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Bioorganic & Medicinal Chemistry 12 (2004) 5777–5784
Bioorganic & Medicinal Chemistry

Chlamydocin–hydroxamic acid analogues as histone deacetylase inhibitors

Norikazu Nishino,[a,b,*] Binoy Jose,[b] Ryuzo Shinta,[a] Tamaki Kato,[a] Yasuhiko Komatsu[b,c] and Minoru Yoshida[b,d]

[a]*Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology, Kitakyushu 808-0196, Japan*
[b]*CREST Research Project, Japan Science and Technology Agency, Saitama 332-0012, Japan*
[c]*Pharmaceuticals and Biotechnology Laboratory, Japan Energy Corporation, Saitama 335-8502, Japan*
[d]*RIKEN, Saitama 351-0198, Japan*

Received 21 June 2004; revised 26 August 2004; accepted 27 August 2004
Available online 23 September 2004

Abstract—Chlamydocin–hydroxamic acid analogues were designed and synthesized as histone deacetylase (HDAC) inhibitors based on the structure and HDAC inhibitory activity of chlamydocin and trichostatin A. Chlamydocin is a cyclic tetrapeptide containing an epoxyketone moiety in the side chain that makes it an irreversible inhibitor of HDAC. We replaced the epoxyketone moiety of chlamydocin with hydroxamic acid to design potent and reversible inhibitors of HDAC. In addition, a number of amino-cycloalkanecarboxylic acids (Acc) are introduced instead of the simple amino-isobutric acid (Aib) for a variety of the series of chlamydocin analogues. The compounds synthesized were tested for HDAC inhibitory activity and the results showed that many of them are potent inhibitors of HDAC. The replacement of Aib residue of chlamydocin with an aromatic amino acid enhances the in vivo and in vitro inhibitory activity. We have carried out circular dichroism and molecular modeling studies on chlamydocin–hydroxamic acid analogue and compared it with the solution structure of chlamydocin.

1. Introduction

Post-translational modification of proteins by acetylation and deacetylation of ε-amino groups of lysine residues by histone acetyl transferase (HAT) and histone deacetylase (HDAC) enzymes is involved in chromatin remodeling and plays a crucial role in gene expression.[1–4] Modification of the level of histone acetylation and its consequences have received enormous interest in recent years and increasing evidence supports their importance for basic cellular functions such as DNA replication, transcription, differentiation, and apoptosis. Acetylation of lysine residues of histones results in more open chromatin structure and therefore activation of transcription, where as deacetylation of histone is associated with a condensed chromatin structure resulting in the repression of gene transcription. Deregulation of HAT and HDAC has been implicated in the formation and development of certain human cancers by changing the expression pattern of various genes. It is therefore proposed that HDACs are a potential target for the development of small molecule anticancer agent.

A number of natural and synthetic HDAC inhibitors have been reported, and in recent years the importance of HDAC inhibitors has increased due to their efficacy against many malignant diseases.[5] Several of these HDAC inhibitors inhibit tumor growth and many of them are under clinical trials. Trichostatin A, (TSA),[6] a cyclic tetrapeptide family including trapoxins (TPX),[7] chlamydocin,[8] HC toxin,[9] Cyl-1, Cyl-2,[10] WF-3161,[11] apicidin,[12,13] and the recent depsipeptide FK228 (formerly FR901228)[14–16] are naturally occurring HDAC inhibitors and inhibitors such as butyrate,[17] valproate,[18] suberoyl anilide hydroxamic acid,[19] analogues of TSA,[20–22] and benzamide derivatives of MS-275[23,24] are synthetic compounds. Trichostatin A is a natural product that contains a hydroxamic acid functional group and is a reversible inhibitor of HDAC. On the other hand, cyclic tetrapeptides, trapoxin, chlamydocin, etc. contain an epoxyketone moiety, and

Keywords: Histone deacetylase; Chlamydocin; Cyclic tetrapeptide; Inhibitor.
* Corresponding author. Tel./fax: +81 93 695 6061; e-mail: nishino@life.kyutech.ac.jp

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doi:10.1016/j.bmc.2004.08.041

