Importance of the backbone conformation of (–)-ternatin in its fat-accumulation inhibitory activity against 3T3-L1 adipocytes†

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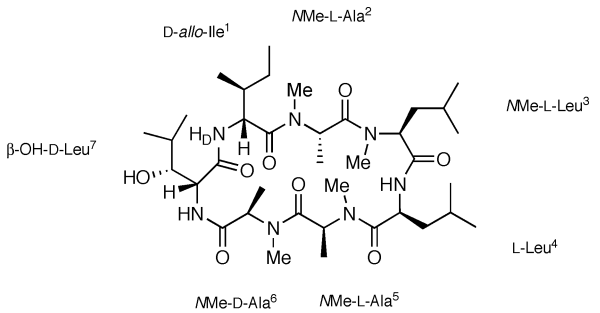
Received 24th October 2008, Accepted 19th November 2008
First published as an Advance Article on the web 7th January 2009
DOI: 10.1039/b818903j

Key relationships between the intramolecular H-bond-derived backbone conformation and the bioactivity of the novel fat-accumulation inhibitor (–)-ternatin are examined by analyses of the NMR spectroscopic data and CD spectra of designed analogues. The results reveal that the β-turn structure of (–)-ternatin is responsible for its potent fat-accumulation inhibitory effect against 3T3-L1 murine adipocytes.

Introduction

Intramolecular H-bonds are the most important chemical interactions that form active structures/foldings of biomolecules and provide critical functions. In particular, proteins are important representatives of such molecules and show wide structural diversity. Key components that determine their structures are their secondary structures, *e.g.*, helix, sheet, and turn structures, all of which are constructed by intramolecular H-bonds. Interestingly, these unique features are also found in naturally occurring cyclic peptides, such as cyclosporin A[1] and gramicidin S.[2] To date, considerable effort has been devoted to studying intramolecular H-bond-derived conformations, in peptides[3,4] as well as peptidomimetics.[5]



(–)-Ternatin (**1**)

Our research group recently isolated (–)-ternatin (**1**), a novel cyclic peptide with the primary structure cyclo [D-*allo*-Ile[1]-*N*Me-L-Ala[2]-*N*Me-L-Leu[3]-L-Leu[4]-*N*Me-L-Ala[5]-*N*Me-D-Ala[6]-(2*R*,3*R*)-3-hydroxy-Leu[7]], from the mushroom *Coriolus versicolor* as a novel fat-accumulation inhibitor against 3T3-L1 murine adipocytes.[6] Recently, we examined the *in vivo* biological activity of **1** in high-fat-fed mice,[6b] and studied its structure–activity relationships (SARs),[7] which enabled the installation of probe tags, *e. g.*, biotin and fluorescent units, in the correct positions.

Apart from our on-going bioorganic investigation to clarify the mode of action of **1**, we also focused on its unique physical and conformational properties. The X-ray crystal structure of **1** reported by Miller *et al.* has a type II β-turn structure in the region between the L-Leu[4] and the β-OH-D-Leu[7] [(2*R*,3*R*)-3-hydroxy-Leu[7]] moieties, which was formed by intramolecular H-bond B with the assistance of two additional H-bonds A and C in the solid state (Fig. 1).[8] Similarly, **1** also shows a single conformer in solution, which can be observed in the ^{1}H NMR spectrum of **1**. Our preliminary NMR-based study of **1** supported the existence of H-bonds A and B in solution by a H–D exchange experiment.[7a] In addition, H-bond C may be important for a single conformation of **1** in solution as well as for its potent bioactivity. For the unique *cis*-amide bond region, a ROESY correlation was observed between H_α(*N*Me-L-Ala[2]) and H_α(*N*Me-L-Leu[3]). Therefore, in solution, **1** should have the same conformation as that found in an X-ray crystal structure. However, a better understanding of the importance of each H-bond to both the conformation and fat-accumulation inhibitory activity of **1** is needed. In this paper, we describe the interrelationships between the intramolecular H-bonds, backbone conformation, and fat-accumulation inhibitory activity of **1**.

Results and discussion

Design and synthesis of analogues

In order to examine the importance of each H-bond, we designed three ternatin analogues, [*N*Me-L-Leu[4]]ternatin (**2**) which lacks the NH proton H_A, [*N*Me-β-OH-D-Leu[7]]ternatin (**3**) which lacks the NH proton H_B, and [D-Leu[7]]ternatin (**4**) which lacks the OH proton H_C (Fig. 2). Synthetic accesses to these analogues were achieved by using our synthetic route developed for the large-scale preparation of **1**, which is summarized in Scheme 1. The left fragment **6**, which was prepared from the unusual amino-acid ester β-OH-D-Leu-OEt (**5**), and the right fragment **8** which was prepared from L-Leu-OMe (**7**), are the common intermediates used for the solution-phase synthesis of **1** and its analogues.[6b,7]

