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Interaction of aliphatic cap group in inhibition of histone deacetylases by cyclic tetrapeptides

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Abstract—Inhibitors of histone deacetylases (HDACs) are a promising class of anticancer agents that effect gene regulation. To know the interaction of aliphatic cap groups with HDACs, cyclic tetrapeptide and bicyclic peptide disulfide hybrids were synthesized without aromatic ring in their macrocyclic framework. Benzene ring of L-Phe in chlamydocin was replaced with several aliphatic amino acids and also a fused bicyclic tetrapeptide was synthesized by ring closing metathesis using Grubb's first generation catalyst. The inhibitory activities of the cyclic peptides against histone deacetylase enzymes were evaluated, which demonstrated most of them are interesting candidates as anticancer agents.
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1. Introduction

Reversible acetylation and deacetylation of the ε-amino groups of lysine residues on core histone tails by histone acetyl transferase (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 DNA and histones to transcriptional regulators.[1–4] Acetylation of lysine residues in N-terminal tails of core histones reduces the interaction with DNA. 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, while deacetylation is associated with a 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 status either due to HAT mutation or abnormal recruitment of HDACs is found to be 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. It is therefore proposed that HDACs are potential targets for the development of low molecular weight anticancer drugs.

Several structurally unrelated natural and synthetic compounds have been reported so far as HDAC inhibitors. Among them trichostatin A (TSA),[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-hydroxy-chlamydocin,[18] HC toxins,[19–23] Cyl-1, Cyl-2,[24–26] WF-3161,[27] apicidin,[28–31] FR-225497[32] are examples of 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. Chlamydocin is a naturally occurring cyclic tetrapeptide containing an aromatic ring in its cyclic framework, and inhibits histone deacetylases potently,[40] whereas HC-toxins[19–23] are also cyclic tetrapeptides which do not have aromatic ring in their cyclic frameworks are still inhibiting the HDACs. Recently we have reported cyclic tetrapeptide based HDAC inhibitors (CHAPs),[41,42] and SCOPs[43] (Fig. 1).

Keywords: Histone deacetylases; Chlamydocin; Grubb's alkene metathesis; Bicyclic tetrapeptide; Inhibitors.
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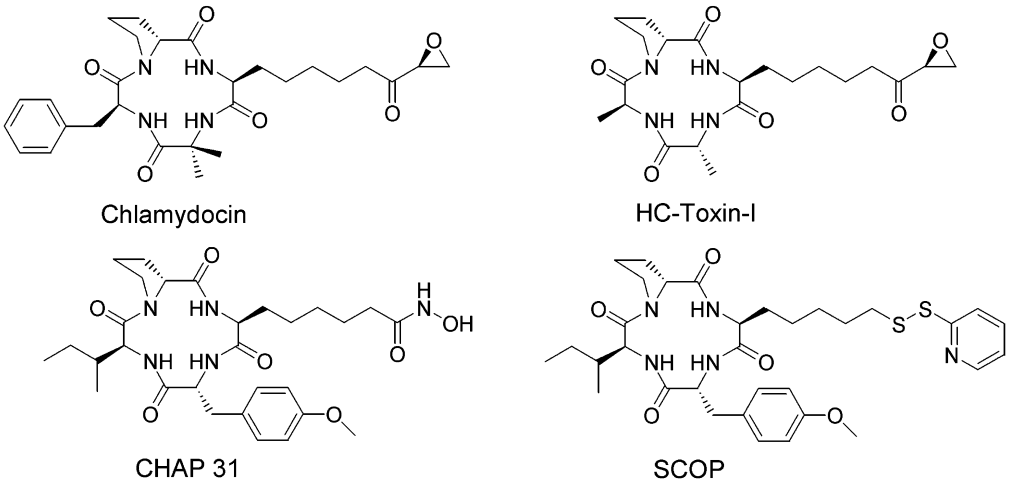


Figure 1. Natural and synthetic inhibitors.

In the present study, to know the interaction of aliphatic cap group with the surface of HDAC enzymes, we synthesized cyclic tetrapeptide and bicyclic peptide disulfide hybrids without aromatic ring in their capping group. For this, we initially focused on the benzene ring of L-Phe in chlamydocin to replace it by several aliphatic amino acids. Recently, ring closing metathesis of ω-unsaturated amino acids to synthesize constrained peptides catalyzed by ruthenium complexes has been reported.[44–48] On the basis of these reports, to increase the size of aliphatic cap group we designed and synthesized a fused bicyclic peptide. We synthesized a cyclic tetrapeptide by successive introduction of Boc-L-2-amino-8-nonenoate (L-Ae9) and Boc-D-2-amino-8-nonenoate (D-Ae9) in the peptide macrocycle, and incorporated a second aliphatic ring by the well-known ring closing alkene metathesis reaction between these two amino acids. We herein describe the account on the synthesis of chlamydocin analogues, bicyclic peptide by alkene metathesis, and a brief description of interesting biological results.

2. Results and discussion

2.1. Chemistry

Our aim was to synthesize potent inhibitors of HDACs. Initially, we synthesized chlamydocin analogues by replacing the phenyl alanine in the cyclic framework of compound **1**[49] (Fig. 2). The compound **1** has similar cyclic framework as in chlamydocin, with the L-2-amino -9,10-epoxy-8-oxodecanoic acid (L-Aoe) being replaced by L-2-amino-7-(2-pyridyl)-disulfidyl heptanoyl (L-Am7 (S2Py)), equipped with a thiol function protected as disulfide hybrid.

