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ORIGINAL ARTICLE

Cyclic tetrapeptides with –SS– bridging between amino acid side chains for potent histone deacetylases' inhibition

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Abstract Cyclic depsipeptide FK228 with an intramolecular disulfide bond is a potent inhibitor of histone deacetylases (HDAC). FK228 is stable in blood because of its prodrug function, whose –SS– bond is reduced within the cell. Here, cyclic peptides with –SS– bridges between a variety of amino acids were synthesized and assayed for HDAC inhibition. Cyclic peptide **3**, *cyclo*(-L-amino acid-L-amino acid-L-Val-D-Pro-), with an –SS– bridge between the first and second amino acids, was found to be a potent HDAC inhibitor. Cyclic peptide **7**, *cyclo*(-L-amino acid-D-amino acid-L-Val-D-Pro-), with an –SS– bridge between the first and second amino acids, was also a potent HDAC inhibitor.

Keywords Cyclic peptide · Histone deacetylase inhibitor · Disulfide bridge · FK228 analog

Introduction

FK228 (FR901228) and some natural cyclic depsipeptides contain intramolecular disulfide bridges (Fig. 1; Ueda et al. 1994; Masuoka et al. 2001). These depsipeptides potently inhibit histone deacetylase (HDAC) after the reductive cleavage of the –SS– bonds. The –SH group coordinates to zinc ion at the active site of class I and II HDACs (Furumai et al. 2002). For practical use, the anticancer drugs should be stable in the body before their delivery to the target sites. But the digestive system and blood deactivate some HDAC inhibitors, although they are potent in vitro. FK228 is useful prodrug because of the stable disulfide group.

Other natural cyclic peptidyl HDAC inhibitors include chlamydocin, trapoxins (Kijima et al. 1993), and HC-toxins (Miller et al. 2003), tethering epoxyketone moieties that interact with the active site. We have discovered that cyclic tetrapeptides with artificial zinc ligating groups are potent and specific HDAC inhibitors (Komatsu et al. 2001; Islam et al. 2011). Here, we present cyclic tetrapeptides that contain disulfide moieties as prodrug candidates, which are easier to synthesize than FK228 and analogs (Hoque et al. 2012).

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Results and discussion

Cyclo(-L-Am7(Ac)-Aib-L-Am7(Ac)-D-Pro-) (**1**) with a chlamydocin framework (-***L**L***D-) and attempted intramolecular disulfide bond formation

The aminoisobutylic acid (Aib) in chlamydocin, *cyclo*(-L-Aoe-Aib-L-Phe-D-Pro-) (Aoe, (2*S*,9*S*)-2-amino-8-oxo-9,10-epoxydecanoic acid), preferably has a β-turn structure and fixes cyclic peptide framework. Using this -L*LD- peptide sequence and using hydroxamic acid as a zinc-ligating group, a chlamydocin–hydroxamic acid analog was successfully synthesized as a potent HDAC inhibitor (Fig. 2, Nishino et al. 2004). An intramolecular disulfide bridge

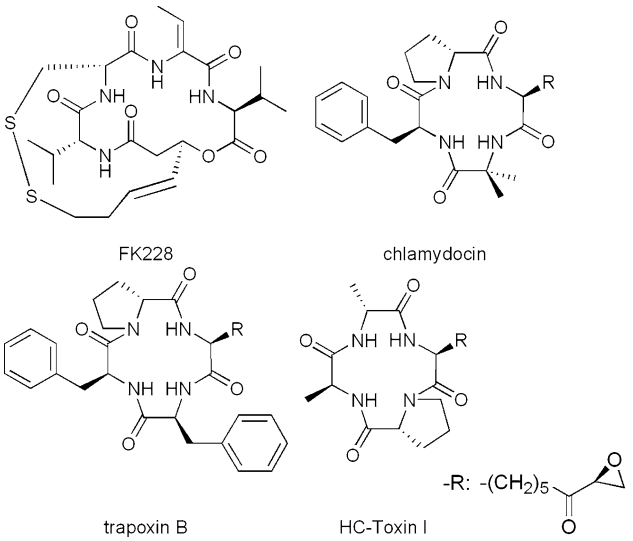


Fig. 1 Cyclic depsipeptidyl HDAC inhibitor FK228 with –SS– bond and cyclic tetrapeptidyl HDAC inhibitors with epoxyketone

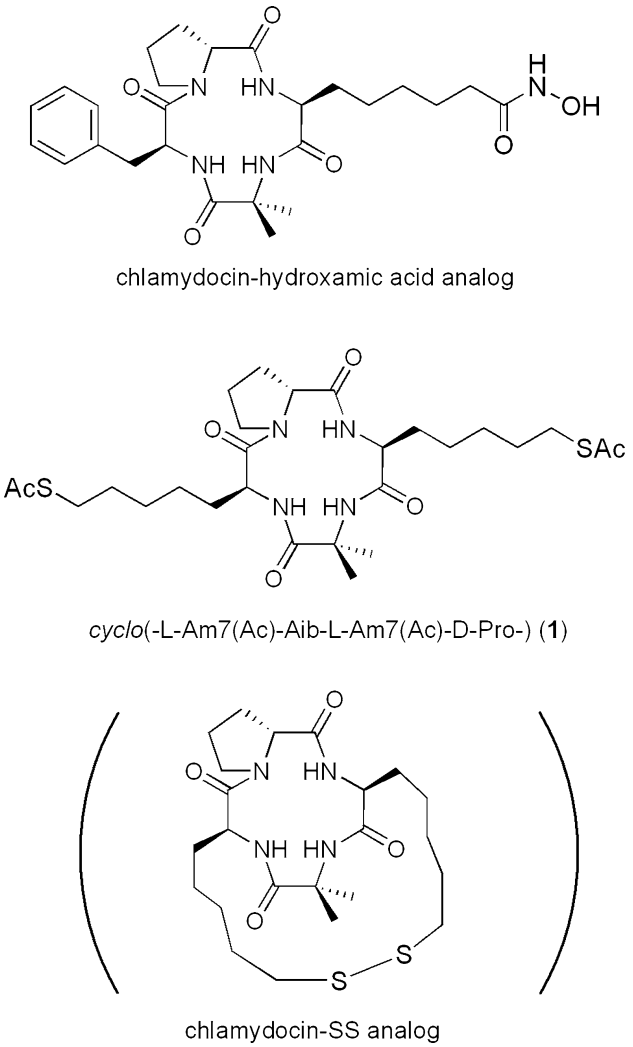


Fig. 2 HDAC inhibitor with chlamydocin framework (-***L**LD**-)

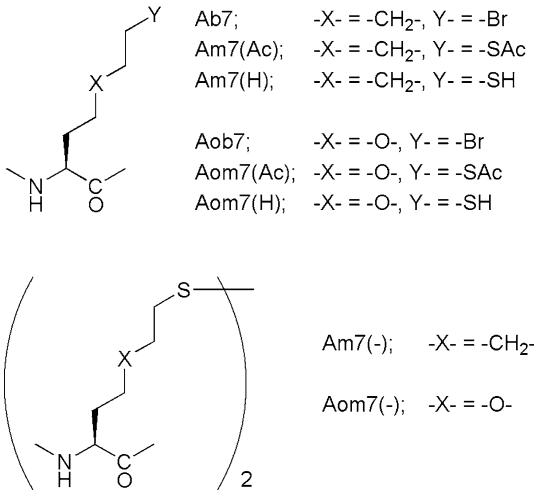


Fig. 3 Unnatural amino acids prepared for –SS–

was added between the facing amino acids, i.e., between the –SH groups written in italics in -***L**LD**-. *Cyclo*(-L-Am7(Ac)-Aib-L-Am7(Ac)-D-Pro-) (**1**) was synthesized expecting –SS– formation between the two Am7(H)s (Figs. 2, 3).