The synthesis of **2** began with preparation of the modified right fragment **11**. Starting from *N*Me-L-Leu-OMe (**9**), sequential

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† Electronic supplementary information (ESI) available: 1D- and 2D-NMR spectra of analogues **1-4**; H/D exchange experiment; measurement of temperature coefficients of amide protons. See DOI: 10.1039/b818903j

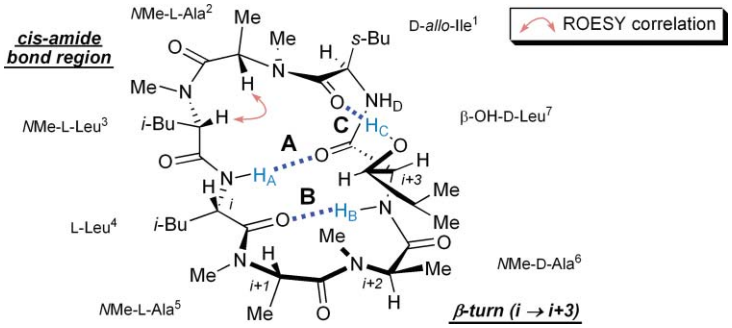


Fig. 1 Proposed stereostructure of (–)-ternatin (**1**) fixed by three intramolecular H-bonds A, B, and C.

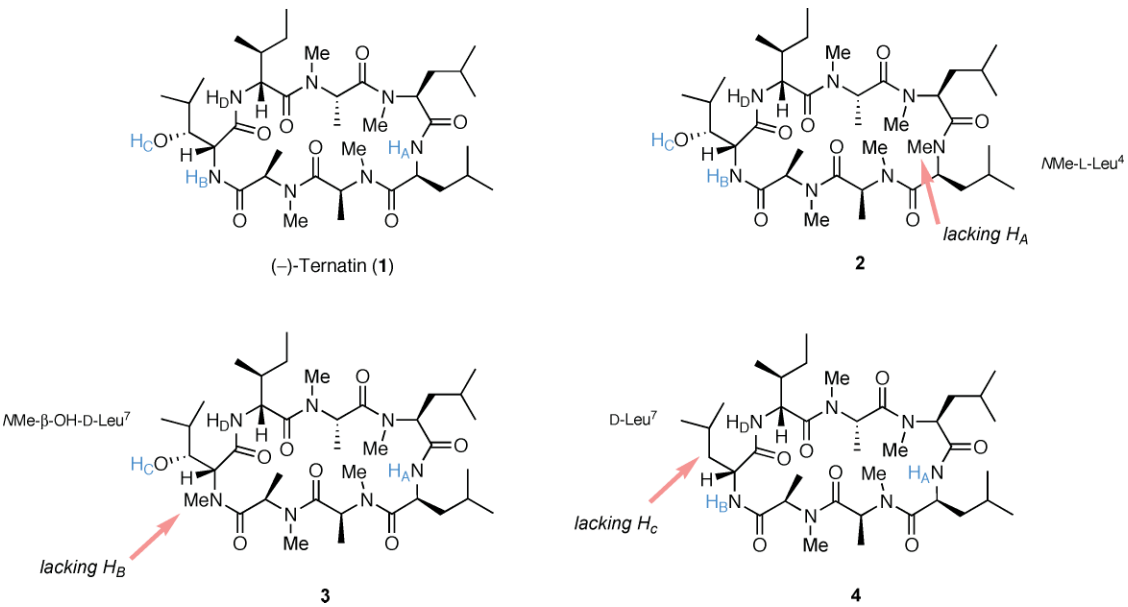


Fig. 2 Design of H-bond-lacking ternatin analogues.

couplings with Boc-amino acids and Boc deprotections were conducted from C- to N-terminus to provide tetrapeptide **10**. Removal of the Boc group of **10** with 50% TFA/CH_2Cl_2 gave **11**. Construction of the macrocyclic structure began with fragment coupling between the left fragment **6** and **11** to give heptapeptide **12** in moderate yield. Methyl ester hydrolysis followed by Boc deprotection of **12** provided the cyclic precursor. Finally, the key HATU/HOAt-mediated macrolactamization was performed at a low concentration (1.5 mM). After HPLC purification of the crude materials, analogue **2** was obtained in 10% yield. Analogue **3** was synthesized in the same manner. First, *N*-methylation of amine **5** was carried out in accordance with White's protocol.[9] The sequential reductive alkylation with benzaldhyde and formaldehyde was followed by the removal of the resulting benzyl group to afford *N*Me-β-OH-D-Leu-OEt (**13**). Next, **13** was coupled with Boc-amino acids to provide tripeptide **14**. **14** was then subjected to alkaline hydrolysis, which gave the modified left fragment **15**. Fragment coupling between **15** and the right fragment **8** provided heptapeptide **16**. Methyl ester hydrolysis and Boc deprotection followed by macrolactamization under dilute conditions afforded analogue **3** in 33% yield after HPLC purification. The synthesis of **4** was reported previously.[7a]