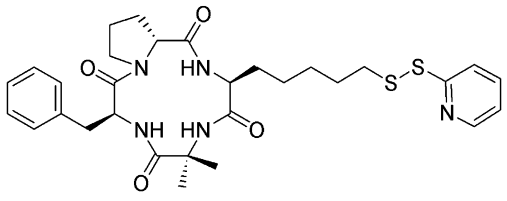
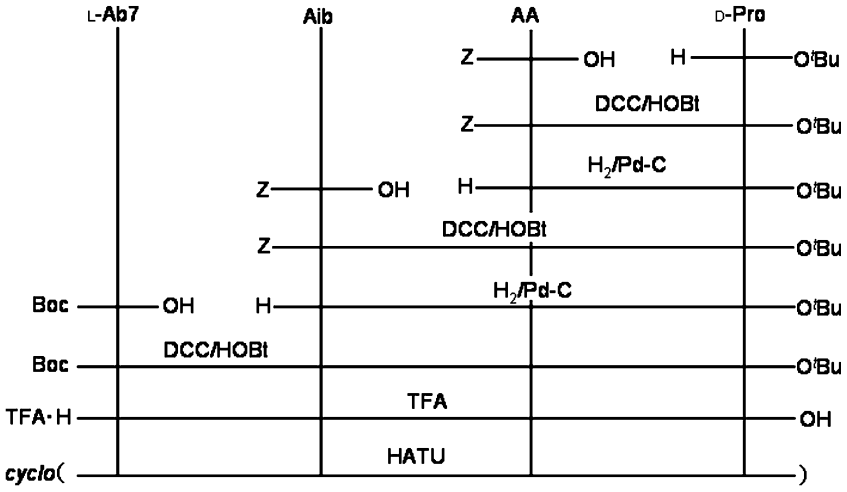


Figure 2. Structure of *cyclo*(-L-Am7(S2Py)-Aib-L-Phe-D-Pro-) (**1**).[49]

Cyclic tetrapeptides were prepared according to general Scheme 1 by the conventional solution phase method, starting from the *Z*-imino acid *tert*-butyl ester. After the removal of Z-protection by catalytic hydrogenation, the free amine was extracted and used for condensation with a *Z*-amino acid using DCC/HOBt. Boc-L-2-amino-7-bromoheptanoic acid (Ab7)[50] was incorporated to prepare the linear tetrapeptide. After removal of both the side protections by treating with trifluoroacetic acid, cyclization reaction was carried out by 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 to thioacetate ester. Subsequent treatment with methylamine in the presence of 2,2′-dipyridyl disulfide gave the desired cyclic tetrapeptides.

To explore the role of aromatic ring in the chlamydocin framework, we replaced its L-Phe residue by incorporating several aliphatic hydrophobic amino acids like L-alanine (L-Ala), L-leucine (L-Leu), L-isoleucine (L-Ile), and L-norleucine (L-Nle) at the AA position in Scheme 1. Following cyclic tetrapeptides were synthesized according to the general method described above: *cyclo*(-L-Am7 (S2Py)-Aib-L-Ala-D-Pro-) (**2**), *cyclo*(-L-Am7(S2Py)-Aib-L-Leu-D-Pro-) (**3**), *cyclo*(-L-Am7(S2Py)-Aib-L-Ile-D-Pro-) (**4**), *cyclo*(-L-Am7(S2Py)-Aib-L-Nle-D-Pro-) (**5**).

Further, to increase the size of capping group and bury the amide group between L-Phe and Aib in chlamydocin by a hydrophobic jacket, we attempted to synthesize a fused bicyclic peptide inhibitor. In a recently published paper,[50] we demonstrated the synthesis and use of α-amino acids with an easily replaceable bromo group at the ω-position in the side chain of α-carbon atom. In light of this view, we synthesized Boc-L-Ae9 and Boc-D-Ae9 with a terminal double bond. Our syn-



Scheme 1. Synthetic strategy of cyclic tetrapeptides 2–5.

thesis strategy starts from diethyl tertiarybutoxycarbonylaminomalonate with 7-bromo-1-heptene as illustrated in Scheme 2. 7-Bromo-1-heptene was coupled with diethyl Boc-aminomalonate by using NaOEt in ethanol. One of the ethyl ester groups of the adduct is hydrolyzed by one equivalent of 1 M NaOH at low temperature (0–5 °C). The oily monoacid monoester after work-up is then subjected to the decarboxylation by refluxing in toluene. After evaporation of toluene, the resulting oily Boc-DL-Ae9-OEt (**6**) was purified by silica gel column chromatography. Finally Boc-DL-Ae9-OEt was subjected to the action of subtilisin Carlsberg from *Bacillus licheniformis* (Sigma) in a mixture of DMF and water (1:3, v/v). Boc-L-2-Ae9-OH (**7**) was isolated as colorless oil. The recovered Boc-D-Ae9-OEt (**8**) was hydrolyzed by 1 M NaOH in EtOH to get Boc-D-Ae9-OH (**9**) with excellent purity in quantitative yield. Boc-protected α-amino acids containing alkyl side chains of different lengths with a terminal double bond can be prepared by this method. The double bond in the side chain is stable enough and variety of functional group transformation is possible in the double bond of these amino acids. Recently, we reported the synthesis of chlamydocin analogues bearing carbonyl group[51] by incorporating Boc-L-Ae9-OH in the cyclic scaffold and various functional group transformations to give potent HDAC inhibitors.