The linear tetrapeptide Boc-L-Ab7-Aib-L-Ab7-D-Pro-OtBu was prepared by the stepwise elongation of D-Pro-OtBu (Scheme 1), taking advantage of the fact that the cyclic imino acid (Pro) hardly racemizes during couplings. Coupling Boc-L-Ab7-OH ((*S*)-*N*-^{t}butoxycarbonyl-2-amino-7-bromoheptanoic acid, Watanabe et al. 2004) and D-Pro-OtBu with *N*,*N*'-dicyclohexylcabodiimide (DCC)/1-hydroxybenzotriazole (HOBt) gave Boc-L-Ab7-D-Pro-OtBu. Boc was selectively removed with HCl/dioxane without cleaving the –COOtBu (Han et al. 2008), then the residue was coupled with Z-Aib-OH to afford Z-Aib-L-Ab7-D-Pro-OtBu. Z-removal with H_2-Pd/C and coupling the product with Boc-L-Ab7-OH gave Boc-L-Ab7-Aib-L-Ab7-D-Pro-OtBu. After TFA treatment, the product TFA·H–D-Ab7-Aib-L-Ab7-D-Pro-OH was cyclized in DMF ([peptide] = 4.1 mM) with *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HATU)/diisopropylethylamine (DIEA). The precursor cyclic tetrapeptide *cyclo*(-L-Ab7-Aib-L-Ab7-D-Pro-) was obtained in 74 % yield based on the linear tetrapeptide after silica gel column chromatography. The bromoalkyl side chains were transformed to AcS– with potassium thioacetate (KSAc) in DMF to yielded *cyclo*(-L-Am7(Ac)-Aib-L-Am7(Ac)-D-Pro-) (**1**). All the new compounds were characterized by FAB-MS, HR-FAB-MS, and ^{1}H-NMR. C_{18} HPLC was used to determine the peptides purity (Table 1).

The –SAc side chains of **1** were deprotected using excess $MeNH_2$ in MeOH (r.t. 2 h). The cyclic tetrapeptide with –SH groups (*cyclo*(-L-Am7(H)-Aib-L-Am7(H)-D-Pro-), not isolated) was mixed with I_2 in DMF-EtOH ([peptide] = 5.0 mM, r.t. 30 min). However, the expected

Scheme 1 Reagents and conditions: (*a*) Boc-L-Ab7-OH, DCC/HOBt; (*b*) HCl/dioxane; (*c*) Z-Aib-OH, DCC/HOBt; (*d*) Pd/C,H_2; (*e*) TFA; (*f*) HATU/DIEA; (*g*) KSAc

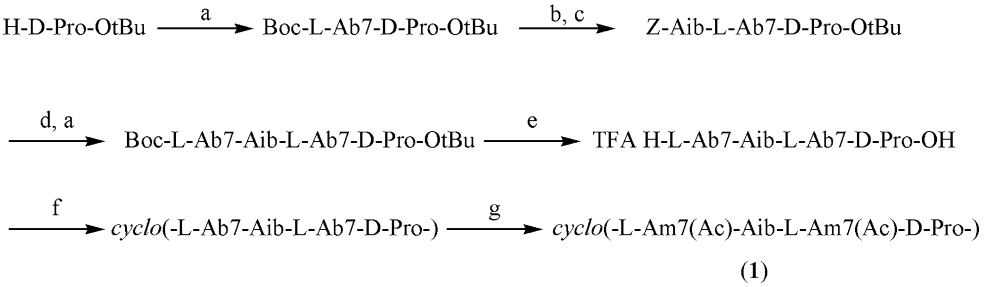


Table 1 Yields and HPLC retention times for the intramolecular –SS– bridging of the cyclic peptide inhibitors

Model	Chlamydocin	Trapoxin B	HC-toxin I					
Configuration	-***L**L***D-	-***LL***LD-	-***L***DL***D***-	-***LD***LD-				
–SAc precursor	**1**	**2**	**4**	**6**	**8**	**10**	**12**	**14**	**16**
HPLC[a]	7.28	7.45	8.33	7.82	6.50	7.98	5.84	7.18	7.51
–SS– formation	Not obtained	**3**	**5**	**7**	**9**	**11**	**13**	**15**	**17**
Yield/%[b]	–	31	23	45	28	28	34	36	36
HPLC[b]	–	6.58	7.90	7.03	5.33	7.22	4.92	6.43	7.04

[a] Retention time/min, C_{18} column (see experimental)

[b] Based on –SAc precursor

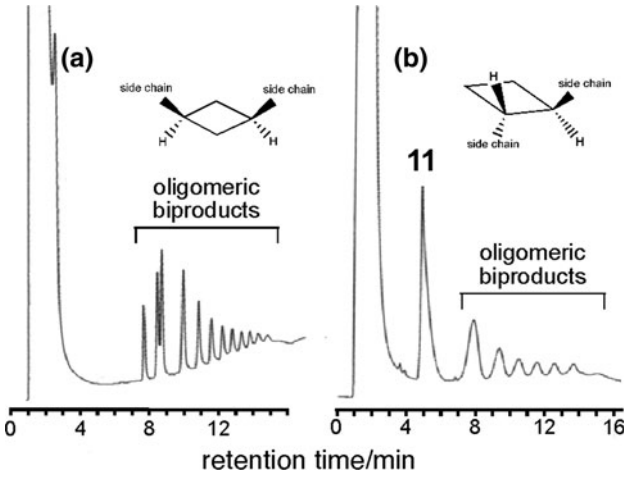


Fig. 4 HPLC profiles of the reaction mixture of the I_2 oxidation of **1** (**a**) **and 10** (**b**)

intramolecular –SS– bridged product was not formed. The C_{18} HPLC chromatogram of the reaction mixture (Fig. 4a), using H_2O-CH_3CN as the mobile phase, showed no signals corresponding to the bicyclic tetrapeptide, but did show oligomeric byproducts with the intermolecular –SS– bridges (determined by MS). For comparison, Fig. 4b shows the intramolecular –SS– bridging from **10** to **11**, which was successful (see below) and led to a large **11** signal appearing. The two –SH groups facing each other in the chlamydocin framework (-***L**L***D-) appeared to be inappropriate for intramolecular –SS– formation.