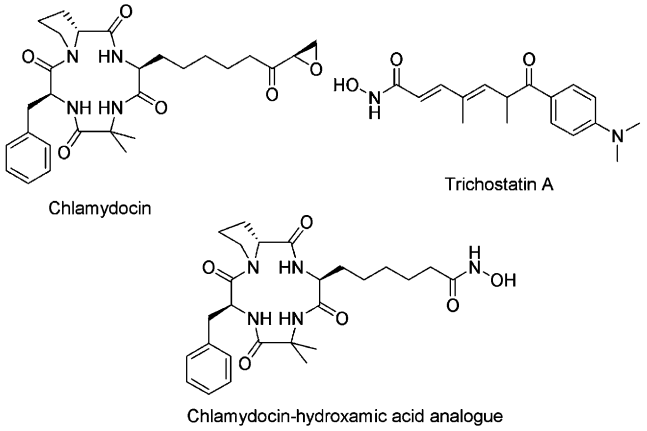


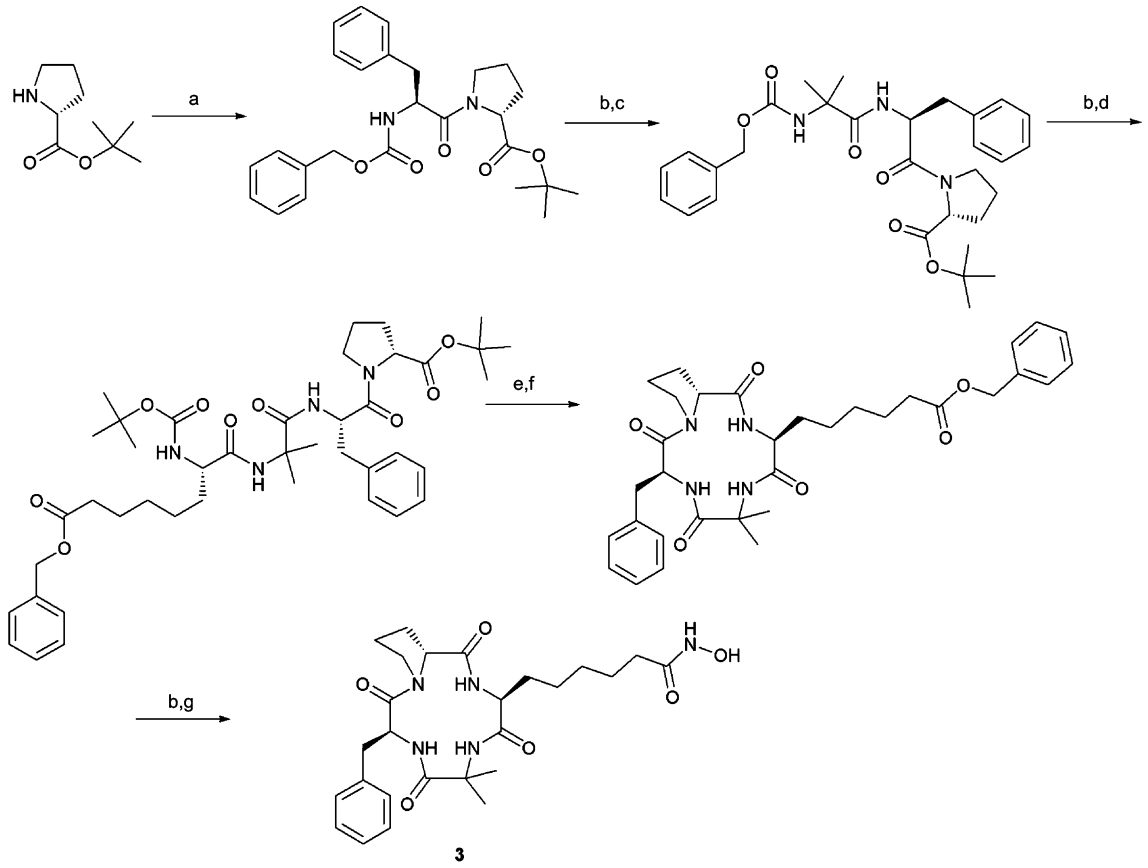
Figure 1. The structure of chlamydocin, trichostatin A, and proposed chlamydocin–hydroxamic acid analogue.

are irreversible inhibitors of HDAC. Chlamydocin was originally isolated from the fungus *Diheterospora chlamydosporia*, and has been shown to exhibit potent anticancer activity in vitro.[8] Chlamydocin belongs to a small family of hydrophobic cyclic tetrapeptides containing the unusual amino acid, 2-amino-8-oxo-9,10-epoxy decanoic acid (Aoe), which is essential for their biological activity. Recently, we have reported a new group of synthetic HDAC inhibitors, CHAPs, by the use of hydroxamic acid as a functional group instead of epoxyketone of naturally occurring cyclic tetrapeptides and our initial studies showed that these inhibitors have HDAC inhibitory activity in nanomolar range (Fig. 1).[25,26]

In the present study, we proposed to synthesize chlamydocin–hydroxamic acid analogues as HDAC inhibitors. During the development of potent inhibitors of HDAC, we found that amino acid residues in the cyclic tetrapeptides, and their conformations are very important for the activity. Therefore we proposed to compare the activities of several CHAPs with different amino acid combinations. We herein describe the full account on synthesis of chlamydocin–hydroxamic acid analogues and a brief description of the interesting biological activity. A comparison of the structural features of chlamydocin and the synthetic chlamydocin–hydroxamic acid analogues is also described.

2. Results and discussion

Our aim is to synthesize potent inhibitors of HDACs based on the natural product chlamydocin. Therefore, we proposed to synthesize chlamydocin–hydroxamic acid analogue by replacing the epoxyketone moiety of chlamydocin by hydroxamic acid. For this, we initially synthesized chlamydocin–hydroxamic acid analogue using solution-phase peptide coupling method as depicted in Scheme 1. *tert*-Butyl protected D-Pro was



Scheme 1. Synthesis of cyclic tetrapeptide. Reagents and conditions: (a) HBTU, DIEA, Z-L-Phe; (b) Pd–C, H_2; (c) HBTU, DIEA, Z-Aib; (d) HBTU, DIEA, Boc-L-Asu(OBzl); (e) TFA; (f) HATU, DIEA; (g) NH_2OH, BOP, HOBt.

coupled with Z-L-Phe to give the dipeptide Z-L-Phe-D-Pro-O^{t}Bu. The Z group of dipeptide was deprotected by catalytic hydrogenation and coupled with Z-Aib. The Z group of the resulted tripeptide Z-Aib-L-Phe-D-Pro-O^{t}Bu was deprotected by catalytic hydrogenation. It was then coupled with Boc protected amino suberic acid benzyl ester, Boc-L-Asu(OBzl) to give Boc-L-Asu(OBzl)-Aib-L-Phe-D-Pro-O^{t}Bu. The *C*-terminal and *N*-terminal protections of the tetrapeptide were removed by treatment with trifluoroacetic acid (TFA) and the resulted linear peptide was cyclized in DMF under high dilution condition using HATU as a coupling reagent to give *cyclo*(-L-Asu(OBzl)-Aib-L-Phe-D-Pro-) in 60% yield. The side chain was deprotected by catalytic hydrogenation and then coupled with hydroxylamine hydrochloride to yield the desired compound **3** and finally purified using gel filtration.

We replaced Aib residue of **3** with many other natural and nonnatural amino acids to study the effect on the HDAC inhibitory activity. The amino acids used for the replacement of Aib are 1-aminocyclopentanecarboxylic acid (Acc5), 1-aminocyclohexanecarboxylic acid (Acc6), 1-aminocycloheptanecarboxylic acid (Acc7), 1-aminocyclooctanecarboxylic acid (Acc8), 2-aminoindane-2-carboxylic acid (A2in), L-Ala, D-Ala, DL-1-aminoindane-1-carboxylic acid (DL-A1in), and L-Pro. The cyclic tetrapeptides chlamydocin–hydroxamic acid analogues were synthesized according to the method described for compound **3** using the corresponding amino acids instead of Aib to give the following compounds *cyclo*(-L-Asu(NHOH)-Acc5-L-Phe-D-Pro-) (**4**), *cyclo*(-L-Asu(NHOH)-Acc6-L-Phe-D-Pro-) (**5**), *cyclo*(-L-Asu(NHOH)-Acc7-L-Phe-D-Pro-) (**6**), *cyclo*(-L-Asu(NHOH)-Acc8-L-Phe-D-Pro-) (**7**), *cyclo*(-L-Asu(NHOH)-A2in-L-Phe-D-Pro-) (**8**), *cyclo*(-L-Asu(NHOH)-L-Ala-L-Phe-D-Pro-) (**9**), *cyclo*(-L-Asu(NHOH)-D-Ala-L-Phe-D-Pro-) (**10**), *cyclo*(-L-Asu(NHOH)-L-A1in-L-Phe-D-Pro-) (**11**), *cyclo*(-L-Asu(NHOH)-D-A1in-L-Phe-D-Pro-) (**12**), and *cyclo*(-L-Asu(NHOH)-L-Pro-L-Phe-D-Pro-) (**13**).