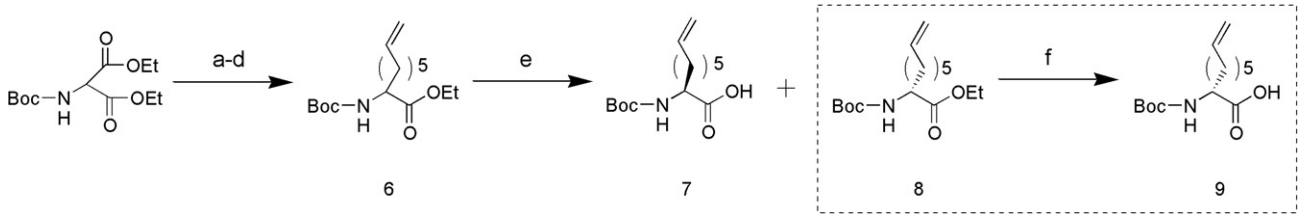
The interesting application of ω-unsaturated amino acids (Aens), synthesis of constrained peptides by ring closing alkene metathesis (RCM) reaction, was done as the methodology development for bicyclic peptides. These cyclic peptides can also mimic the β-turns in the naturally occurring bioactive proteins and peptides. And also it is possible to get information about the rotation of C—N (ϕ) and C—C (ψ) σ-bonds. We synthesized a dipeptide Boc-D-Ae9-L-Ae9-OBzl (**10**) using the solution phase peptide synthesis protocol (Scheme 3). Cyclization was done by Grubb's first generation Ru catalyst and the resulting cyclized product was a mixture of two isomers. Two diastereomers *cis* (**11**) and *trans* (**12**) were separated by HPLC and found in 1:2 ratio in 50% total yield. The major one was obtained as *trans*- and minor as *cis*-, identified by calculating the coupling constant of olefinic protons (Fig. 3).

On the basis of above observations, we synthesized a bicyclic peptide disulfide hybrid. The cyclic tetrapeptide *cyclo*(-L-Ab7-D-Ae9-L-Ae9-D-Pro-) (**13**) was synthesized according to general method explained in Scheme 1. The second ring was synthesized by ring closing metathesis between D-Ae9 and L-Ae9 using Grubb's first generation Ru catalyst. Further, the alkene group in (**14**), which is a mixture of *cis* and *trans*, was catalytically hydrogenated to give aliphatic fused bicyclic peptide containing Ab7 (**15**). The bicyclic tetrapeptide **15** was reacted with potassium thioacetate (Wako, Japan) to convert the bromide into thioacetate ester (**16**). Further treatment with methylamine in the presence of 2,2′-dipyridyl disulfide gave the desired product (**17**) (Scheme 4). All the synthesized compounds were characterized by ^{1}H NMR and high resolution FAB-MS. Purity of the compounds was determined by HPLC analysis and all the compounds showed purity above 97%.

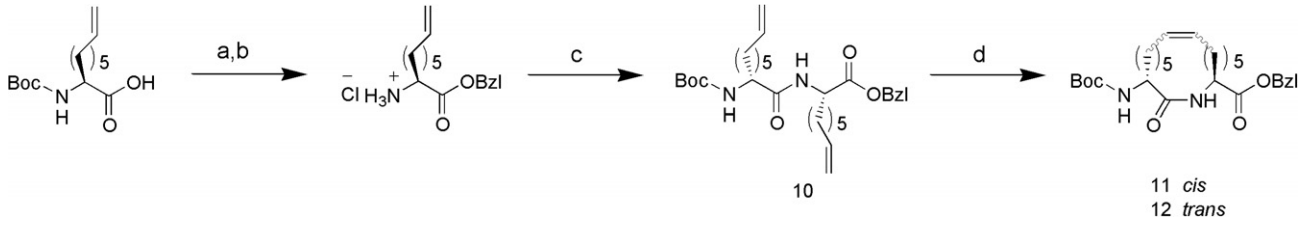
2.2. Enzyme inhibition and biological activity

The synthesized compounds were assayed for HDAC inhibitory activity using HDAC1, HDAC4, and HDAC6 enzymes prepared by 293T cells with the aid of 0.1 mM of dithioltritol (DTT), which is known to reduce S—S bond, but does not contribute to the enzyme activity.[13] In the presence of DTT, 2,2′-dipyridyl disulfide did not effect the enzyme inhibition (unpublished data). Detailed experimental procedure for the preparation and assay were performed according to the reported methods.[13,51] In addition, to know the inhibitory activity of these compounds in cell based condition, we carried out p21 promoter assay according to the literature. The results of HDAC inhibitory activity and the p21[51] promoter assay of the compounds are shown in Table 1.

These compounds inhibit within nanomolar range. For comparison, inhibitory activity of trichostatin A is also shown. The compound **1** which showed good activity



Scheme 2. Synthesis of Boc-L-Ae9-OH and Boc-D-Ae9-OEt. Reagents and conditions: (a) Na, anhyd EtOH, reflux, 30 min; (b) $Br(CH_2)_5{-}CH{=}CH_2$, reflux, 4 h; (c) NaOH aq, EtOH, 4 h; (d) toluene, reflux, 3 h; (e) subtilisin Carlsberg, H_2O/DMF (1:3), 4 h; (f) 1 M NaOH aq, EtOH, 4 h.