Conformational analysis

The key NMR parameters needed for the current study were obtained by detailed analyses of 1D- and 2D-NMR spectra (see ESI†). The measurement of ^{1}H NMR spectra in C_6D_6 revealed that **1** and **2** existed as single conformers, **3** as a mixture of conformers, and **4** as a 4:1 mixture of two conformers. The same tendency was observed in the measurements in DMSO-d_6. Based on the results, the significant loss of the intramolecular H-bond B, a key interaction for the β-turn structure, might be an important cause of the multiple conformations of **3**. Meanwhile, H-bond C is thought to contribute toward tightening the β-turn structure of **1**, since **4**, which lacks the hydroxy proton H_C, existed as a mixture of conformers (see the ^{1}H NMR spectra of **1** and **4** in the ESI†).

We then evaluated the H-bonding properties of **1** and its analogues except for **3** using NMR spectroscopy. First, the H–D exchange properties of the NH and OH protons were examined in C_6D_6 with the addition of D_2O[10] (Table 1, and see also the ESI†). These experiments demonstrated a common trend in compounds **1** and **4**, showing slow (or no) H–D exchanges of the two NH protons H_A and H_B and a rapid exchange of H_D. In addition, NH protons H_A and H_B in **1** and **4** gave rise to sharp signals at quite a low field (the chemical shift values: >7.5 ppm), which

Table 1 Key NMR parameters for substrates **1**, **2**, and **4** in C_6D_6[a]

Parameter	Substrate		
	1	**2**	**4**
NH_A (^3J Leu$^4_{\alpha,NH}$)	7.57 (8.0 Hz)	–	7.89 (8.0 Hz)
NH_A H–D exchange	S	–	M
NH_B (^3J β-OH-Leu$^7_{\alpha,NH}$)	7.92 (8.0 Hz)	6.80 (4.0 Hz)	7.98 (8.5 Hz)
NH_B H–D exchange	S	S	S
OH_C (^3J β-OH-Leu$^7_{\beta,OH}$)	5.87 (s)	3.23 (s)	–
OH_C H–D exchange	F	F	–
NH_D (^3J Ile$^1_{\alpha,NH}$)	6.22 (7.2 Hz)	6.64 (9.5 Hz)	6.92–6.88 (br d)
NH_D H–D exchange	W	M	F

[a] The H–D exchange experiment was conducted in C_6D_6 with the addition of D_2O (20 μL in C_6D_6 solution). The tendency of H–D exchange was classified as follows; 50% H–D exchange within 1 h (F; fast) 5 h (W; weak), and 24 h (M; medium), and no exchange within 24 h (S; strong).

were expected to be involved in intramolecular H-bonds. On the other hand, slow H–D exchanges of the two NH protons H_B and H_D were observed in compound **2**. Unfortunately, the hydroxy protons in **1** and **2** could not be examined due to their flexible nature toward H–D exchanges. Next, the temperature coefficients ($-\Delta\delta/\Delta T$) of NH protons were investigated in DMSO-d_6. The data