Cyclo(-L-Am7(Ac)-L-Am7(Ac)-L-Val-D-Pro-) (**2**) with a trapoxin B (-***LL***LD-) framework and intramolecularly disulfide bridged *cyclo*(-L-Am7(-)-L-Am7(-)-L-Val-D-Pro-) (**3**)

Next, the trapoxin B -***LL***LD- sequence, *cyclo*(-L-Aoe-L-Phe-L-Phe-D-Pro-), was used to arrange two L-Am7(Ac) residues at neighboring positions in *cyclo*(-L-Am7(Ac)-L-Am7(Ac)-L-Val-D-Pro-) (**2**, Fig. 5). The third amino acid L-Phe in trapoxin B was replaced by L-Val. The linear tetrapeptide Boc-L-Ab7-L-Ab7-L-Val-D-Pro-OtBu was synthesized from H-D-Pro-OtBu, Z-L-Val-OH, and Boc-L-Ab7-OH (two times), deprotected, cyclized with HATU/DIEA (77 % yield from the linear tetrapeptide), and transformed to **2** with KSAc. Treatment with $MeNH_2$/MeOH followed by I_2 oxidation ([peptide] = 5.0 mM) gave the intramolecularly –SS– bridged cyclic tetrapeptide *cyclo*(-L-Am7(-)-L-Am7(-)-L-Val-D-Pro-) (**3**) with some oligomeric byproducts. LH-20 gel filtration chromatography with DMF afforded **3** in 31 % yield based on **2**. The –SS– bond formation between neighboring amino acids in **2** seems to be easier than that between facing amino acids in **1**. The –SS–bridged **3** eluted earlier than **2** in the HPLC, probably because of its compact molecular structure.

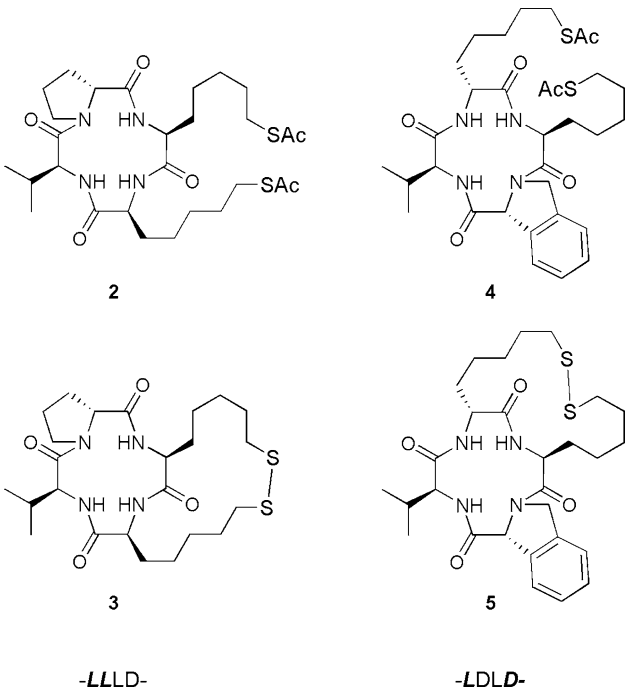
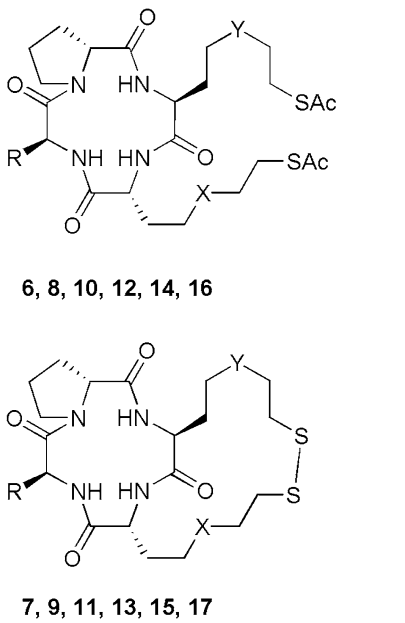


Fig. 5 HDAC inhibitors with trapoxin and HC-toxin frameworks



6, **7**; -R = -iPr, -X- = -Y- = -CH_2-
8, **9**; -R = -Me, -X- = -Y- = -CH_2-
10, **11**; -R = -CH_2Ph, -X- = -Y- = -CH_2-

12, **13**; -R = -Me, -X- = -O-, -Y- = -CH_2-
14, **15**; -R = -CH_2Ph, -X- = -O-, -Y- = -CH_2-
16, **17**; -R = -CH_2Ph, -X- = -CH_2-, -Y- = -O-

Fig. 6 HDAC inhibitors with -***LD***LD- configuration

Cyclo(-L-Am7(Ac)-D-Tic-L-Val-D-Am7(Ac)-) (**4**) with an HC-toxin (-***L***DL***D***-) framework and the intramolecularly disulfide bridged *cyclo*(-L-Am7(-)-D-Tic-L-Val-D-Am7(-)-) (**5**)

The third cyclic tetrapeptide motif was the -***L***DL***D***- sequence in the HC-toxin, *cyclo*(-L-Aoe-D-Pro-L-Ala-D-Ala-). *Cyclo*(-L-Am7(Ac)-D-Tic-L-Val-D-Am7(Ac)-) (**4**; Tic = 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) was designed with increased hydrophobicity by modifying -D-Pro-L-Ala- to -D-Tic-D-Val- (Fig. 5). The linear tetrapeptide Boc-L-Val-D-Ab7-L-Ab7-D-Tic-OtBu was prepared, deprotected, and cyclized with HATU/DIEA to give *cyclo*(-L-Ab7-D-Tic-L-Val-D-Ab7-) in a modest yield (58 %), which was then transformed to *cyclo*(-L-Am7(Ac)-D-Tic-L-Val-D-Am7(Ac)-) (**4**). Deprotecting **4** with $MeNH_2$/MeOH and oxidation with I_2/EtOH afforded *cyclo*(-L-Am7(-)-D-Tic-L-Val-D-Am7(-)-) (**5**) in a rather low yield (23 %).

Cyclo(-L-Am7(Ac)-D-Am7(Ac)-L-Val-D-Pro-) (**6**) with an -***LD***LD- configuration and the intramolecularly disulfide bridged *cyclo*(-L-Am7(-)-D-Am7(-)-L-Val-D-Pro-) (**7**)

The intramolecular –SS– formation of the trapoxin-modeled peptide with an -***LL***LD- configuration **2**, *cyclo*(-L-Am7(Ac)-L-Am7(Ac)-L-Val-D-Pro-), gave a moderate yield of **3** (31 %). *Cyclo*(-L-Am7(Ac)-D-Am7(Ac)-L-Val-D-Pro-) (**6**), a cyclic peptide with an -***LD***LD- configuration, was tested instead of **2** (Fig. 6). The linear tetrapeptide Boc-L-Ab7-D-Ab7-L-Val-D-Pro-OtBu was deprotected, cyclized with HATU/DIEA (95 % yield), and transformed to **6** with KSAc. Treatment with $MeNH_2$/MeOH and I_2 gave the intramolecularly –SS– bridged cyclic peptide **7** in 45 % yield after LH-20 purification. This was the highest yield for the intramolecular –SS– formation found in this study (Table 1). The structure of **7** with an -***LD***LD- configuration might be less restricted than the structure of **3** (-***LL***LD-configuration); **6** and **7** with -***LD***LD- configurations eluted later than **2** and **3** with -***LL***LD- configuration in C_{18} HPLC analyses, respectively, although they contained the same amino acid residues. The later C_{18} HPLC elution suggests that **6** and **7** were less polar than **2** and **3**, respectively, possibly because of the effective intramolecular hydrogen bonds in **6** and **7**.