Scheme 3. Synthesis of cyclic dipeptide. Reagents and conditions: (a) Bzl-Br, DMF, TEA, 6 h; (b) 2 M HCl/dioxane, 3 h; (c) Boc-D-Ae9-OH (**9**), DMF, TEA, HBTU, HOBt, 4 h; (d) Grubb's first generation Ru catalyst, 48 h, CH_2Cl_2.

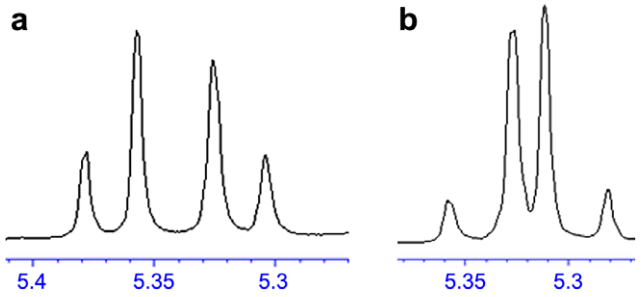
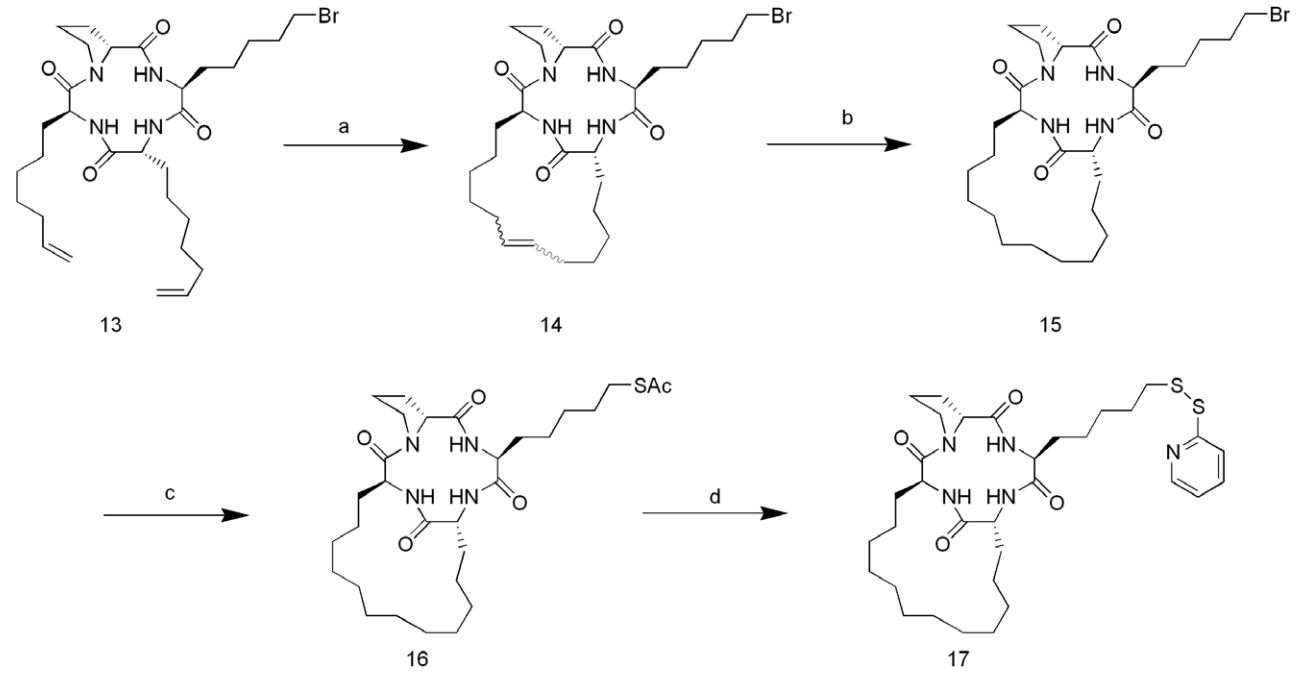


Figure 3. Selected ^1H NMR spectra of (a) *cis* and (b) *trans* compounds.

is taken as standard for this study. In compounds **2**–**5**, when L-Phe is replaced by several aliphatic amino acids, we expected decrease in activity as compared to **1**. They showed almost comparable in vitro activity. As the p21 promoter assay needs the cell permeability of the inhibitors, therefore compounds with more hydrophobicity should exhibit greater activity toward the HDACs. Compounds **2**–**5** show good activity in p21 assay but they were 10-fold less active than **1**. This explains the fact that, although these compounds do not contain aromatic ring in their cap group, still there is favorable interaction of the macrocyclic cap group with residues at the rim of HDACs active site pocket.



Scheme 4. Synthesis of bicyclic peptide disulfide hybrid. Reagents: (a) Grubb's first generation catalyst, DCM; (b) AcOH, Pd–C, H_2; (c) AcSK, DMF; (d) $MeNH_2$/MeOH, 2,2′-dipyridyl disulfide, DMF.

Table 1. HDAC inhibitory activity and p21 promoter assay data

Compound	IC_{50} (nM)			p21 promoter assay
	HDAC1	HDAC4	HDAC6	EC_{1000} (nM)
TSA[52]	190	200	280	190
1[49]	39	18	404	45
2	94	52	430	750
3	58	33	230	630
4	38	23	310	260
5	38	24	280	270
17	35	20	4900	160

To increase the size of macrocyclic cap group, we synthesized compound **17**. This compound showed good activity in both cell free and cell based conditions in nanomolar range. This is probably because of the presence of a big flexible aliphatic fused ring which interacts extensively with the rim residues of HDACs. Also cell permeability is enhanced in this case, which is reflected in its p21 activity data. Further, compound **17** was poorly inhibited by HDAC6 compared to HDAC1 and HDAC4 (HDAC6/HDAC1 = 140) and its selectivity was better as compared to **1** (HDAC6/HDAC1 = 10.3). Thus, **17** shows better selectivity than the other compounds listed in Table 1.