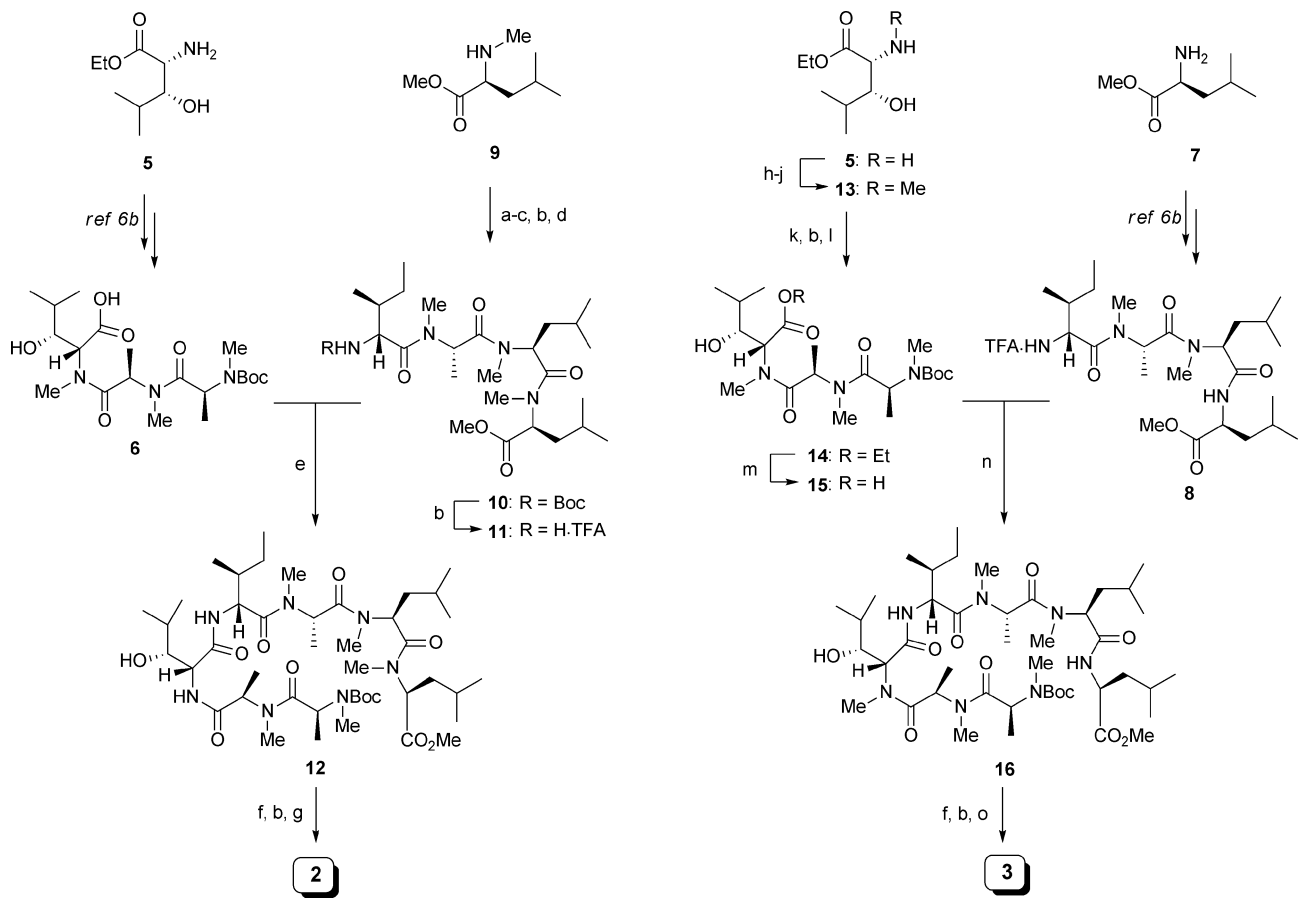
Table 2 Temperature shift coefficients ($-\Delta\delta/\Delta T$) of NH protons in DMSO-d_6 (ppb/K) for substrates **1**, **2**, and **4**[a]

Substrate	$-\Delta\delta/\Delta T$ (ppb/K)			
	NH_A	NH_B	OH_C	NH_D
1	2.0	0.1	2.0	4.6
2	–	3.2	3.0	0.8
4	1.1	0.8	–	5.0

[a] ^{1}H NMR spectra of substrates were taken in the range of 25–60 °C.

showed low values for NH protons H_A and H_B in **1** and **4**, and for NH protons H_B and H_D in **2**, which indicates the H-bonding or solvent-shielding of each NH proton (Table 2). Since the results are consistent with the H–D exchange profiles, the existence of H-bonds A and B in compounds **1** and **4** and their β-turn structures are strongly suggested. In compound **2**, however, it is possible that H-bonds may involve the NH protons H_B and H_D.

The conformations of compounds **1–4** in solution were then investigated by circular dichroism (CD) spectroscopy (Fig. 3).[11] Consistent with the former NMR studies, compounds **1** and **4** showed evidence of a type II β-turn structure with a maximum



Scheme 1 Synthesis of analogues **2** and **3**. *Reagents and Conditions*: (a) Boc-*N*Me-L-Leu-OH, HATU, DIPEA, CH_2Cl_2, DMF, 97%; (b) 50% TFA/CH_2Cl_2; (c) Boc-*N*Me-L-Ala-OH, HATU, DIPEA, CH_2Cl_2, DMF, 67% in 2 steps; (d) Boc-D-*allo*-Ile-OH, HATU, DIPEA, CH_2Cl_2, DMF, 100% in 2 steps; (e) **6** (1.0 eq.), HATU, DIPEA, CH_2Cl_2, DMF, 79% in 2 steps; (f) LiOH, THF, *t*-BuOH, H_2O; (g) HATU, HOAt, DIPEA, CH_2Cl_2 (1.5 mM), DMF, 10% in 3 steps; (h) PhCHO, MeOH; $NaBH_3CN$; (i) $(CH_2O)_n$, MeOH; $NaBH_3CN$; (j) H_2, $Pd(OH)_2/C$, 47% in 3 steps; (k) Boc-*N*Me-D-Ala-OH, HATU, DIPEA, CH_2Cl_2, DMF, 100%; (l) Boc-*N*Me-L-Ala-OH, HATU, DIPEA, CH_2Cl_2, DMF, 77%; (m) NaOH, 1,4-dioxane, H_2O; (n) **8** (1.0 eq.), HATU, DIPEA, CH_2Cl_2, DMF, 70% in 2 steps; (o) HATU, HOAt, DIPEA, CH_2Cl_2 (1.5 mM), DMF, 33% in 3 steps.