Effect of amino acid hydrophobicity and ether-linked side chains on the analogs of **6** and **7** with -***LD***LD-configuration

Using the -***LD***LD- configuration, the effect of hydrophobicity was examined by introducing L-Ala and L-Phe instead of L-Val in **6** and **7**. Therefore, *cyclo*(-L-Am7(Ac)

-D-Am7(Ac)-L-Ala-D-Pro-) (**8**), *cyclo*(-L-Am7(-)-D-Am7(-)-L-Ala-D-Pro-) (**9**), *cyclo*(-L-Am7(Ac)-D-Am7(Ac)-L-Phe-D-Pro-) (**10**), and *cyclo*(-L-Am7(-)-D-Am7(-)-L-Phe-D-Pro-) (**11**) were synthesized (Fig. 6). The HPLC retention times showed that the order of hydrophobicities of these products was **10** and **11** (Phe) > **6** and **7** (Val) > **8** and **9** (Ala).

We also introduced a new amino acids Aob7 (2-amino-5-oxy-7-bromoheptanoic acid) and Aom7(Ac) (2-amino-5-oxy-7-acetylmercaptoheptanoic acid), which has an ether moiety in the side chains (Fig. 3). This ether linkage may affect the solubility, synthetic yield, and biological activity. *Cyclo*(-L-Am7(Ac)-D-Aom7(Ac)-L-Ala-D-Pro-) (**12**), *cyclo*(-L-Am7(-)-D-Aom7(-)-L-Ala-D-Pro-) (**13**), *cyclo*(-L-Am7(Ac)-D-Aom7(Ac)-L-Phe-D-Pro-) (**14**), *cyclo*(-L-Am7(-)-D-Aom7(-)-L-Phe-D-Pro-) (**15**), *cyclo*(-L-Aom7(Ac)-D-Am7(Ac)-L-Phe-D-Pro-) (**16**), and *cyclo*(-L-Aom7(-)-D-Am7(-)-L-Phe-D-Pro-) (**17**) were synthesized. These ether-linked peptides were more hydrophilic and eluted from the HPLC faster than the corresponding non-ether-containing peptides. For instance, **12** and **13** eluted faster than **8** and **9**, respectively.

^{1}H-NMR investigation into the peptide structure before and after the –SS– bridging reaction

Figure 7 shows the ^{1}H-NMR spectra (in $CDCl_3$, low-field region) of (a) **8**, which has -SAc tails, and (b) **9**, with an –SS– bridge. The amide NH protons were slightly low-field shifted comparing **9** (δ 7.12, d, 10 Hz, L-Am7(-); δ 6.42, d, 10 Hz, Ala, and δ 6.14, d, 10 Hz, D-Am7(-)) with **8** (δ 7.09, d, 10 Hz; δ 6.30, d, 10 Hz; δ 6.05, d, 10 Hz). The 10 Hz coupling constant of every amide protons in **8** and **9** indicated that the cyclic peptide conformation was little altered by the –SS– bridge (Pardi et al. 1984). The *J* value (coupling constant) between NH and $C^{\alpha}H$ reflects the dihedral angle of the amino acid. The Ala and D-Pro C^{α} protons in **8** (δ 4.93, dq, Ala; δ 4.71, dd, D-Pro) were not shifted in **9**, but the C^{α} protons of L-Am7(Ac) (δ 4.35, dt) and D-Am7(Ac) (δ 4.29, dt) in **8** were shifted to δ 4.46 (m, intensity 2 H), due to the change from SAc to –SS–.

Enzyme inhibition and biological activity

The cyclic peptides were assayed for HDAC activity in vitro using three enzymes, HDAC1, HDAC4, and HDAC6, which were prepared using 293T cells and a substrate attaching a fluorophore (Furumai et al. 2002). In the presence of 0.1 mM dithiothreitol (DTT), the cyclic peptides with the intramolecular –SS– bridges (**3**, **5**, **7**, **13**, and **15**) showed modest activities with IC_{50} concentrations lower than 0.1 μM for HDAC1 and HDAC4. Compound **7** gave an IC_{50} of 0.0081 μM (8.1 nM), making it more potent than trichostatin A or FK228. It is interesting that the cyclic peptide **7** with an

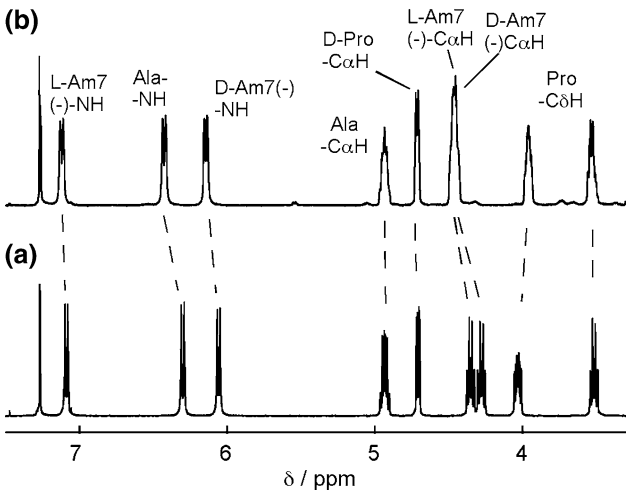


Fig. 7 ^{1}H-NMR spectra of **8** (**a**) **and 9** (**b**) in $CDCL_3$

-***LD***LD- configuration gave a higher activity than **3** with an -***LL***LD- configuration. The cyclic peptides with –SAc tails (**1**, **2**, **4**, **6**, **12**, and **14**) inhibited the enzymes almost one order of magnitude less effectively than **3** did. It should be noted here that these cyclic peptides were hardly effective in the absence of the reductant DTT, indicating that the active moiety for inhibition is –SH (Table 2).

The cyclic peptides with –SAc and –SS– groups were both ineffective inhibitors of HDAC6. For instance, the IC_{50} for **7** was ~1,000 times higher for HDAC6 than that for HDAC1. However, the effect of **3** was less different on HDAC1 and HDAC6. Selectivity for HDAC1 over HDAC4 was not observed probably because of their similar surface binding regions. The p21 promoter assay was performed in vivo without DTT. The cyclic peptides with –SAc tails, **2** and **6**, had higher activity (EC_{1000} below 0.1 μM). The disulfide bond might be reduced to –SH in the cell medium in this assay. The reason why some peptides with –SAc tails (**2** and **6**) showed higher activity than those with –SS– (**3** and **7**) is not yet clear. The conformation and the hydrogen-bondings in the cyclic peptides may have some influence on the membrane permeability of the peptides, for instance. The effect of amino acid structure, for instance replacing Val (**6** and **7**) with Ala (**8** and **9**), did not show clear effect in the p21 promoter activity. The effect of ether linkage (**12** and **13**) in the alkyl side chain gave little difference compared to **8** and **9**.