Next we changed the D-Pro residue of chlamydocin to D-pipecolic acid (D-Pip). This compound was synthesized using the same method reported for compound **3** using DL-Pip instead of D-Pro. After cyclization, the compound containing D-Pip was purified by column chromatography and characterized by HPLC and NMR spectroscopy. Thus, we synthesized compounds, *cyclo*(-L-Asu(NHOH)-Aib-L-Phe-D-Pip-) (**14**), *cyclo*(-L-Asu(NHOH)-Acc5-L-Phe-D-Pip-) (**15**), *cyclo*(-L-Asu(NHOH)-Acc8-L-Phe-D-Pip-) (**16**), and *cyclo*(-L-Asu(NHOH)-A2in-L-Phe-D-Pip-) (**17**). We then synthesized *cyclo*(-L-Asu(NHOH)-A2in-L-Ile-D-Pip-) (**18**) by replacing L-Phe of **17** by L-Ile. This compound was synthesized by using the same method for **17** with L-Ile instead of L-Phe.

To know the effect of retro-enantio-analogues, we synthesized two compounds, such as *cyclo*(D-Asu(NHOH)-L-Pro-D-Phe-Aib-) (**19**) and *cyclo*(-L-Asu(NHOH)-L-Pro-D-Phe-Aib-) (**20**) using the same method reported for **3** using different amino acids. Finally we synthesized *cyclo*(-L-Asu(NHOH)-Aib-L-Phe-L-Pro-) (**21**). All the synthesized compounds were characterized by ^{1}H NMR and high-resolution FAB-MS. The purity of the compounds was determined by HPLC analysis and all the synthesized cyclic tetrapeptides showed purity above 97%.

We evaluated the ability of chlamydocin–hydroxamic acid analogues to inhibit a partially purified mouse HDAC in vitro by comparing their IC_{50}s. To evaluate their cellular activity, we determined the concentration required for 2-fold increased expression of MHC class-I molecules in B16/BL6 melenoma cells ($C_{\times 2}$). Table 1 shows the HDAC inhibitory activity of

Table 1. HDAC inhibitory data for reported compounds

No	Compound	IC_{50} (nM) HDAC1	MHC $C_{\times 2}$ (nM)
1	TSA	6.0	2.8
2	Chlamydocin	0.15	4.6
3	*cyclo*(-L-Asu(NHOH)-Aib-L-Phe-D-Pro-)	5.2	33.2
4	*cyclo*(-L-Asu(NHOH)-Acc5-L-Phe-D-Pro-)	3.02	5.67
5	*cyclo*(-L-Asu(NHOH)-Acc6-L-Phe-D-Pro-)	2.19	5.38
6	*cyclo*(-L-Asu(NHOH)-Acc7-L-Phe-D-Pro-)	2.14	2.76
7	*cyclo*(-L-Asu(NHOH)-Acc8-L-Phe-D-Pro-)	3.99	1.88
8	*cyclo*(-L-Asu(NHOH)-A2in-L-Phe-D-Pro-)	0.98	1.29
9	*cyclo*(-L-Asu(NHOH)-L-Ala-L-Phe-D-Pro-)	1.95	325
10	*cyclo*(-L-Asu(NHOH)-D-Ala-L-Phe-D-Pro-)	3.23	NT
11	*cyclo*(-L-Asu(NHOH)-L-A1in-L-Phe-D-Pro-)	1.12	2.69
12	*cyclo*(-L-Asu(NHOH)-D-A1in-L-Phe-D-Pro-)	1.95	2.29
13	*cyclo*(-L-Asu(NHOH)-L-Pro-L-Phe-D-Pro-)	3303	NT
14	*cyclo*(-L-Asu(NHOH)-Aib-L-Phe-D-Pip-)	2.98	NT
15	*cyclo*(-L-Asu(NHOH)-Acc5-L-Phe-D-Pip-)	12.4	37.8
16	*cyclo*(-L-Asu(NHOH)-Acc8-L-Phe-D-Pip-)	2.75	NT
17	*cyclo*(-L-Asu(NHOH)-A2in-L-Phe-D-Pip-)	1.2	NT
18	*cyclo*(-L-Asu(NHOH)-A2in-L-Ile-D-Pip-)	2.68	NT
19	*cyclo*(-D-Asu(NHOH)-L-Pro-D-Phe-Aib-)	8.57	3950
20	*cyclo*(-L-Asu(NHOH)-L-Pro-D-Phe-Aib-)	31.7	57,800
21	*cyclo*(-L-Asu(NHOH)-Aib-L-Phe-L-Pro-)	45.7	717

chlamydocin–hydroxamic acid analogue compounds. These compounds inhibited the HDACs at nanomolar concentrations. For comparison, the inhibitory activities of TSA and chlamydocin are also shown. Most of these chlamydocin–hydroxamic acid analogues are potent inhibitors of HDAC. The replacement of epoxyketone moiety of chlamydocin by hydroxamic acid affects the in vitro and in vivo activities. The activity of chlamydocin–hydroxamic acid analogue **3** is lower than chlamydocin **2**. This fact could be attributed to irreversible mechanism of **2**, though the straightforward comparison is difficult. When the amino acid residue Aib is replaced by Acc5, Acc6, Acc7, and Acc8, the activity increased, and out of these, replacement with Acc7 seems optimum. The replacement of Aib residue with L-Ala gives good inhibitory activity in vitro; however, cellular activity was low. Chlamydocin–hydroxamic analogues of Aib replaced with aminoindanecarboxylic acids show very good activity in vitro and in vivo. This high activity of inhibitors containing an aminoindanecarboxylic acid residue shows the importance of an aromatic ring for the activity instead of Aib. This result indicates the presence of a favorable interaction between the benzene ring and the residues at the rim of the HDAC binding channel. Out of these, three compounds **8**, **11**, and **12**, inhibitor with 2-amino-2-indanecarboxylic acid **8** showed more potency. On the other hand, the replacement of Aib with L-Pro in **13** resulted in a significant decrease in the activity.