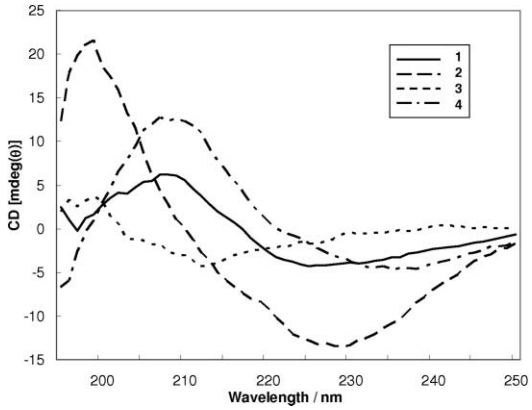


Fig. 3 CD spectra of compounds **1–4** in MeOH.

ellipticity at nearly 208 nm.[12] Hence, **2** gave a curve with a different shape (a maximum ellipticity at 199 nm and a negative band around 228 nm), which was recognized as a β-turn structure similar to type I[11a] and/or another conformation derived from H-bond B with an additional intramolecular H-bond that involves NH proton H_D. The low intensity and non-defined shape of the curve found for **3** may be attributed to an equilibrium of conformers.

In summary, these spectroscopic data suggested that compounds **1** and **4** form the β-turn structure in solution, while **2** forms another type of conformation and **3** exists as multiple conformers. The key interactions for this β-turn structure are both transannular H-bonds A and B, while H-bond C can stabilize its conformation.

Bioactivity

To understand the relationships between conformation and bioactivity, the fat-accumulation inhibitory activities of the analogues were evaluated with 3T3-L1 murine adipocytes (Table 3). The bioassay consisted of the treatment of confluent 3T3-L1 preadipocytes with each sample and insulin (an inducer of adipogenesis), and further incubation for 7 days. After this period, the control cells were differentiated into mature adipocytes. Both the rate of fat accumulation and the cell viability were calculated. Among the samples tested, no cytotoxicity was observed at the concentration that gave 50% fat accumulation inhibition (IC_{50}).

Based on the results, none of the samples showed more potent bioactivity than the natural compound **1** which showed an IC_{50} value for fat-accumulation inhibition of 0.027 μM. In particular, the bioactivities of analogues **2** and **3** were quite weak with IC_{50} values of 22 μM and 77 μM, respectively. The potencies of bioactivity were in the order **1** > **4** (1/8-fold less potent) > **2** (1/815-fold) > **3** (1/2850-fold), which were consistent with the trend in the β-turn structure observed in our conformational studies. Therefore, these results clearly indicate that the β-turn structure is absolutely responsible for potent bioactivity.

Table 3 Inhibitory effects on fat accumulation of compounds **1–4** against 3T3-L1 murine adipocytes and cell viability[a]

Compound	Fat accumulation-inhibitory effect: IC_{50} (μM)	Relative potency	Cell viability[b]: IC_{50} (μM)
(–)-Ternatin (**1**)	0.027 ± 0.003	1	0.28 ± 0.03
2	22 ± 3	1/815	> 130
3	77 ± 5	1/2850	> 130
4	0.22 ± 0.02	1/8	3.3 ± 0.2

[a] Values are the means of quadruplicate determinations. [b] Cell viability was calculated independently to exclude undesired fat-accumulation inhibition arising from the toxicity of the tested compounds. At the respective IC_{50} values, no cell toxicities were observed for any of the compounds.

Conclusions

In conclusion, we evaluated (–)-ternatin (**1**) and its analogues **2–4** which were designed to understand the interrelationships between intramolecular H-bonds, conformation, and bioactivity. This study clarified that intramolecular H-bonds A and B directly contribute to the β-turn conformation of **1** as well as to its potent bioactivity (fat-accumulation inhibition against 3T3-L1 adipocytes). Meanwhile, H-bond C is also a key interaction that is responsible for tightening the bioactive conformation of **1**. Further studies on this bioactive molecule are underway.

Experimental section

Materials and methods

Reagents and solvents were purchased from commercial sources. All reactions were performed under a nitrogen atmosphere unless otherwise noted. Column chromatography was performed with Fuji Silysia silica gel FL-60D (Aichi, Japan). High-pressure liquid chromatography (HPLC) was performed with Develosil ODS HG-5 reversed-phase column (Nomura Chemical Co. Ltd., Aichi, Japan). Optical rotations were measured with a JASCO DIP-1000 polarimeter. IR spectra were recorded on a JASCO FT/IR-230 spectrometer. CD measurements were obtained on a JASCO J-720 spectrometer. The ^1H, ^{13}C and 2D NMR spectra were recorded on JEOL JNM-A400 and JNM-A600. The chemical shifts are referenced to the solvent peaks δ_H 7.26 and δ_C 77.0 for chloroform-*d* ($CDCl_3$), δ_H 3.30 and δ_C 49.0 for methanol-d_4 (CD_3OD), δ_H 7.16 and δ_C 128.0 for benzene-d_6 (C_6D_6), and δ_H 2.49 and δ_C 39.5 for dimethylsulfoxide-d_6 (DMSO-d_6). Mass spectra were determined on a JEOL JMS-700 spectrometer operating in the positive FAB mode (*m*-nitrobenzyl alcohol as a matrix).