Experimental

General

All solvents and reagents were reagent grade. Silica gel 60 (230–400 mesh) eluting with $CHCl_3$-1 % MeOH (v/v) or

Table 2 Activity of cyclic peptides for the HDAC inhibition in vitro and in vivo

Compound/active tail	HDAC inhibition (IC_{50}/μM)[a]			p21 promoter activity	
	HDAC1	HDAC4	HDAC6	EC_{1000}/μM[b]	EC_{10000}/μM[b]
Trichostatin A[c]	0.023	0.034	0.065	0.020	0.031
FK228	0.001		0.62		
1/–SAc				0.017	0.049
2/–SAc	0.26	0.18	0.92	0.036	0.071
3/–SS–	0.0139	0.0093	0.083	0.23	0.53
4/–SAc	0.15	0.20	3.7	0.23	0.43
5/–SS–	0.027	0.037	0.59	0.14	0.23
6/–SAc	0.30	0.16	47	0.042	0.079
7/–SS–	0.0081	0.0083	7.3	0.41	0.60
8/–SAc				0.14	0.39
9/–SS–				0.29	0.86
12/–SAc	0.31	0.13	5.4	0.26	0.47
13/–SS–	0.016	0.011	0.32	0.19	0.33
14/–SAc	0.19	0.099	6.5	0.16	0.29
15/–SS–	0.010	0.0059	0.33	0.15	0.32

[a] 50 % inhibitory concentration, in the presence of 0.1 mM dithiothreitol

[b] EC_{1000}; effective concentration of 10× increased induction, EC_{10000}; 100× increased induction

[c] In the absence of dithiothreitol

LH-20 (gel filtration) eluting with DMF was used for chromatography. Merck Chromolith Performance RP-18e column (4.6 × 100 mm) was used for HPLC, with a solvent gradient of (H_2O-0.1 % TFA)/(CH_3CN-0.1 % TFA) over 15 min, 2.0 mL/min flow rate, and 220 nm detection to note the retention time (Rt). FAB-mass spectra including the high resolution spectra (HR-MS) were measured on a JEOL JMS-SX 102A. NMR spectra were recorded on a JEOL JNM α-500 in $CDCl_3$.

Boc-L-2-amino-5-oxy-7-bromoheptanoic acid (Boc-L-Aob7-OH) and Boc-D-2-amino-5-oxy-7-bromoheptanoic acid (Boc-D-Aob7-OH)

Na (0.46 g, 20 mmol) were dissolved in EtOH (20 mL), to which diethyl (Boc-amino) malonate (5.5 g, 20 mmol) was added and refluxed for 30 min. Bis(2-bromoethyl) ether (14 g, 60 mmol) was added and refluxed for further 6 h. Oily diester was obtained by diethyl ether extraction, which was monohydrolysed in 20 mL EtOH/20 mL of 1 M aqueous NaOH (0 °C, 5 h). After evaporation and acidification (citric acid), the monoacid monoester product was extracted with AcOEt to give an oil. Refluxing the oil in 20 mL toluene for 7 h yielded Boc-DL-Aob7-OEt (16 mmol, 82 %) as a solid. This solid was suspended in DMF (17 mL)/H_2O (51 mL) at 38 °C with pH 7–8 (1 M NH_3). Subtilisin Carlsberg from *B. licheniformis* (Sigma, 17 mg, 1 mg enzyme per mmol substrate) was added with adding 1 M NH_3 to maintain pH 7–8 for 3 h. After evaporation, Boc-D-Aob7-OEt was extracted with diethyl ether under basic conditions. After acidification, Boc-L-Aob7-OH was extracted with AcOEt as colorless oil (2.5 g, 7.7 mmol, 46 % based on Boc-D-L-Aob7-OEt). Hydrolysis of Boc-D-Aob7-OEt (1 M NaOH) afforded Boc-D-Aob7-OH as colorless oil (2.5 g, 7.8 mmol, 48 %).

*Cyclo(-L-Am7(Ac)-Aib-L-Am7(Ac)-D-Pro-) (**1**)*

The mixture of Boc-L-Ab7-OH (1.7 g, 5.2 mmol), H-D-Pro-O^{t}Bu (0.89 g, 5.2 mmol), DCC (1.3 g, 6.2 mmol), HOBt·H_2O (0.80 g, 5.2 mmol) was mixed in 11 mL DMF (0 °C to r.t. 5 h). After evaporation, the residue taken up in AcOEt was filtered, washed, concentrated, and chromatographed (silica gel) to yield Boc-L-Ab7-D-Pro-O^{t}Bu as an oil (2.1 g, 4.4 mmol, 85 %). This dipeptide (4.4 mmol) was dissolved in ice-cold 4 M HCl/dioxane (11 mL) and was kept at room temperature (30 min) to obtain H-L-Ab7-D-Pro-O^{t}Bu as a syrup (1.5 g, 4.0 mmol, 91 %), which was reacted with Z-Aib-OH (1.1 g, 4.8 mmol) to give Z-Aib-L-Ab7-D-Pro-O^{t}Bu as an oil (1.6 g, 2.7 mmol, 67 %). This tripeptide (2.7 mmol) was stirred with 0.13 g Pd/C in 13 mL CH_3COOH under H_2 overnight. After filtration and evaporation, the residue was dissolved in AcOEt, washed, and concentrated to obtain H-Aib-L-Ab7-D-Pro-O^{t}Bu (1.2 g, 2.6 mmol, 95 %), which was reacted with Boc-L-Ab7-OH (0.83 g, 2.6 mmol) to afford Boc-L-Ab7-Aib-L-Ab7-D-Pro-O^{t}Bu as white foam (1.3 g, 1.7 mmol, 66 %). This tetrapeptide (0.63 g, 0.82 mmol) was dissolved in

4 mL TFA (0 °C) and kept at room temperature (3 h) to yield TFA·H-L-Ab7-Aib-L-Ab7-D-Pro-OH as a solid (0.58 g, 0.82 mmol, 100 %). This TFA salt (0.82 mmol), HATU (0.47 g, 1.2 mmol), and DIEA (0.56 mL, 2.1 mmol) were added in two portions to DMF (0.20 L) in every 30 min. After evaporation, the residue was washed and chromatographed (silica gel) to yield cyclic tetrapeptide, *cyclo*(-L-Ab7-Aib-L-Ab7-D-Pro-) (0.36 g, 0.61 mmol, 74 %). This cyclic tetrapeptide (0.59 mmol) was reacted with KSAc (0.20 g, 1.8 mmol) in 5.0 mL DMF (r.t. 7 h) to obtain *cyclo*(-L-Am7(Ac)-Aib-L-Am7(Ac)-D-Pro-) (**1**) as heavy oil after silica gel chromatography (0.21 g, 61 %, HPLC: Rt 7.28 min). HR-MS, calcd for $C_{27}H_{45}N_4O_6S_2$ ($[M + H]^+$) 585.2781, found 585.2792. ^{1}H NMR: δ 7.35 (d, $J = 6$ Hz, 1H), 7.14 (dd, $J = 3$ Hz, 1H), 5.95 (d, $J = 3$ Hz, 1H), 4.84 (m, 1H), 4.75 (m, 1H), 4.19 (m, 1H), 3.92 (m, 1H), 3.54 (m, 1H), 2.67 (m, 4H), 2.39 (m, 1H), 2.26 (m, 1H), 1.85 (m, 8H), 1.66 (m, 10H), 1.37 (m, 12H).