3. Conclusion

In order to develop HDAC inhibitors with aliphatic capping group, we replaced aromatic ring of phenylalanine in the chlamydocin framework by several aliphatic amino acids. We also successfully developed a method to synthesize bicyclic tetrapeptide by ring closing metathesis using Grubb's first generation catalyst. Our synthetic strategy allowed the convenient preparation of several analogues of chlamydocin. The cell free and cell based assay studies showed that most of the synthesized compounds were potently inhibited HDACs and compound **17** has some selectivity among HDAC1 and HDAC6.

4. Experimental

4.1. General

Unless otherwise noted, all solvents and reagents were reagent grade and used without purification. Flash chromatography was performed using silica gel 60 (230–400 mesh) eluting with solvents as indicated. All compounds were routinely checked by thin layer chromatography (TLC) and/or high performance liquid chromatography (HPLC). TLC was performed on aluminum-backed silica gel plates (Merck DC-Alufolien Kieselgel 60 F_{254}) with spots visualized by UV light or charring. Analytical HPLC was performed on a Hitachi instrument equipped with a chromolith performance RP-18e column (4.6 × 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–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 million (s = singlet; d = doublet; t = triplet; m = multiplet). Assignments of proton resonances were confirmed, when possible, by correlated spectroscopy. Amino acids were coupled using standard solution-phase chemistry with dicyclohexyl-carbodiimide (DCC), 2-(1H-benzatriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), or *O*-(7-azabenzotriazoyl-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU).

4.1.1. Synthesis of *cyclo*(-L-Am7(S2Py)-Aib-L-Ala-D-Pro-) (2). To a cooled solution of H-D-Pro-O^{t}Bu (256 mg, 1.50 mmol), Z-L-Ala-OH (336 mg, 1.50 mmol), and HOBt·H_2O (230 mg, 1.50 mmol) in dimethylformamide (DMF) (3 mL) was added DCC (375 mg, 1.80 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 (471 mg, 80%) as colorless oil. To the protected dipeptide (471 mg, 1.25 mmol) dissolved in acetic acid (5 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 2 M Na_2CO_3 solution and dried over anhydrous Na_2CO_3. Evaporation of ethyl acetate gave H-L-Ala-D-Pro-O^{t}Bu (300 mg, 99%). To a cooled solution of H-L-Ala-D-Pro-O^{t}Bu (300 mg, 1.22 mmol) and Z-Aib-OH (300 mg, 1.22 mmol) in DMF (4 mL) were successively added DCC (302 mg, 1.50 mmol), HOBt·H_2O (190 mg, 1.22 mmol). The product Z-Aib-L-Ala-D-Pro-O^{t}Bu (460 mg, 80%) was obtained in the same manner as described earlier as white foam. The tripeptide Z-Aib-L-Ala-D-Pro-O^{t}Bu (460 g, 1.00 mmol) was subjected to catalytic hydrogenation with Pd–C (50 mg) in acetic acid (5 mL). The free amine was taken into ethyl acetate (25 mL) by the aid of 2 M Na_2CO_3 solution (10 mL). After drying over anhydrous Na_2CO_3, ethyl acetate solution was evaporated to obtain H-Aib-L-Ala-D-Pro-O^{t}Bu (327 mg, 99%). The N-terminal free tripeptide H-Aib-L-Ala-D-Pro-O^{t}Bu (327 mg, 1.00 mmol) was coupled with Boc-L-Ab7-OH (330 mg, 2.30 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-Aib-L-Ala-D-Pro-O^{t}Bu (473 mg, 80%) as a white foam. Boc-L-Ab7-Aib-L-Ala-D-Pro-O^{t}Bu (474 mg, 0.75 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-Aib-L-Ala-D-Pro-OH (460 mg, 99%). To a volume of DMF (100 mL) TFA·H-L-Ab7-Aib-L-Ala-D-Pro-OH (460 mg, 0.75 mmol), HATU (342 mg, 0.90 mmol), and DIEA (0.24 mL, 1.80 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-Aib-L-Ala-D-Pro-) (200 mg, 60%) as a white foam after drying in vacuo. The cyclic tetrapeptide *cyclo*(-L-Ab7-Aib-L-Ala-D-Pro-) (200 mg, 0.45 mmol) was dissolved in DMF (2 mL), then potassium thioacetate (80 mg, 0.70 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)-Aib-L-Ala-D-Pro-) (197 mg, 89%). To a solution of *cyclo*(-L-Am7(acetyl)-Aib-L-Ala-D-Pro-) (197 mg, 0.40 mmol) in DMF (2 mL), under argon atmosphere, 2,2′-dipyridyldisulfide (200 mg, 0.90 mmol) and 40% solution of CH_3NH_2/MeOH (0.22 mL, 2.00 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)-Aib-L-Ala-D-Pro-) as white foam (50 mg, 30%), HPLC, t_R 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, $CDCl_3$): δ_H 1.27 (m, 2H), 1.34 (s, 3H), 1.36 (d, J = 7 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).