Most of the naturally occurring cyclic tetrapeptides containing a D-Pro residue, an analogue with D-Pip is also observed as in trapoxin A, Cyl-2, etc. However, no chlamydocin analogue with a D-Pip residue was reported. Therefore, we replaced the D-Pro residue of **3** with D-Pip to study the effect of the increase in ring size on the inhibitory activity. The replacement of D-Pro of chlamydocin–hydroxamic acid inhibitor to D-Pip, showed no significant differences in the activity. The retro-enantio-analogues of chlamydocin **19** and **20** showed lower inhibitory activity than **3**. This decrease in activity indicates the importance of position and chirality of Pro residue in the cyclic tetrapeptide. The change from L-Asu to D-Asu affected adversely to the in vitro and in vivo activity.

CD spectra of some of the chlamydocin–hydroxamic acid analogues are shown in Figure 2. The CD spectrum of chlamydocin **2** and **3** are similar up to 260 nm. At the longer wavelength than 260 nm, **2** shows a negative ellipticity due to the chiral ketone in **2**,[8] which is absent in **3**. Other analogues, except **13**, **19**, and **21** showed similar CD spectra as **3** indicating that these compounds have similar conformations in methanol. Changes in the Pro position and chirality give different conformations for **13**, **19**, and **21**.

Conformation analysis of chlamydocin–hydroxamic acid analogues was studied using ^{1}H NMR in DMSO-d_6 and in $CDCl_3$. Complete assignments of the chemical shifts were made by using COSY, HOHAHA, and NOESY spectra. Comparison of the conformations of hydroxamic acid analogue **3** and chlamydocin **2** showed

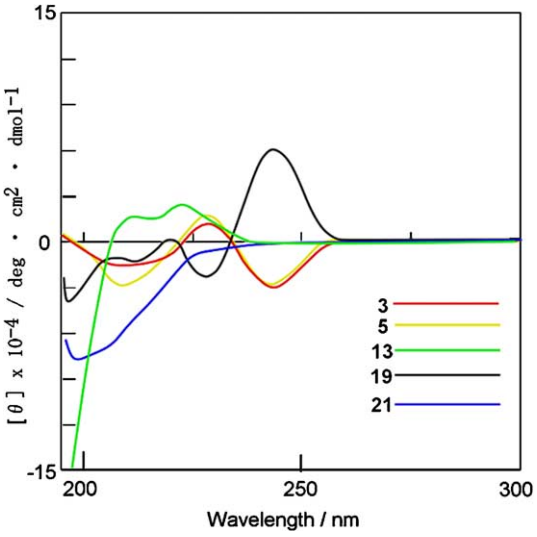


Figure 2. CD spectra of chlamydocin–hydroxamic acid analogues.

that the synthetic compound and natural product have similar structural features.[27,28] The conformation of the cyclic peptide framework of chlamydocin is remarkably sensitive to solvents. In chloroform, the framework of chlamydocin exists with bis-γ-turn structure with four transoid amide bonds. On the other hand in DMSO, it exists as the equilibrium of bis-γ-turn structure and β-turn structure.[29] We observed the same behavior for chlamydocin–hydroxamic acid analogue also. The molecular modeling study on **3** showed that the existence of bis-γ-turn structure in chloroform (Fig. 3a) and an equilibrium of bis-γ-turn structure and β-turn structure (Fig. 3b) in DMSO. Similar behavior was observed for compounds **4**–**8**. On the other hand, compounds **9**–**13** showed only bis-γ-turn conformation in chloroform and also in DMSO indicating that the presence of Aib is the reason for the two different conformations.

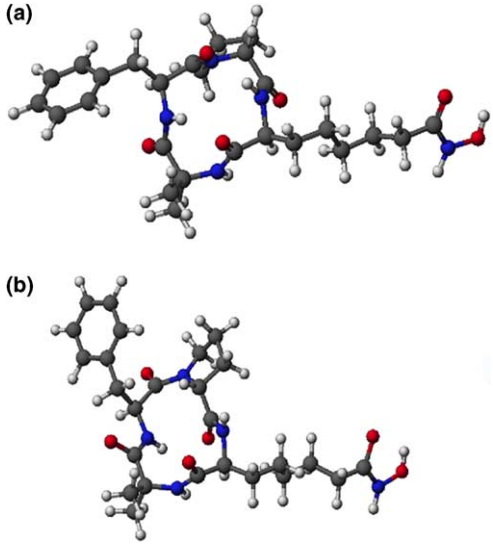


Figure 3. Energy minimized structures of the two conformers of chlamydocin–hydroxamic acid analogue: (a) bis-γ-turn and (b) β-turn.

3. Conclusion

We have successfully synthesized several chlamydocin–hydroxamic acid analogues as HDAC inhibitors. The in vitro and in vivo HDAC inhibitory activity of the inhibitors was evaluated and it was found that most of the inhibitors are potent toward HDAC. The results indicate that the change of the Aib residue of chlamydocin to aromatic amino acids can increase the inhibitory activity.

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) or high-performance liquid chromatography (HPLC). TLC was performed on aluminum-backed silica gel plates (Merck DC-Alufolien Kieselgel 60 F_{254}) with spots visualized by UV light. Analytical HPLC were performed on a Hitachi instrument equipped with a Wako pak C4 column (4.6 mm × 150 mm). 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 30 min with detection at 220 nm with a flow rate of 1 mL/min. NMR spectra were recorded on a JEOL JNM A500 MHz spectrometer. NMR spectra were measured in $CDCl_3$ or DMSO-d_6 solutions containing TMS as reference. All ^1H shifts are given in ppm (s = singlet; d = doublet; m = multiplet). Assignments of proton resonances were confirmed, when possible, by selective homonuclear decoupling experiments or by correlated spectroscopy. FAB-mass spectra and high-resolution mass spectra (HRMS) were measured on a JEOL JMS-SX 102A Instrument. Amino acids were coupled using standard solution-phase chemistry with dicyclohexylcarbodimide (DCC), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), or *O*-(7-azabenzotriazoyl-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) as the coupling reagent. BOP (benzotriazol-1-yloxytris-(dimethylamino)-phosphonium hexafluorophosphate) was used for the reaction with hydroxylamine.