Boc-D-*allo*-Ile-*N*Me-L-Ala-*N*Me-L-Leu-*N*Me-L-Leu-OMe (10). To a stirred solution of *N*Me-L-Leu-OMe (**9**) (216 mg, 1.35 mmol) in dry CH_2Cl_2 (3 mL) and DMF (0.6 mL) was added Boc-*N*Me-L-Leu-OH (332 mg, 1.35 mmol) and HATU (567 mg, 1.49 mmol). The solution was cooled to 0 °C and DIPEA (0.71 mL, 4.05 mmol) was then added dropwise. The solution was warmed to room temperature and allowed to stir for 2 h. The reaction was quenched with 0.1 M HCl aq. and extracted with EtOAc (5 mL × 2). The extracts were washed with saturated aqueous $NaHCO_3$ (5 mL), water (5 mL), and brine (5 mL × 2), dried with Na_2SO_4, and then concentrated in vacuo. The resulting oil was purified by column chromatography on silica-gel (hexane/ether, 9/1→4/1→) to give Boc-*N*Me-L-Leu-*N*Me-L-Leu-OMe (508 mg, 97%) as a colorless oil. ^1H NMR (400 MHz, $CDCl_3$, a major rotamer) δ 5.28–5.18 (m, 1 H), 4.88 (dd, J = 8.6, 6.1 Hz, 1 H), 3.66 (s, 3 H), 2.94 (s, 3 H), 2.71 (s, 3 H), 1.72–1.56 (m, 6 H), 1.45 (s, 9 H), 1.01–0.84 (m, 12 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.8, 171.3, 155.3, 79.4, 56.8, 56.3, 54.2, 52.0, 51.6, 37.9, 37.2, 30.7, 29.0, 27.8 (3 C), 22.5 (2 C), 21.8 (2 C); HRMS (FAB) calcd for $C_{20}H_{39}N_2O_5$ $(M + H)^+$ 387.2859, found 387.2875.

To a stirred solution of Boc-*N*Me-L-Leu-*N*Me-L-Leu-OMe (213 mg, 0.55 mmol) in dry CH_2Cl_2 (1 mL) cooled to 0 °C was added TFA (1 mL). After s.m. consumption was indicated by TLC, the solution was concentrated in vacuo. The resulting oil was lyophilized three times with toluene to afford amine as a colorless paste. To a stirred solution of amine in dry CH_2Cl_2 (0.4 mL) and DMF (0.15 mL) was added Boc-*N*Me-L-Ala-OH (112 mg, 0.55 mmol) and HATU (231 mg, 0.61 mmol). After the solution was cooled to 0 °C, DIPEA (290 μL, 1.66 mmol) was added dropwise. The solution was warmed to room temperature and allowed to stir for 2 h. The reaction was quenched with 0.1 M HCl aq., and extracted with EtOAc (5 mL × 2). The extracts were washed with saturated aqueous $NaHCO_3$ (5 mL), water (5 mL), and brine (5 mL × 2), dried with Na_2SO_4, and then concentrated in vacuo. The resulting oil was purified by column chromatography on silica-gel (hexane/ether, 9/1→4/1→1/1→) to give Boc-*N*Me-L-Ala-*N*Me-L-Leu-*N*Me-L-Leu-OMe (174 mg, 67%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$, a major rotamer) δ 5.52–5.42 (m, 1 H), 5.15 (dd, J = 10.0, 5.8 Hz, 1 H), 4.73–4.64 (m, 1 H), 3.63 (s, 3 H), 2.88 (s, 3 H), 2.75 (s, 3 H), 2.71 (s, 3 H), 1.74–1.29 (m, 6 H), 1.40 (s, 9 H), 1.21 (d, J = 6.8 Hz, 3 H), 0.92–0.81 (m, 12 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.4, 171.2, 170.3, 155.0, 79.7, 56.8, 54.3, 51.7, 50.2, 37.9, 37.7 (2 C), 37.2, 36.7, 30.8, 29.2, 27.9 (3 C), 24.5 (2 C), 24.3 (2 C), 22.8; HRMS (FAB) calcd for $C_{24}H_{46}N_3O_6$ $(M + H)^+$ 472.3387, found 472.3386.