4.1.2. Synthesis of *cyclo*(-L-Am7(S2Py)-Aib-L-Leu-D-Pro-) (3). These compounds were synthesized using L-leucine instead of L-Phe according the method described earlier, to yield *cyclo*(-L-Am7(S2Py)-Aib-L-Leu-D-Pro-) (154 mg, 39%) HPLC, t_R 8.10 min. HR-FAB-MS $[M+H]^+$ 564.2665 for $C_{27}H_{42}O_4N_5S_2$ (calcd 564.2678). ^1H NMR (500 MHz, $CDCl_3$): δ_H 0.92 (d, J = 7 Hz, 3H), 1.01 (d, J = 7.2 Hz, 3H) 1.15 (m, 1H), 1.27 (m, 1H), 1.38 (s, 3H), 1.41 (m, 3H), 1.59–1.67 (m, 6H), 1.78 (s, 1H), 1.86 (m, 1H), 1.91 (m, 1H), 2.02 (m, 1H), 2.28 (m, 1H), 2.41 (m, 1H), 2.77 (t, 2H), 3.53 (m, 1H), 3.91 (m, 1H), 4.18 (ddd, J = 10, 10.1, 7.7 Hz, 1H), 4.53 (t, 1H), 4.71 (dd, J = 2, 2.5 Hz, 1H), 5.98 (s, 1H), 7.08 (m, 1H), 7.21 (d, J = 7.2 Hz, 1H), 7.31 (d, J = 8 Hz, 1H), 7.72 (m, 1H), 8.46 (dd, J = 4 Hz, 1H).

4.1.3. Synthesis of *cyclo*(-L-Am7(S2Py)-Aib-L-Ile-D-Pro-) (4). These compounds were synthesized using DL-2MePhe instead of Aib according the procedure described for **3**, to yield *cyclo*(-L-Am7(S2Py)-Aib-L-Ile-D-Pro-) (40 mg, 22%) as a white foam. HPLC, t_R 7.88 min. HR-FAB-MS $[M+H]^+$ 564.2659 for $C_{27}H_{42}O_4N_5S_2$ (calcd 564.2678). ^1H NMR (500 MHz, $CDCl_3$): δ_H 0.86 (d, J = 7 Hz, 3H), 0.92 (t, 3H) 1.16 (m, 1H), 1.29 (m, 1H), 1.35 (s, 3H), 1.41 (m, 2H), 1.61–1.69 (m, 7H), 1.78 (s, 1H), 1.84 (m, 1H), 1.93 (m, 1H), 2.02 (m, 1H), 2.26 (m, 1H), 2.38 (m, 1H), 2.77 (t, 2H), 3.51 (m, 1H), 3.89 (m, 1H), 4.17 (ddd, J = 10.2, 10.2, 7.6 Hz, 1H), 4.55 (t, 1H), 4.74 (dd, J = 2, 2 Hz),1H), 5.97 (s, 1H), 7.08 (m, 1H), 7.19 (d, J = 10.2 Hz, 1H), 7.31 (d, J = 10.5 Hz, 1H), 7.72 (d, J = 8 Hz, 1H), 8.46 (dd, J = 5 Hz, 1H).

4.1.4. Synthesis of *cyclo*(-L-Am7(S2Py)-Aib-L-Nle-D-Pro-) (5). This compound was synthesized according to the procedure described to yield *cyclo*(-L-Am7(S2Py)-Aib-L-Nle-D-Pro-) (80 mg 64%). HPLC, t_R 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, $CDCl_3$): δ_H 0.90 (t, 3H), 1.29–1.13 (m, 6H), 1.34 (s, 3H), 1.40 (m, 3H), 1.60–1.69 (m, 8H), 1.76 (s, 3H), 1.84 (m, 1H), 1.90 (m, 1H), 2.50 (m, 1H), 2.38 (m, 1H), 2.77 (t, 2H), 3.52 (m, 1H), 3.92 (m, 1H), 4.17 (ddd, J = 10.0, 10.1, 8 Hz, 1H), 4.72 (dd, J = 2, 2.5 Hz, 1H), 4.84 (dd, J = 8.5, 8 Hz, 1H), 5.97 (s, 1H), 7.09 (m, 2H), 7.30 (d, J = 10 Hz, 1H), 7.65 (m, 1H), 7.72 (d, J = 8 Hz, 1H), 8.46 (dd, J = 5 Hz, 1H).

4.1.5. Synthesis of Boc-L-2-amino-8-nonenoic acid (7). Metallic sodium (4.60 g, 200 mmol) was dissolved in absolute ethanol (200 mL) and diethyl *tert*-butoxycarbonylaminomalonate (55.06 g, 200 mmol) was added to the solution. The reaction mixture was refluxed for 30 min for the complete removal of the labile hydrogen and then 7-bromo-1-heptene (35.4 g, 200 mmol) was added and refluxed for 4 h. The reaction mixture was cooled on an ice bath and hydrolysis was carried out by the treatment of 1 M aq NaOH (200 mL, 200 mmol). After selective hydrolysis for 3 h at 0 °C, ethanol was evaporated and the unreacted 7-bromo-1-heptene was removed by extraction with diethyl ether under basic condition. Then the aqueous solution was extracted with ethyl acetate at pH 3–4, by adding solid citric acid. Finally ethyl acetate layer was washed with brine and dried over anhydrous $MgSO_4$. Ethyl acetate was evaporated to obtain a colorless oil of monoester monoacid (59.0 g, 87%). HPLC, t_R 10.12 min. It was then dissolved in toluene (175 mL) and refluxed for 3 h. The oily ester after evaporation of toluene is then subjected to silica-gel column chromatographic purification to get Boc-DL-Ae9-OEt (**6**) as colorless oil (43.5 g, 83%). The resulting oily mass was suspended into a mixture of water (450 mL) and DMF (150 mL) (1:3, v/v) solvent system at 37 °C using a mechanical stirrer and pH was adjusted at about 7–8 by adding 1 M aq ammonia solution. Then