4.1.1. Synthesis of *cyclo*(-L-Asu(NHOH)-Aib-L-Phe-D-Pro-) (3). To a solution of Z-L-Phe-OH (2.25 g, 7.5 mmol) and H-D-Pro-O^{t}Bu (0.862 g, 5.0 mmol) in DMF (10 mL) HOBt·H_2O (766 mg, 5.0 mmol), HBTU (2.84 g, 7.5 mmol), and triethylamine (1.75 mL, 12.5 mmol) were added and the mixture was stirred for 2 h. DMF was evaporated and the residue was dissolved in ethyl acetate and washed with citric acid solution (10%), sodium bicarbonate solution (4%), and brine, respectively, and then dried over anhydrous $MgSO_4$. After evaporation of ethyl acetate, the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield Z-L-Phe-D-Pro-O^{t}Bu (1.40 g, 62%). The protected dipeptide (1.40 g, 3.1 mmol) was dissolved in acetic acid (5 mL). Pd–C (150 mg) was added and the mixture was stirred under H_2 pressure for 10 h. After filtration, acetic acid was evaporated. The residue was dissolved in ethyl acetate and washed with sodium bicarbonate solution (4%) and dried over sodium carbonate. Evaporation of ethyl acetate gave H-L-Phe-D-Pro-O^{t}Bu (0.853 g, 2.68 mmol, 86%), which was coupled with Z-Aib-OH (954 mg and 4.02 mmol) according to the method described above to yield Z-Aib-L-Phe-D-Pro-O^{t}Bu (1.23 g, 85%). Z-Aib-L-Phe-D-Pro-O^{t}Bu (1.23 g, 2 mmol) was dissolved in acetic acid (5 mL) and Pd–C (100 mg) was added. The mixture was stirred under H_2 for 10 h. After filtration, acetic acid was evaporated. The residue was dissolved in ethyl acetate and washed with sodium bicarbonate solution (4%) and dried over sodium carbonate. Evaporation of ethyl acetate yielded H-Aib-L-Phe-D-Pro-O^{t}Bu (0.726 g, 79%). H-Aib-L-Phe-D-Pro-O^{t}Bu (0.726 g, 1.80 mmol) was coupled with Boc-L-Asu-(OBzl)-OH (1.02 g, 2.70 mmol) as described earlier to yield Boc-L-Asu(OBzl)-Aib-L-Phe-D-Pro-O^{t}Bu (993 mg, 72%). Boc-L-Asu(OBzl)-Aib-L-Phe-D-Pro-O^{t}Bu (993 mg, 1.30 mmol) was dissolved in TFA (3 mL) at 0 °C and kept for 2 h. After evaporation of TFA, the residue was solidified by titration with ether to yield H-L-Asu(OBzl)-Aib-L-Phe-D-Pro-OH as TFA salt (737 mg, 78%). HPLC, rt 8.66 min. To a DMF (400 mL) solvent, H-L-Asu(OBzl)-Aib-L-Phe-D-Pro-OH·TFA (300 mg, 0.42 mmol), DIEA/DMF (3.0 mL, 1.7 mmol), and HATU (250 mg, 0.65 mmol) were added in separate five portions in every 30 min with stirring, for the cyclization reaction. After the reaction, DMF was evaporated under vacuo, the residue being dissolved in ethyl acetate and washed with citric acid (10%) solution, sodium bicarbonate (4%) solution, and brine, respectively. It was then dried over anhydrous $MgSO_4$ and filtered. After evaporation of ethyl acetate, the residue was purified by column chromatography using a mixture of chloroform and methanol (99:1) to yield *cyclo*(-L-Asu(OBzl)-Aib-L-Phe-D-Pro-) (151 mg, 61%) HPLC rt 18.1 min. *cyclo*(-L-Asu(OBzl)-Aib-L-Phe-D-Pro-) (151 mg, 0.256 mmol) was dissolved in methanol (5 mL) and Pd–C (50 mg) was added. The solution was stirred under H_2 for 10 h. After the filtration of Pd–C, methanol was evaporated to yield *cyclo*(-L-Asu-Aib-L-Phe-D-Pro-) (124 mg, 97%). HPLC, rt 6.32 min. The *cyclo*(-L-Asu-Aib-L-Phe-D-Pro-) (124 mg, 0.248 mmol) was dissolved in DMF (3 mL) at 0 °C and hydroxylamine hydrochloride (86 mg, 1.24 mmol) HOBt·H_2O (57 mg, 0.372 mmol), BOP (165 mg, 0.372 mmol), and triethylamine (0.24 mL, 1.74 mmol) were added. The mixture was stirred for 2 h. Gel filtration of the reaction mixture using Sephadex LH-20 was followed by lyophilization to yield *cyclo*(-L-Asu(NHOH)-Aib-L-Phe-D-Pro-) as white powder (95 mg, 74%) HPLC, rt 14.3 min, ^1H NMR (500 MHz, $CDCl_3$): δ 7.56 (d, 1H), 7.11–7.29 (m, 7H), 6.31 (d, 1H), 5.12 (m, 1H), 4.68 (d, 1H), 4.22 (d, 1H), 3.86 (m, 1H), 3.16 (m, 2H), 2.94 (m, 3H), 2.31 (m, 1H), 2.13 (m, 2H), 1.77–1.25 (m, 16H); CD (methanol) λ ($[\theta]_M$) 243 (−31,000), 228 (12,000), 210 (−15,000) nm; HR-FAB MS $[M+H]^+$ 516.2819 for $C_{26}H_{38}O_6N_5$ (calcd 516.2822).

4.1.2. Synthesis of *cyclo*(-L-Asu(NHOH)-Acc5-L-Phe-D-Pro-) (4). This compound was synthesized according to the procedure reported for **3** using Acc5 instead of Aib. HPLC, rt 15.9 min, ^{1}H NMR (500 MHz, DMSO-d_6) δ: Conformer 1, 8.36 (s, 1H), 7.63 (d, 1H), 7.17–7.28 (m, 5H), 7.00 (d, 1H), 4.95 (m, 1H), 4.74 (m, 1H), 4.26 (m, 1H), 3.05–3.35 (m, 2H), 2.89 (m, 2H), 1.24–2.27 (m, 22H); Conformer 2, 8.43 (d, 1H), 7.96 (d, 1H), 7.17–7.28 (m, 5H), 6.85 (d, 1H), 4.84 (m, 1H), 4.55 (m, 1H), 4.18 (m, 1H), 3.05–3.35 (m, 2H), 2.89 (m, 2H), 1.24–2.27 (m, 22H); HR FAB-MS $[M+H]^+$ 542.2982 for $C_{28}H_{40}O_6N_5$ (calcd 542.2979).