To a stirred solution of Boc-*N*Me-L-Ala-*N*Me-L-Leu-*N*Me-L-Leu-OMe (38 mg, 81 μmol) in dry CH_2Cl_2 (0.6 mL) cooled to 0 °C was added TFA (0.6 mL). After s.m. consumption was indicated by TLC, the solution was concentrated in vacuo. The resulting oil was lyophilized three times with toluene to afford amine as a colorless paste. To a stirred solution of amine in dry CH_2Cl_2 (0.2 mL) and DMF (0.1 mL) was added Boc-D-*allo*-Ile-OH·1/2H_2O (20 mg, 81 μmol; after co-evaporation with toluene (3 mL × 3)) and HATU (34 mg, 89 μmol). After the solution was cooled to 0 °C, DIPEA (42 μL, 0.24 mmol) was dropwise added. The solution was warmed to room temperature and allowed to stir for 2 h. The reaction was quenched with 0.1 M HCl aq., and extracted with EtOAc (5 mL × 2). The extracts were washed with saturated aqueous $NaHCO_3$ (5 mL), water (5 mL), and brine (5 mL × 2), dried with Na_2SO_4, and then concentrated in vacuo. The resulting oil was purified by column chromatography on silica-gel (hexane/ether, 8/1→4/1→1/1→) to give **10** (41.3 mg, 83%) as a colorless oil. **10**: $[\alpha]_D^{24}$ −94.4 (c = 1.0, $CHCl_3$); IR ($CHCl_3$) 3424, 2959, 2363, 1735, 1702, 1630, 1499 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, a major rotamer) δ 5.56–5.44 (m, 2 H), 5.23–5.17 (m, 1 H), 5.15 (d, J = 9.3 Hz, 1 H) 4.50 (dd, J = 9.3, 5.8 Hz, 1 H), 3.68 (s, 3 H), 2.99 (s, 3 H), 2.93 (s, 3 H), 2.87 (s, 3 H), 1.84–1.33 (m, 9 H), 1.40 (s, 9 H), 1.27–1.21 (m, 3 H), 0.98–0.81 (m, 18 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.1, 171.9, 170.9, 169.8, 156.0, 79.6, 54.8, 53.8, 52.1, 51.5, 49.7, 37.7, 37.4, 37.1, 31.2, 30.2, 29.6, 28.3 (3 C), 26.3, 25.0, 24.8, 23.2, 23.0, 22.0, 21.3, 14.7, 14.2, 11.8; HRMS (FAB) calcd for $C_{30}H_{57}N_4O_7$ $(M + H)^+$ 585.4227, found 585.4253.

Boc-*N*Me-L-Ala-*N*Me-D-Ala-β-OH-D-Leu-D-*allo*-Ile-*N*Me-L-Ala-*N*Me-L-Leu-*N*Me-L-Leu-OMe (12). To a stirred solution of **10** (49 mg, 80 μmol) in dry CH_2Cl_2 (1 mL) cooled to 0 °C was added TFA (1 mL). After s.m. consumption was indicated by TLC, the solution was concentrated in vacuo. The resulting oil was lyophilized three times with toluene to afford amine **11** as a colorless paste. **11** was dissolved in dry CH_2Cl_2 (0.19 mL) and DMF (40 μL), and carboxylic acid **6** (33 mg, 80 μmol) and HATU (33 mg, 88 μmol) were added. After the solution was cooled to 0 °C, DIPEA (42 μl, 0.24 mmol) was then added dropwise. The solution was warmed to room temperature and allowed to stir for 2 h. The reaction was quenched with 0.1 M HCl aq. and extracted with EtOAc (5 mL × 2). The extracts were washed with saturated aqueous $NaHCO_3$ (5 mL), water (5 mL), and brine (5 mL × 2), dried with Na_2SO_4, and then concentrated in vacuo. The resulting oil was purified by column chromatography on silica-gel (hexane/ether 1/2→0/1→ ether/EtOAc 1/1) to give **12** (56 mg, 79%) as a colorless paste. **12**: $[\alpha]_D^{24}$ −51.7 (c = 2.0, $CHCl_3$); IR ($CHCl_3$) 3410, 3018, 2959, 2926, 2318, 1637, 1460, 1395 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$, a major rotamer) δ 6.89 (d, J = 8.0 Hz, 1 H), 6.77 (d, J = 8.4 Hz, 1 H), 5.44 (dd, J = 8.5, 6.6 Hz, 1 H), 5.15 (dd, J = 10.3, 5.1 Hz, 1 H) 5.06 (q, J = 5.7 Hz, 1 H), 4.95 (q, J = 6.2 Hz, 1 H), 4.73–4.64 (m, 2 H), 4.37–4.30 (m, 1 H), 3.63 (s, 3 H), 3.21 (m, 1 H), 2.88 (s, 6 H), 2.73 (s, 9 H), 2.69–2.65 (s, 1 H) 1.76–1.09 (m, 19 H), 1.39 (s, 9 H), 0.94–0.78 (m, 24 H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.1, 171.8, 171.7, 171.4, 171.1, 170.9, 169.6, 154.1, 80.3, 57.3, 54.7, 54.2, 52.3, 52.1 (2 C), 51.5, 50.7, 50.1, 38.6 (3 C), 37.7, 37.1, 31.2, 30.1, 29.6, 28.3 (3 C), 26.5, 24.9, 24.7, 23.2 (2 C), 22.8, 22.1, 21.6, 21.3, 19.6, 14.6, 14.1 (2 C), 11.7 (2 C); HRMS (FAB) calcd for $C_{44}H_{81}N_7O_{11}Na$ $(M + Na)^+$ 906.5892, found 906.5870.