subtilisin Carlsberg from *B. licheniformis* (60 mg, 1 mg enzyme per mmol of substrate) was added and pH was maintained at 7–8 by continuous addition of 1 M aq ammonia solution. The reaction was completed within 4 h. Water and DMF were evaporated and Boc-D-Ae9-OEt (**8**) (22.2 g, 51%) was extracted with diethyl ether under basic condition. Then the aqueous solution was extracted with ethyl acetate as described above to get Boc-L-Ae9-OH (**7**) as colorless oil (19.2 g, 49%). HPLC, t_R 8.38 min. $[\alpha]_D^{22.4}$ 3.0 (c = 1, MeOH). HRMS $[M+H]^+$ 272.1893 for $C_{14}H_{26}NO_4$ (calcd 272.1862) ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 1.29–1.39 (m, γ, δ and ε, 6H), 1.49 (s, 9H), 1.67 (m, 1H), 1.86 (m, 1H), 2.06 (m, 2H), 4.29 (m, 1H), 4.92–5.01 (m, 3H), 5.79 (ddt, J = 17.2, 10.2, 6.5 Hz, 1H).

4.1.6. Synthesis of Boc-D-2-amino-8-nonenoic acid (9). The ester Boc-D-Ae9-OEt (**8**) (22.2 g, 74.1 mmol) was re-resoluted using 5 mg of subtilisin Carlsberg from *B. licheniformis* using the procedure described in experimental Section 4.1.5. The pure D-ester (20.0 g, 66.8 mmol) was dissolved in ethanol (70 mL) and hydrolyzed by 1 M NaOH (70 mL, 70 mmol) for 4 h at 0 °C. After completion of the reaction, ethanol was evaporated and the aqueous solution of Boc-D-Ae9-OH (**9**) was acidified to pH 3 by solid citric acid. Then the aqueous solution was extracted with ethyl acetate. Finally ethyl acetate layer was washed with brine, dried over anhydrous $MgSO_4$, and evaporated to obtain Boc-D-Ae9-OH (**9**) as colorless oil (17.9 g, 99%). HPLC, t_R 8.33 min. $[\alpha]_D^{22.1}$ 2.8 (c = 1, MeOH).

4.1.7. Synthesis of cyclic dipeptide by alkene ring closing metathesis (11) and (12). To a cooled solution of HCl·H-L-Ae9-OBzl (900 mg, 3.00 mmol), Boc-D-Ae9-OH (820 mg, 3.00 mmol) in DMF (10 mL), HBTU (1.71 g, 4.50 mmol), HOBt·H_2O (459 mg, 3.00 mmol), and triethyl amine (1.30 mL, 9.00 mmol) were added successively. The mixture was stirred at room temperature for 4 h. After completion of the reaction, DMF was evaporated and the residue was dissolved in ethyl acetate and washed with 10% citric acid, 4% sodium bicarbonate, and brine, respectively. The ethyl acetate solution was dried over anhydrous $MgSO_4$ and concentrated to remain an oily substance, which was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield Boc-D-Ae9-L-Ae9-OBzl (**10**) (1.40 g, 89%) as colorless foam. HPLC, t_R 12.68 min. HR-FAB-MS $[M+H]^+$ 515.3453 for $C_{30}H_{47}O_5N_2$ (calcd 515.3485). To a solution of linear dipeptide Boc-D-Ae9-L-Ae9-OBzl (107 mg, 0.20 mmol) in anhydrous and degassed dichloromethane (25 mL), a solution of Grubb's first generation ruthenium catalyst (33 mg, 0.04 mmol) in anhydrous and degassed dichloromethane (5 mL) was added. The reaction mixture was stirred at room temperature for 48 h. After the completion of reaction, dichloromethane was evaporated. Finally diastereomers were separated by HPLC [column: YMC pack, ODS-A (250 × 10 mm), condition: Gradient condition from solvent A to B 75–80% over 25 min with 220 nm detection, Sol. A; 10% acetonitrile with 0.1% TFA and Sol. B; 100% acetonitrile with 0.1% TFA] to yield *cis* isomer (**11**) (17 mg, 18%). HPLC, t_R 13.22 min. HR-FAB-MS $[M+H]^+$ 487.3214 for $C_{28}H_{43}O_5N_2$ (calcd 487.3172). ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 1.17, 1.25, 1.33 and 1.64 (m, 12H) 1.42 (s, 9H), 1.78 and 1.62 (m, 2H), 1.87 and 1.65 (m, 2H), 1.94 and 2.02 (m, 4H), 4.15 (m, 1H), 4.78 (m, 1H), 5.17 (ABq, J = 12.5 Hz, 2H), 5.24 (d, J = 7.5 Hz, 1H), 5.34 (m, 2H), 6.49 (d, J = 7.5 Hz, 1H), 7.35 (m, 5H) and *trans* isomer (**12**) (30.5 mg, 32%). HPLC, t_R 15.02 min. HR-FAB-MS $[M+H]^+$ 487.3151 for $C_{28}H_{43}O_5N_2$ (calcd 487.3172). ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 1.07, 1.23 and 1.49 (m, 12H) 1.43 (s, 9H), 1.79 (m, 2H), 1.95 (m, 2H), 1.93 and 2.03 (m, 4H), 4.23 (m, 1H), 4.67 (m, 1H), 5.17 (ABq, J = 12.2 Hz, 2H), 5.23 (d, J = 8 Hz, 1H), 5.32 (m, 2H), 6.51 (d, J = 7 Hz, 1H), 7.35 (m, 5H).