4.1.3. Synthesis of *cyclo*(-L-Asu(NHOH)-Acc6-L-Phe-D-Pro-) (5). This compound was synthesized according to the procedure reported for **3** using Acc6 instead of Aib. HPLC, rt 16.9 min, ^{1}H NMR (500 MHz, DMSO-d_6) δ: Conformer 1, 7.99 (br, 1H), 7.56 (d, 1H), 7.18–7.31 (m, 5H), 7.07 (d, 1H), 4.97 (m, 1H), 4.73 (m, 1H), 4.35 (m, 1H), 3.30–3.60 (m, 2H), 2.82–3.10 (m, 2H), 1.10–2.25 (m, 24H); Conformer 2, 8.41 (d, 1H), 7.48 (br, 1H), 7.18–7.31 (m, 5H), 6.81 (d, 1H), 4.80 (m, 1H), 4.55 (m, 1H), 4.26 (m, 1H), 3.30–3.60 (m, 2H), 2.82–3.10 (m, 2H), 1.10–2.25 (m, 24H); CD (methanol) λ ($[\theta]_M$) 241 (−30,000), 227 (19,000), 210 (−28,000) nm; HR-FAB MS $[M+H]^+$ 556.3156 for $C_{29}H_{42}O_6N_5$ (calcd 556.3135).

4.1.4. Synthesis of *cyclo*(-L-Asu(NHOH)-Acc7-L-Phe-D-Pro-) (6). This compound was synthesized according to the procedure reported for **3** using Acc7 instead of Aib. HPLC, rt 17.4 min, ^{1}H NMR (500 MHz, DMSO-d_6) δ: Conformer 1, 7.85 (d, 1H), 7.72 (d, 1H), 7.16–7.31 (m, 5H), 6.95 (d, 1H), 4.96 (m, 1H), 4.73 (d, 1H), 4.33 (m, 1H), 3.40–3.52 (m, 2H), 3.03–3.25 (m, 2H), 2.80–2.94 (m, 2H), 2.18–2.46 (m, 2H), 1.26–1.98 (m, 22H); Conformer 2, 8.60–8.65 (m, 1H), 7.16–7.31 (m, 5H), 6.82 (d, 1H), 4.85 (br, 1H), 4.50 (m, 1H), 4.33 (m, 1H), 3.40–3.52 (m, 2H), 3.03–3.25 (m, 2H), 2.80–2.94 (m, 2H), 2.18–2.46 (m, 2H), 1.26–1.98 (m, 22H); HR-FABMS $[M+H]^+$ 570.3264 for $C_{30}H_{44}O_6N_5$ (calcd 570.3292).

4.1.5. Synthesis of *cyclo*(-L-Asu(NHOH)-Acc8-L-Phe-D-Pro-) (7). This compound was synthesized according to the procedure reported for **3** using Acc8 instead of Aib. HPLC, rt 18.4 min, ^{1}H NMR (500 MHz, DMSO-d_6) δ: Conformer 1, 7.67–7.92 (br, 1H), 7.13–7.28 (m, 6H), 6.84–6.93 (br, 1H), 4.92 (br, 1H), 4.81 (br, 1H), 4.24 (br, 1H), 3.34–3.49 (br, 2H), 2.98–3.12 (br, 2H), 2.69–2.85 (br, 2H), 2.21–2.41 (br, 4H), 1.21–1.90 (br, 22H); Conformer 2, 7.67–7.92 (br, 1H), 7.13–7.28 (m, 6H), 6.84–6.93 (br, 1H), 4.68 (br, 1H), 4.45 (br, 1H), 4.24 (br, 1H), 3.34–3.49 (br, 2H), 2.98–3.12 (br, 2H), 2.69–2.85 (br, 2H), 2.21–2.41 (br, 4H), 1.21–1.90 (br, 22H); HR-FABMS $[M+H]^+$ 584.3455 for $C_{31}H_{46}O_6N_5$ (calcd 584.3448).

4.1.6. Synthesis of *cyclo*(-L-Asu(NHOH)-A2in-L-Phe-D-Pro-) (8). This compound was synthesized according to the procedure reported for **3** using A2in instead of Aib. HPLC, rt 17.8 min, ^{1}H NMR (500 MHz, DMSO-d_6) δ: Conformer 1, 8.65 (br, 1H), 8.29 (br, 1H), 7.80 (d, 1H), 7.06–7.35 (m, 9H), 4.92 (m, 1H), 4.74 (d, 1H), 4.27 (m, 1H), 3.40–3.58 (m, 2H), 2.80–3.35 (m, 6H), 2.20 (m, 2H), 1.10–1.95 (m, 12H); Conformer 2, 8.75 (br, 1H), 8.65 (br, 1H), 7.06–7.35 (m, 10H), 4.98 (d, 1H), 4.58 (m, 1H), 4.08 (m, 1H), 3.40–3.58 (m, 2H), 2.80–3.35 (m, 6H), 2.20 (m, 2H), 1.10–1.95 (m, 12H); HR FAB-MS $[M+H]^+$ 590.2970 for $C_{32}H_{40}O_6N_5$ (calcd 590.2979).

4.1.7. Synthesis of *cyclo*(-L-Asu(NHOH)-L-Ala-L-Phe-D-Pro-) (9). This compound was synthesized according to the procedure reported for **3** using L-Ala instead of Aib. HPLC, rt 12.1 min, ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.34 (s, 1H), 8.67 (s, 1H), 8.16 (br, 1H), 7.90 (m, 1H), 7.72 (m, 1H), 7.19–7.38 (m, 5H), 5.05 (m, 1H), 4.62 (m, 1H), 4.10 (m, 1H), 3.81 (m, 1H), 2.60–3.20 (m, 4H), 0.96–2.18 (m, 17H); HR FAB-MS $[M+H]^+$ 502.2642 for $C_{25}H_{36}O_6N_5$ (calcd 502.2666).

4.1.8. Synthesis of *cyclo*(-L-Asu(NHOH)-D-Ala-L-Phe-D-Pro-) (10). This compound was synthesized according to the procedure reported for **3** using D-Ala instead of Aib. HPLC, rt 13.9 min, ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.31 (br, 1H), 8.98 (br, 1H), 8.53 (m, 1H), 7.15–7.32 (m, 7H), 4.92 (m, 1H), 4.68 (m, 1H), 4.41 (m, 1H), 4.21 (m, 1H), 3.67 (m, 1H), 3.30 (m, 1H), 3.02 (m, 1H), 2.88 (m, 1H), 1.07–2.18 (m, 17H); HR FAB-MS $[M+H]^+$ 502.2658 for $C_{25}H_{36}N_5O_6$ (calcd 502.2666).