*Cyclo(-L-Am7(Ac)-L-Am7(Ac)-L-Val-D-Pro-) (**2**)*

Z-L-Val-D-Pro-O^{t}Bu was synthesized from Z-L-Val-OH (3.3 g, 13 mmol), HCl·H-D-Pro-O^{t}Bu (2.5 g, 12 mmol), DCC (3.0 g, 14 mmol), HOBt·H_2O (1.8 g, 12 mmol), and triethylamine (12 mmol, 1.7 mL) to yield 4.3 g (11 mmol, 88 %) solid after chromatography. This dipeptide (11 mmol) was deprotected with H_2-Pd/C to yield H-L-Val-D-Pro-O^{t}Bu (2.5 g, 9.3 mmol, 88 %), which was reacted with Boc-L-Ab7-OH (3.0 g, 9.3 mmol) to obtain Boc-L-Ab7-L-Val-D-Pro-O^{t}Bu as white foam (4.2 g, 7.3 mmol, 78 %). This tripeptide (7.3 mmol) was treated with 4 M HCl/dioxane to generate H-L-Ab7-L-Val-D-Pro-O^{t}Bu (2.8 g, 5.8 mmol, 80 %), which was again coupled with Boc-L-Ab7-OH (1.9 g, 5.8 mmol) to obtain the tetrapeptide Boc-L-Ab7-L-Ab7-L-Val-D-Pro-O^{t}Bu as white foam after silica gel chromatography (3.6 g, 4.6 mmol, 80 %). After TFA treatment, TFA·H-L-Ab7-L-Ab7-L-Val-D-Pro-OH (4.6 mmol), HATU (2.6 g, 7.0 mmol), and DIEA (2.0 mL, 12 mmol) were added in five portions to 0.77 L DMF in every 10 min. After work-up and silica gel chromatography, *cyclo*(-L-Ab7-L-Ab7-L-Val-D-Pro-) was obtained (0.48 g, 0.78 mmol, 77 %). This cyclic peptide (0.78 mmol) was reacted with KSAc (0.27 g, 2.4 mmol) in 6.2 mL DMF to afford *cyclo*(-L-Am7(Ac)-L-Am7(Ac)-L-Val-D-Pro-) (**2**) as a solid after silica gel chromatography (0.40 g, 0.67 mmol, 85 %, HPLC: Rt 7.45 min). HR-MS, calcd for $C_{28}H_{47}N_4O_6S_2$ ($[M + H]^+$) 599.2915, found 599.2977. ^{1}H NMR: δ 7.10 (d, $J = 10$ Hz, 2H), 6.41 (d, $J = 5$ Hz, 1H), 4.75 (dd, $J = 2$ Hz, 1H), 4.43 (t, $J = 10$ Hz, 1H), 4.2 (m, 1H), 3.9 (m, 1H), 3.5 (m, 2H), 2.84 (t, 4H), 2.4 (m, 1H), 2.3 (m, 8H), 2.2 (m, 1H), 1.9 (m, 1H), 1.69 (m, 2H), 1.6 (m, 5H), 1.3 (m, 9H), 0.99 (d, 3H), 0.90 (d, 3H).

*Cyclo(-L-Am7(-)-L-Am7(-)-L-Val-D-Pro-) (**3**)*

The cyclic peptide **2** (0.15 g, 0.25 mmol) in 2.0 mL DMF was mixed with $MeNH_2$ (2.6 mmol) in 0.27 mL MeOH under Ar (r.t. 2 h). After evaporation, the residue was dissolved in 52 mL DMF and I_2 (67 mg, 0.26 mmol) in EtOH was added dropwise (r.t. 30 min). The oxidized peptide was purified by LH-20 chromatography to obtain *cyclo*(-L-Am7(-)-L-Am7(-)-L-Val-D-Pro-) (**3**) as a solid (40 mg, 78 μmol, 31 %, HPLC: Rt 6.58 min). HR-MS, calcd for $C_{24}H_{41}N_4O_4S_2$ ($[M + H]^+$) 513.2569, found 513.2558. ^{1}H NMR: δ 7.19 (d, $J = 10$ Hz, 2H), 6.45 (d, $J = 5$ Hz, 1H), 4.80 (dd, $J = 2$ Hz, 1H), 4.55 (t, $J = 10$ Hz, 1H), 4.28 (m, 1H), 3.92 (m, 1H), 3.5 (m, 2H), 2.86 (m, 4H), 2.3 (m, 4H), 1.9 (m, 1H), 1.8 (m, 2H), 1.7 (m, 5H), 1.3 (m, 9H), 1.00 (d, 3H), 0.91 (d, 3H).

*Cyclo(-L-Am7(Ac)-D-Tic-L-Val-D-Am7(Ac)-) (**4**)*

Boc-L-Ab7-D-Tic-O^{t}Bu was synthesized from Boc-L-Ab7-OH, H–D-Tic-O^{t}Bu, DCC/HOBt in DMF (78 % after silica gel chromatography). Treatment with 4 M HCl/dioxane gave H-L-Ab7-D-Tic-O^{t}Bu (70 %), which was reacted with Boc-D-Ab7-OH to obtain Boc-D-Ab7-L-Ab7-D-Tic-O^{t}Bu as a white foam (72 % after silica gel chromatography). Treatment again with 4 M HCl/dioxane gave H-D-Ab7-L-Ab7-D-Tic-O^{t}Bu (75 %), which was condensed with Boc-L-Val-OH to obtain Boc-L-Val-D-Ab7-L-Ab7-D-Tic-O^{t}Bu as a white foam (60 % after silica gel chromatography). This linear tetrapeptide was deprotected with TFA to yield TFA·H-L-Val-D-Ab7-L-Ab7-D-Tic-OH (91 %). This tetrapeptide (0.58 g, 0.73 mmol), HATU (0.42 g, 1.1 mmol), and DIEA (0.32 mL, 1.8 mmol) were added in two portions to 0.18 L DMF in every 30 min. After work-up and silica gel chromatography, *cyclo*(-L-Ab7-D-Tic-L-Val-D-Ab7-) was obtained as a white foam (0.28 g, 0.42 mmol, 58 %). This cyclic tetrapeptide (0.42 mmol) was reacted with KSAc (1.3 mmol) to yield *cyclo*(-L-Am7(Ac)-D-Tic-L-Val-D-Am7(Ac)-) (**4**) as a heavy oil (0.19 g, 69 % after silica gel chromatography, HPLC: Rt 8.33 min). HR-MS, calcd for $C_{33}H_{49}N_4O_6S_2$ ($[M + H]^+$) 661.3094, found 661.2859. ^{1}H NMR: δ 7.2 (m, 1H), 7.14 (d, $J = 8$ Hz, 1H), 6.84 (d, $J = 10$ Hz, 1H), 6.50 (d, $J = 10$ Hz, 1H), 6.14 (d, $J = 10$ Hz, 1H), 5.1 (m, 2H), 4.93 (d, $J = 15$ Hz, 1H), 4.4 (m, 2H), 3.89 (t, $J = 10$ Hz, 1H), 3.4 (m, 1H), 3.0 (m, 1H), 2.8 (m, 4H), 2.31(d, $J = 5$ Hz, 6H), 2.2 (m, 1H), 1.9 (m, 2H), 1.7 (m, 4H), 1.5 (m, 6H), 1.4 (m, 6H), 0.96 (d, $J = 10$ Hz, 3H), 0.90 (d, $J = 5$ Hz, 3H).

