ryu, B.; Kern, S. E. *Cancer Res.* **2000**, *60*, 3137–3142.
38. Suzuki, T.; Ando, T.; Tsuchiya, K.; Fukazawa, N.; Saito, A.; Mariko, Y.; Yamanashi, T.; Nakanishi, O. *J. Med. Chem.* **1999**, *42*, 3001–3003.
39. Saito, A.; Yamashita, T.; Mariko, Y.; Nosaka, Y.; Tsuchiya, K.; Ando, T.; Suzuki, T.; Tsuruo, T.; Nakanishi, O. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 4592–4597.
40. Finnin, M. S.; Donigian, J. R.; Cohen, A.; Richon, V. M.; Riftkind, R. A.; Marks, P. A.; Breslow, R.; Pavletich, N. P. *Nature* **1999**, *401*, 188–193.
41. Kim, S.-D. *J. Biochem. Mol. Biol.* **1995**, *28*, 227–231.

42. De Shepper, S.; Bruwiere, H.; Steller, U.; Andries, L.; Wouters, W.; Janicot, M.; Arts, J.; Van Heusden, J. *J. Pharmacol. Exp. Ther.* **2003**, *304*, 881–888.
43. Nishino, N.; Jose, B.; Shinta, R.; Kato, T.; Komatsu, Y.; Yoshida, M. *Bioorg. Med. Chem.* **2004**, *12*, 5777–5784.
44. Hirashima, Y.; Kato, T.; Nishino, N.; Maeda, S.; Nishino, T. G.; Yoshida, M. *Proceedings of 29th European Peptide Symposium (J. Pep. Sci.)* (in press).
45. Kahnberg, P.; Lucke, A. J.; Glenn, M. P.; Boyle, G. M.; Tyndall, J. D. A.; Parsons, P. G.; Fairlie, D. P. *J. Med. Chem.* **2006**, *49*, 7611–7622.
46. Watanabe, L. A.; Jose, B.; Kato, T.; Nishino, N.; Yoshida, M. *Tetrahedron Lett.* **2004**, *45*, 491–494.
47. Bhuiyan, M. P. I.; Kato, T.; Okauchi, T.; Nishino, N.; Maeda, S.; Nishino, T. G.; Yoshida, M. *Bioorg. Med. Chem.* **2006**, *14*, 3438–3446.
48. Nishino, N.; Yoshikawa, D.; Watanabe, L. A.; Kato, T.; Jose, B.; Komatsu, Y.; Sumida, Y.; Yoshida, M. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2427–2431.
49. Kawai, M.; Jasensky, R. D.; Rich, D. H. *J. Am. Chem. Soc.* **1983**, *105*, 4456–4462.