4.1.9. Synthesis of *cyclo*(-L-Asu(NHOH)-L-A1in-L-Phe-D-Pro-) (11) and *cyclo*(-L-Asu(NHOH)-D-A1in-L-Phe-D-Pro-) (12). These compounds were synthesized using DL-A1in instead of Aib for **3** to yield Boc-L-Asu-(OBzl)-DL-A1in-L-Phe-D-Pro-O^{t}Bu. Then Boc-L-Asu-(OBzl)-DL-A1in-L-Phe-D-Pro-O^{t}Bu (889 mg and 1.00 mmol) was deprotected using TFA to yield H-L-Asu(OBzl)-DL-A1in-L-Phe-D-Pro-OH-TFA 717 mg (90%). It was cyclized according to the method described earlier and purified by column chromatography using a mixture of chloroform and methanol (9:1) to yield *cyclo*(-L-Asu(OBzl)-L-A1in-L-Phe-D-Pro-) (77 mg, 26%) (HPLC, rt 16.00 min) and *cyclo*(-L-Asu(OBzl)-D-A1in-L-Phe-D-Pro-) (225 mg, 75%) HPLC, rt 17.04 min. *cyclo*(-L-Asu(OBzl)-L-A1in-L-Phe-D-Pro-) (77 mg and 0.116 mmol) was deprotected by catalytic hydrogenation using Pd–C (50 mg) to yield *cyclo*(-L-Asu-L-A1in-L-Phe-D-Pro-) (63 mg, 94%). It was then coupled with hydroxylamine hydrochloride as reported earlier to yield *cyclo*(-L-Asu(NHOH)-L-A1in-L-Phe-D-Pro-) (**11**) (48 mg, 82%). HPLC, rt 16.0 min, ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.32 (s, 1H), 8.65 (s, 1H), 8.16 (d, 1H), 7.65 (br, 1H), 7.09–7.29 (m, 9H), 6.67 (d, 1H), 4.94 (m, 1H), 4.58–4.84 (m, 1H), 4.22–4.31 (m, 1H), 3.42 (m, 1H), 3.35 (m, 1H), 3.20 (m, 1H), 3.16 (m, 1H), 2.79–2.95 (m, 2H), 1.15–2.50 (m, 16H); HR FAB-MS $[M+H]^+$ 590.2957 for $C_{32}H_{40}O_6N_5$ (calcd 590.2979). *cyclo*(-L-Asu(OBzl)-D-A1in-L-Phe-D-Pro-) (225 mg, 0.339 mmol) was deprotected and then coupled with hydroxylamine hydrochloride as reported earlier to yield *cyclo*(-L-Asu(NHOH)-D-A1in-L-Phe-D-Pro) (**12**) (179 mg, 0.312 mmol). 129 mg (70%) HPLC, rt 17.0 min, ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.32 (s, 1H), 8.95 (d, 1H), 8.83 (br, 1H), 8.64 (br, 1H), 7.16–7.29

(m, 10H), 4.99 (d, 1H), 4.48 (m, 1H), 4.35 (m, 1H), 3.48 (br, 1H), 2.73–3.32 (m, 4H), 1.28–2.29 (m, 17H); HR FAB-MS $[M+H]^+$ 590.2978 for $C_{32}H_{40}O_6N_5$ (calcd 590.2979).

4.1.10. Synthesis of *cyclo*(-L-Asu(NHOH)-L-Pro-L-Phe-D-Pro-) (13). This compound was synthesized according to the procedure reported for **3** using L-Pro instead of Aib. HPLC, rt 15.4 min, ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.61 (br, 1H), 8.15 (br, 1H), 7.13–7.29 (m, 5H), 3.80–4.75 (m, 6H), 2.61–3.05 (m, 4H), 1.14–1.94 (m, 18H); CD (methanol) λ ($[\theta]_M$) 222 (25,000), 211 (17,000) nm; HR FAB-MS 528.2853 $[M+H]^+$ for $C_{27}H_{38}O_6N_5$ (calcd 528.2822).

4.1.11. Synthesis of *cyclo*(-L-Asu(NHOH)-Aib-L-Phe-D-Pip-) (14). This compound was synthesized as reported for compound **3** using DL-Pip instead of D-Pro. After cyclization reaction, the cyclic peptide containing D-Pip was separated using column chromatography. The benzyl protection of Asu was removed by catalytic hydrogenation as earlier and coupled with hydroxyl amine to yield *cyclo*(-L-Asu(NHOH)-Aib-L-Phe-D-Pip-) 125 mg (74%), ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.33 (s, 1H), 8.65 (br, 1H), 7.90 (d, 1H), 7.15–7.38 (m, 6H), 6.20 (d, 1H), 5.12 (m, 2H), 4.19 (m, 1H), 3.65 (m, 1H), 3.03 (m, 1H), 2.90 (m, 2H), 2.72 (m, 2H), 1.91–1.95 (m, 4H), 1.26–1.56 (m, 16H); HR FAB-MS $[M+H]^+$ 530.2960 for $C_{27}H_{40}O_6N_5$ (calcd 530.2979).

4.1.12. Synthesis of *cyclo*(-L-Asu(NHOH)-Acc5-L-Phe-D-Pip-) (15). This compound was synthesized according to the procedure reported for **3** using Acc5 instead of Aib. HPLC, rt 17.4 min, ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.82 (d, 1H), 7.66 (br, 1H), 7.17–7.24 (m, 5H), 6.18 (d, 1H), 5.12 (m, 2H), 4.22 (m, 1H), 3.70 (m, 1H), 2.88–3.05 (m, 4H), 2.60 (m, 1H), 2.33 (m, 2H), 1.27–1.94 (m, 20H); HR FAB-MS $[M+H]^+$ 556.3179 for $C_{29}H_{42}O_6N_5$ (calcd 556.3135).

4.1.13. Synthesis of *cyclo*(-L-Asu(NHOH)-Acc8-L-Phe-D-Pip-) (16). This compound was synthesized according to the procedure reported for **3** using Acc8 instead of Aib. HPLC, rt 20.0 min, ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.34 (br, 1H), 8.66 (br, 1H), 9.84 (d, 1H), 7.12–7.28 (m, 6H), 6.25 (d, 1H), 5.10–5.16 (m, 2H), 4.22 (m, 1H), 3.67 (m, 1H), 3.04 (m, 1H), 2.86 (m, 1H), 2.72 (m, 1H), 1.20–1.95 (m, 30H); HR FAB-MS $[M+H]^+$ 598.3627 for $C_{32}H_{48}O_6N_5$ (calcd 598.3605).