*Cyclo(-L-Am7(-)-D-Tic-L-Val-D-Am7(-)-) (**5**)*

The solution of **4** (85 mg, 0.13 mmol) in 2.0 mL DMF was treated with $MeNH_2$ (0.13 mL, 1.3 mmol) in MeOH (r.t.

5 h). After evaporation, the residue dissolved in 25 mL DMF was reacted with I_2 (33 mg, 0.13 mmol) in EtOH (r.t. 30 min). After LH-20 chromatography, *cyclo*(-L-Am7(-)-D-Tic-L-Val-D-Am7(-)-) (**5**) was obtained as a solid (17 mg, 30 μmol, 23 %, HPLC: Rt 7.90 min). HR-MS, calcd for $C_{29}H_{43}N_4O_4S_2$ ($[M + H]^+$) 575.2726, found 575.2608. ^{1}H NMR: δ 7.2 (m, 1H), 6.75 (d, $J = 10$ Hz, 1H), 6.53 (d, $J = 10$ Hz, 1H), 6.20 (d, $J = 10$ Hz, 1H), 5.2 (m, 2H), 4.9 (m, 1H), 4.6 (m, 2H), 3.85 (t, $J = 10$, Hz, 1H), 3.39 (dd, $J = 6$ Hz, 1H), 2.97 (dd, $J = 8$ Hz, 1H), 2.7 (m, 4H), 2.2 (m, 2H), 2.1 (m, 1H), 1.7 (m, 6H), 1.5 (m, 11H), 0.95 (d, 3H), 0.89 (d, 3H).

*Cyclo(-L-Am7(Ac)-D-Am7(Ac)-L-Val-D-Pro-) (**6**)*

This compound was synthesized similarly to **2**, using Boc-D-Ab7-OH instead of Boc-L-Ab7-OH in the synthesis of tripeptide. 95 % yield from *cyclo*(-L-Ab7-D-Ab7-L-Val-D-Pro-), HPLC: Rt 7.82 min, HR-MS, calcd for $C_{28}H_{47}N_4O_6S_2$ ($[M + H]^+$) 599.2937, found 599.2977. ^{1}H NMR: δ 7.16 (d, $J = 10$ Hz, 1H), 6.28 (d, $J = 10$ Hz, 1H), 6.06 (d, $J = 10$ Hz, 1H), 4.71 (dd, $J = 3$ Hz, 1H), 4.3 (m, 2H), 4.2 (m, 1H), 4.0 (m, 1H), 3.5 (m, 1H), 2.84 (t, $J = 8$ Hz, 4H), 2.4 (m, 1H), 2.3 (m, 8H), 2.2 (m, 1H), 1.9 (m, 1H), 1.8 (m, 2H), 1.76 (s, 1H), 1.5 (m, 5H), 1.3 (m, 8H), 0.99 (s, 3H), 0.89 (s, 3H).

*Cyclo(-L-Am7(-)-D-Am7(-)-L-Val-D-Pro-) (**7**)*

This compound was synthesized similarly to **3**. 45 % yield from **4**, HPLC: Rt 7.03 min, HR-MS, calcd for $C_{24}H_{41}N_4O_4S_2$ ($[M + H]^+$) 513.2569, found 513.2585. ^{1}H NMR: δ 7.18 (d, $J = 10$ Hz, 1H), 6.38 (d, $J = 10$ Hz, 1H), 6.09 (d, $J = 10$ Hz, 1H), 4.72 (dd, $J = 2$, 2 Hz, 1H), 4.5 (m, 1H), 4.4 (m, 1H), 4.37 (t, $J = 10$ Hz, 1H), 3.9 (m, 1H), 3.5 (m, 1H), 2.8 (m, 2H), 2.6 (m, 2H), 2.3 (m, 2H), 2.1 (m, 2H), 2.0 (m, 1H), 1.8 (m, 2H), 1.7 (m, 2H), 1.6 (m, 3H), 1.4 (m, 9H), 0.98 (d, 3H), 0.88 (d, 3H).

*Cyclo(-L-Am7(Ac)-D-Am7(Ac)-L-Ala-D-Pro-) (**8**)*

85 % yield from *cyclo*(-L-Ab7-D-Ab7-L-Ala-D-Pro-), HPLC: Rt 6.50 min, HR-MS, calcd for $C_{26}H_{43}N_4O_6S_2$ ($[M + H]^+$), 571.2624, found 571.2663. ^{1}H NMR: δ 7.09 (d, $J = 10$ Hz, 1H), 6.30 (d, $J = 10$ Hz, 1H), 6.05 (d, $J = 10$ Hz, 1H), 4.9 (m, 1H), 4.70 (dd, $J = 3$ Hz, 1H), 4.3 (m, 2H), 4.0 (m, 1H), 3.5 (m, 1H), 2.84 (m, 4H), 2.3 (m, 8H), 1.9 (m, 5H), 1.5 (m, 6H), 1.3 (m, 10H).

*Cyclo(-L-Am7(-)-D-Am7(-)-L-Ala-D-Pro-) (**9**)*

28 % yield from **8**, HPLC: Rt 5.33 min, HR-MS, calcd for $C_{22}H_{37}N_4O_4S_2$ ($[M + H]^+$) 485.2256, found 485.2278. ^{1}H NMR: δ 7.12 (d, $J = 6$ Hz, 1H), 6.42 (d, $J = 10$ Hz, 1H), 6.14 (d, $J = 10$ Hz, 1H), 5.0 (m, 1H), 4.71 (d, $J = 8$ Hz, 1H), 4.5 (m, 2H), 4.0 (m, 1H), 3.5 (m, 1H), 2.7 (m, 4H), 2.3 (m, 2H), 2.18 (t, 1H), 2.00 (m, 1H), 1.8 (m, 3H), 1.7 (m, 4H), 1.4 (m, 12H).

*Cyclo(-L-Am7(Ac)-D-Am7(Ac)-L-Phe-D-Pro-) (**10**)*

95 % yield from *cyclo*(-L-Ab7-D-Ab7-L-Phe-D-Pro-), HPLC: Rt 7.98 min, HR-MS, calcd for $C_{33}H_{47}N_4O_6S_2$ ($[M + H]^+$), 647.2937, found 647.2960. ^{1}H NMR: δ 7.21 (m, 5H), 7.13 (d, $J = 10$ Hz, 1H), 6.47 (d, $J = 10$ Hz, 1H), 6.06 (d, $J = 10$ Hz, 1H), 5.08 (m, 1H), 4.63 (m, 1H), 4.38 (m, 1H), 4.27 (m, 1H), 3.50 (m, 2H), 3.07 (m, 2H), 2.85 (m, 4H), 2.32 (m, 6H), 1.97 (m, 2H), 1.70 (m, 4H), 1.56 (m, 4H), 1.34 (m, 10H).

*Cyclo(-L-Am7(-)-D-Am7(-)-L-Phe-D-Pro-) (**11**)*

28 % yield from **10**, HPLC: Rt 7.22 min, HR-MS, calcd for $C_{28}H_{41}N_4O_4S_2$ ($[M + H]^+$) 561.2569, found 561.2546. ^{1}H NMR: δ 7.21 (m, 5H), 7.15 (d, $J = 10$ Hz, 1H), 6.56 (d, $J = 10$ Hz, 1H), 6.10 (d, $J = 10$ Hz, 1H), 5.08 (m, 1H), 4.63 (m, 1H), 4.46 (m, 2H), 3.48 (m, 2H), 3.06 (m, 2H), 2.75 (m, 2H), 2.65 (m, 2H), 2.02 (m, 2H), 1.96 (m, 2H), 1.84 (m, 2H), 1.47 (m, 2H), 1.71 (m, 6H), 1.54 (m, 2H), 1.42 (m, 2H), 1.34 (m, 2H).