4.1.8. Synthesis of bicyclic disulfide hybrid (17). The protected dipeptide Boc-L-Ae9-D-Pro-O^{t}Bu (4.00 g, 9.43 mmol) synthesized according to the procedure as described earlier was dissolved in 4 N HCl/dioxane (40 mL) and the reaction solution was kept at room temperature for 30 min. After evaporation of HCl/dioxane, the residue was dissolved in ethyl acetate and washed with 10% sodium carbonate to get the free amine H-L-Ae9-D-Pro-O^{t}Bu (1.88 g, 61%). HPLC, t_R 5.68 min. MALDI-TOF-MS $[M+Na]^+$ 325.7 for $C_{18}H_{33}O_3N_2$ (calcd 325.2). The free amine H-L-Ae9-D-Pro-O^{t}Bu (1.88 g, 5.80 mmol) was coupled with Boc-D-Ae9-OH (1.63 g, 6.00 mmol) following the same procedure mentioned earlier to obtain Boc-D-Ae9-L-Ae9-D-Pro-O^{t}Bu (3.26 g, 97%). HPLC, t_R 11.60 min. The tripeptide Boc-D-Ae9-L-Ae9-D-Pro-O^{t}Bu (3.26 g, 5.60 mmol) was treated with 4 N HCl/dioxane (20 mL) as described above. The resulting free amine H-D-Ae9-L-Ae9-D-Pro-O^{t}Bu was extracted into ethyl acetate and obtained as a colorless oil (1.91 g, 72%, HPLC, t_R 7.91 min). The N-terminal deprotected tripeptide H-D-Ae9-L-Ae9-D-Pro-O^{t}Bu (1.24 g, 2.59 mmol) and Boc-L-Ab7-OH (1.26 g, 3.90 mmol) were condensed using the DCC/HOBt protocol to afford Boc-L-Ab7-D-Ae9-L-Ae9-D-Pro-O^{t}Bu (1.30 g, 63%), after work-up and flash chromatography. HPLC, t_R 12.55 min. MALDI-TOF-MS $[M+Na]^+$ 805.4 for $C_{39}H_{67}O_7N_4{}^{79}Br$ Na (calcd 805.4). Sequential deprotection, and cyclization yielded *cyclo* (-L-Ab7-D-Ae9-L-Ae9-D-Pro-) **13** (380 mg, 62%). HPLC, t_R 11.43 min. HR-FAB-MS $[M+H]^+$ 609.3038 for $C_{30}H_{50}O_4N_4{}^{79}Br$ (calcd 609.3015) and 611.3006 for $C_{30}H_{50}N_4O_4{}^{81}Br$ (calcd 611.2995). The cyclic tetrapeptide **13** (235 mg, 0.38 mmol) was treated with Grubb's first generation catalyst (64 mg, 0.08 mmol) in anhydrous dichloromethane (50 mL) according to the similar procedure as described earlier to yield an isomeric mixture of fused bicyclic tetrapeptides **14** (100 mg, 45%). HPLC, t_R 10.10 min. HR-FAB-MS $[M+H]^+$ 581.2714 for $C_{28}H_{46}O_4N_4{}^{79}Br$ (calcd 581.2702). To the bicyclic peptide **14** dissolved in acetic acid (10 mL), Pd–C (100 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 to give **15** (90.6 mg, 98%). HPLC, t_R 9.35 min. HR-FAB-MS $[M+H]^+$ 583.2861 for $C_{28}H_{48}O_4N_4{}^{79}Br$ (calcd 583.2859). Further, compound

15 was transformed to its thioester **16** (41 mg, 81%). HPLC, t_R 9.00 min. HR-FAB-MS $[M+H]^+$ 579.3578 for $C_{30}H_{51}O_5N_4S$ (calcd 579.3580). Finally, the compound **16** was converted into the desired disulfide hybrid **17** (25 mg, 55%) according to the procedure described earlier. HPLC, t_R 9.64 min. HR-FAB-MS $[M+H]^+$ 646.3478 for $C_{33}H_{52}O_4N_5S_2$ (calcd 646.3461) ^1H NMR (500 MHz, $CDCl_3$): δ_H 1.14–1.42 (m, 29H), 1.61 (m, 1H), 1.69 (m, 2H), 1.81 (m, 3H), 1.90–1.99 (m, 2H), 2.13 (m, 1H), 2.28 (m, 1H), 2.39 (m, 1H), 2.77 (t, 2H), 3.52 (ddd, J = 11.5, 11.5, 7.7 Hz, 1H), 4.08 (m, 1H), 4.24 (ddd, J = 10.5, 10.25, 7.25 Hz, 1H), 4.84 (m, 1H), 6.12 (d, J = 9.5 Hz, 1H), 6.22 (d, J = 10 Hz, 1H), 7.08 (m, 1H), 7.11 (d, J = 10 Hz, 1H), 7.65 (m, 1H), 7.72 (d, J = 7 Hz, 1H), 8.46 (dd, J = 1, 1 Hz, 1H).

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