4.1.14. Synthesis of *cyclo*(-L-Asu(NHOH)-A2in-L-Phe-D-Pip-) (17). This compound was synthesized according to the procedure reported for **3** using A2in instead of Aib. HPLC, rt 19.0 min, ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.29 (s, 1H), 8.61 (s, 1H), 8.01 (d, 1H), 7.89 (br, 1H), 7.08–7.22 (m, 9H), 6.22 (m, 1H), 5.11 (m, 2H), 4.22 (m, 1H), 3.72–3.76 (br, 2H), 3.38 (m, 1H), 3.04–3.11 (m, 3H), 2.88–2.92 (m, 1H), 2.71–2.73 (m, 1H), 1.86–1.92 (m, 6H), 1.19–1.56 (m, 10H); HR FAB-MS $[M+H]^+$ 604.3126 for $C_{33}H_{42}O_6N_5$ (calcd 604.31351).

4.1.15. Synthesis of *cyclo*(-L-Asu(NHOH)-A2in-L-Ile-D-Pip-) (18). This compound was synthesized according to the procedure reported for **3** using A2in instead of Aib. HPLC, rt 17.9 min, ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.00 (br, 1H), 7.78 (d, 1H), 7.11–7.20 (m, 4H), 6.23 (d, 1H), 5.15 (m, 1H), 4.56 (m, 1H), 4.24 (m, 1H), 3.80–3.88 (m, 2H), 3.08 (m, 1H), 2.88 (m, 1H), 3.09 (m, 1H), 2.72–2.88 (m, 2H), 0.78–1.93 (m, 24H); HR FAB-MS $[M+H]^+$ 570.3309 for $C_{30}H_{44}O_6N_5$ (calcd 570.3292).

4.1.16. Synthesis of *cyclo*(-D-Asu(NHOH)-L-Pro-D-Phe-Aib-) (19). This compound was synthesized according to the procedure reported for **3**. HPLC, rt 15.2 min, ^{1}H NMR (500 MHz, DMSO-d_6) δ: Conformer 1, 7.95–8.07 (m, 1H), 7.56–7.58 (m, 1H), 7.12–7.32 (m, 6H), 4.58–4.74 (m, 3H), 3.41–3.57 (m, 2H), 2.73–3.08 (m, 4H), 1.16–2.23 (m, 18H); Conformer 2, 8.66–8.68 (m, 1H), 7.95–8.07 (m, 1H), 7.12–7.32 (m, 5H), 6.74 (br, 1H), 4.58–4.74 (m, 1H), 4.48 (m, 1H), 4.16 (m, 1H), 3.41–3.57 (m, 2H), 2.73–3.08 (m, 4H), 1.16–2.23 (m, 18H); CD (methanol) λ ($[\theta]_M$) 242 (59,000), 228 (−23,000), 221 (500), 213 (−13,000), 197 (−36,000) nm; HR FAB-MS $[M+H]^+$ 516.2850 for $C_{26}H_{38}O_6N_5$ (calcd 516.2822).

4.1.17. Synthesis of *cyclo*(-L-Asu(NHOH)-L-Pro-D-Phe-Aib-) (20). This compound was synthesized according to the procedure reported for **3**. HPLC, rt 13.1 min, ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.60 (br, 1H), 7.16–7.40 (br, 7H), 6.49 (br, 1H), 3.90–4.50 (br, 3H), 2.60–3.20 (br, 8H), 1.10–2.20 (br, 16H); HR FAB-MS $[M+H]^+$ 516.2827 for $C_{26}H_{38}O_6N_5$ (calcd 516.2822).

4.1.18. Synthesis of *cyclo*(-L-Asu(NHOH)-Aib-L-Phe-L-Pro-) (21). This compound was synthesized according to the procedure reported for **3**. HPLC, rt 13.2 min, ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.60 (br, 1H), 7.90–8.20 (br, 2H), 7.13–7.26 (br, 6H), 4.60–4.90 (br, 2H), 4.22 (br, 1H), 3.40 (br, 2H), 2.60–3.20 (br, 6H), 1.05–1.98 (br, 16H); CD (methanol) λ ($[\theta]_M$) 198 (−76,000) nm; HR FAB-MS $[M+H]^+$ 516.2831 for $C_{26}H_{38}O_6N_5$ (calcd 516.2822).

4.2. MHC class-I molecule up-regulation assay

The activity of cyclic tetrapeptides to induce the expression of MHC class-I molecules was determined by the method reported previously.[25] Briefly, 24 h after 5000 B16/BL6 cells had been introduced into each well of a 96-well microplate (200 μL), test samples were added. After an additional 72 h of incubation, the cell-surface expression of the MHC class-I molecules was measured by a cell ELISA method. The degree of MHC class-I up-regulating activity of the compounds was compared with respect to their concentrations for 2-fold up-regulation ($C_{\times 2}$) of the MHC class-I molecules.

4.3. Circular dichroism

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 cm^2 dmol^{-1}).

4.4. NMR spectroscopy and structure calculation

NMR spectra were recorded on a JEOL spectrometer operating at 500 MHz in DMSO-d_6 or $CDCl_3$ using TMS as internal standard. Spectra were recorded at variable temperatures such as, 298, 303, 313, 323, and 333 K, respectively. Assignments of proton resonances were confirmed, when possible, by COSY, HOHAHA, and NOESY experiments. Mixing times for NOESY experiments were 200 and 400 ms. Number of scans varied between 32 and 64, and the number of points in t_2 dimension was 512. ^{1}H resonances were assigned using standard procedures. $^3J_{NH-C^{\alpha}H}$ coupling constants for nonoverlapping signals were determined from 1D spectra with high digital resolution.

4.5. Molecular modeling studies

All calculations were performed on a Silicon Graphics computer. The distance geometry program was used to generate structures consistent with the distance constraints derived from the NOEs. Temperature coefficient of NH protons indicating hydrogen bonds and ϕ angles calculated from $J_{NH-H^{\alpha}}$ were used to filter out structures that did not meet the experimental data. An error of $\pm 30^{\circ}$ was tolerated for the ϕ angles calculated from $J_{NH-H^{\alpha}}$ at this stage of refinement. Energy minimization and molecular dynamics calculation were carried out using the CHARMm program of Insight II using CHARMm forcefield.

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