*Cyclo(-L-Am7(Ac)-D-Aom7(Ac)-L-Ala-D-Pro-) (**12**)*

77 % yield from *cyclo*(-L-Ab7-D-Aob7-L-Ala-D-Pro-), HPLC: Rt 5.84 min, HR-MS, calcd for $C_{25}H_{41}N_4O_7S_2$ ($[M + H]^+$) 573.2417, found 573.2424. ^{1}H NMR: δ 7.08 (d, $J = 10$ Hz, 1H), 6.42 (d, $J = 10$ Hz, 1H), 6.29 (d, $J = 10$ Hz, 1H), 4.9 (m, 1H), 4.71 (dd, 1H), 4.6 (m, 1H), 4.3 (m, 1H), 4.0 (m, 1H), 3.5 (m, 5H), 3.07 (t, 2H), 2.85 (t, 2H), 2.33 (d, 6H), 2.1 (m, 1H), 1.9 (m, 1H), 1.8 (m, 3H), 1.75 (s, 1H), 1.6 (m, 3H), 1.3 (m, 8H).

*Cyclo(-L-Am7(-)-D-Aom7(-)-L-Ala-D-Pro-) (**13**)*

34 % yield from **12**, HPLC: Rt 4.92 min, HR-MS, calcd for $C_{21}H_{35}N_4O_5S_2$ ($[M + H]^+$) 487.2049, found 487.2045. ^{1}H NMR: δ 7.12 (d, $J = 10$ Hz, 1H), 6.57 (d, $J = 10$ Hz, 1H), 6.45 (d, $J = 10$ Hz, 1H), 4.9 (m, 1H), 4.7 (m, 2H), 4.4 (m, 1H), 3.9 (m, 1H), 3.6 (m, 5H), 2.9 (m, 2H), 2.8 (m, 1H), 2.7 (m, 1H), 2.4 (m, 1H), 2.3 (m, 1H), 2.1 (m, 1H), 2.0 (m, 4H), 1.8 (m, 2H), 1.6 (m, 3H), 1.4 (m, 5H).

*Cyclo(-L-Am7(Ac)-D-Aom7(Ac)-L-Phe-D-Pro-) (**14**)*

71 % from *cyclo*(-L-Ab7-D-Aob7-L-Phe-D-Pro-), HPLC: Rt 7.18 min, HR-MS, calcd for $C_{31}H_{45}N_4O_7S_2$ ($[M + H]^+$)

649.2730, found 649.2756. ^{1}H NMR: δ 7.27 (m, 5H), 7.11 (d, J = 10 Hz, 1H), 6.58 (d, J = 10 Hz, 1H), 6.30 (d, J = 10 Hz, 1H), 5.09 (m, 1H), 4.63 (m, 2H), 4.27 (m, 1H), 3.5 (m, 6H), 3.1 (m, 2H), 3.0 (m, 2H), 2.85 (m, 2H), 2.3 ~ 2.0 (m, 10H), 1.8 (m, 2H), 1.7 (m, 4H), 1.33 (m, 4H).

Cyclo(-L-Am7(-)-D-Aom7(-)-L-Phe-D-Pro-) ***(15)***

36 % yield from **14**, HPLC: Rt 6.43 min, HR-MS, calcd for $C_{27}H_{39}N_4O_5S_2$ ($[M + H]^+$) 563.2362, found 563.2315. ^{1}H NMR: δ 7.27 (m, 5H), 7.15 (d, J = 10 Hz, 1H,), 6.71 (d, J = 10 Hz, 1H), 6.40 (d, J = 10 Hz, 1H), 5.09 (m, 1H), 4.73 (m, 1H), 4.63 (dd, 1H), 4.42 (m, 1H), 3.83 and 3.17 (two m, 2H), 3.57 (m, 2H), 3.47 (m, 2H), 3.0 (m, 2H), 2.88 (m, 2H), 2.7 (m, 2H), 2.1 ~ 1.9 (m, 6H), 1.8 (m, 4H), 1.5 (m, 2H), 1.3 (m, 2H).

Cyclo(-L-Aom7(Ac)-D-Am7(Ac)-L-Phe-D-Pro-) ***(16)***

64 % from *cyclo*(-L-Aob7-D-Ab7-L-Phe-D-Pro-), HPLC: Rt 7.51 min, HR-MS, calcd for $C_{31}H_{45}N_4O_7S_2$ ($[M + H]^+$) 649.2730, found 649.2695. ^{1}H NMR: δ 7.24 (d, 5H), 7.16 (d, J = 10 Hz, 1H), 6.48 (d, J = 10 Hz, 1H), 6.17 (d, J = 10 Hz, 1H), 5.09 (m, 1H), 4.63 (m, 1H), 4.54 (m, 1H), 4.38 (m, 1H), 3.5 (m, 6H), 3.2 ~ 2.9 (m, 6H), 2.3 ~ 2.0 (m, 10H), 1.8 (m, 2H), 1.7 (m, 4H), 1.34 (m, 4H).

Cyclo(-L-Aom7(-)-D-Am7(-)-L-Phe-D-Pro-) ***(17)***

36 % from **16**, HPLC: Rt 7.04 min, HR-MS, calcd for $C_{27}H_{39}N_4O_5S_2$ ($[M + H]^+$) 563.2362, found 563.2354. ^{1}H NMR: δ 7.24 (m, 5H), 7.16 (d, J = 10 Hz, 1H), 6.61 (d, J = 10 Hz, 1H), 6.15 (d, J = 10 Hz, 1H), 5.09 (m, 1H), 4.70 (m, 1H), 4.63 (m, 1H), 4.42 (m, 1H), 3.5 (m, 6H), 3.1 (m, 2H), 2.8 (m, 4H), 2.0 (m, 6H), 1.7 (m, 4H), 1.52 (m, 2H), 1.38 (m, 2H).

Conclusion

Cyclic tetrapeptides with intramolecular –SS– bridges were synthesized by the oxidation of –SH side chains, which were generated from –SAc precursors. *Cyclo*(-L-Am7(Ac)-Aib-L-Am7(Ac)-D-Pro-) (**1**) did not afford intramolecular –SS– bridged products, whereas *cyclo*(-L-Am7(Ac)-L-Am7(Ac)-L-Val-D-Pro-) (**2**), cyclo(-L-Am7(Ac)-D-Tic-L-Val-D-Am7(Ac)-) (**4**), and cyclo(-L-Am7(Ac)-D-Am7(Ac)-L-Val-D-Pro-) (**6**) did afford –SS– bridged products. Various cyclic peptides with intramolecular –SS– bridges were obtained by substituting Val in **6** with Ala and Phe (**8** and **10**, respectively), and by incorporating an ether linkage into the side chains of **6** (to give **12**, **14**, and **16**). Compounds **3**, **7**, and **15** potently inhibited HDAC1 and HDAC4, which were generated by the oxidation of **2**, **6**, and **14**, respectively.

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

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