cyclo[D-*allo*-Ile-*N*Me-L-Ala-*N*Me-L-Leu-*N*Me-L-Leu-*N*Me-L-Ala-*N*Me-D-Ala-β-OH-D-Leu] (2). To a stirred solution of **12** (53 mg, 60 μmol) in *t*-BuOH (1.0 mL), THF (0.25 mL), and H_2O (0.25 mL) was added LiOH·H_2O (13 mg, 0.30 mmol). The solution was stirred at r.t. until TLC indicated s.m. consumption. The reaction was quenched with 0.1 M HCl aq. and extracted with EtOAc (5 mL × 2). The extracts were dried with Na_2SO_4 and concentrated in vacuo. The resulting residue was dissolved in dry CH_2Cl_2 (0.3 mL) and then cooled to 0 °C. To this mixture was added TFA (0.3 mL). After s.m. consumption was indicated by TLC, the solution was concentrated in vacuo. The resulting oil was lyophilized three times with toluene to afford cyclic precursor. To a stirred solution of this compound in dry CH_2Cl_2 (39 mL) and DMF (1.3 mL) was added a solution of HATU (45 mg, 0.12 mmol) and HOAt (16 mg, 0.12 mmol). Afer the solution was cooled to 0 °C, DIPEA (47 μL, 0.27 mmol) was added. The reaction mixture was stirred for 2 d at room temperature. The reaction mixture was quenched with 0.1 M HCl aq. (10 mL) and diluted with EtOAc (100 mL). The extracts were washed with saturated aqueous $NaHCO_3$ (30 mL) and brine (30 mL × 2), dried with Na_2SO_4, and then concentrated in vacuo. The resulting paste was purified by column chromatography on silica-gel ($CHCl_3$/MeOH, 1/0→39/1→) to give crude product. Further purification was conducted by HPLC using Develosil ODS HG-5 column (ϕ 20 × 250 mm) eluting with 55% CH_3CN aq., at a flow rate of 5 mL/min monitoring at 215 nm, to give pure **2** as an amorphous solid (4.6 mg, 10%, t_R = 42.0 min). **2**: $[\alpha]_D^{22}$ −15.6 (c = 0.41, $CHCl_3$); IR ($CHCl_3$) 2953, 2919, 2854, 2363, 2337, 1623 cm^{-1}; ^{1}H NMR (600 MHz, C_6D_6, a single conformer) δ 6.80 (d, J = 4.0 Hz, 1 H), 6.64 (d, J = 9.5 Hz, 1 H), 6.08 (t, J = 7.3 Hz, 1 H), 5.65 (q, J = 6.5 Hz, 1 H), 5.58 (dd, J = 7.7, 5.9 Hz, 1 H), 5.44 (q, J = 6.5 Hz, 1 H), 5.32 (q, J = 7.7 Hz, 1 H), 4.92 (dd, J = 9.5, 5.5 Hz, 1 H), 4.38 (dd, J = 4.7, 3.3 Hz, 1 H), 3.32 (s, 3 H), 3.23 (s, 1 H),

3.16–3.13 (m, 1 H), 2.89 (s, 3 H), 2.794 (s, 3 H), 2.792 (s, 3 H), 2.47 (s, 3 H), 2.30–2.14 (m, 2 H), 1.99–1.93 (m, 1 H), 1.85–1.72 (m, 3 H), 1.63–0.70 (m, 4 H), 1.58 (d, $J = 7.3$ Hz, 3 H), 1.29 (d, $J = 6.6$ Hz, 3 H), 1.23 (d, $J = 6.5$ Hz, 3 H), 1.04 (d, $J = 6.5$ Hz, 3 H), 0.99 (d, $J = 6.6$ Hz, 3 H), 0.97 (d, $J = 6.6$ Hz, 3 H), 0.92 (d, $J = 6.6$ Hz, 3 H), 0.91 (d, $J = 6.6$ Hz, 6 H), 0.84 (d, $J = 6.5$ Hz, 3 H), 0.81 (t, $J = 7.3$ Hz, 3 H); ^{13}C NMR (150 MHz, C_6D_6) δ 172.4, 172.1, 170.1 (2 C), 169.9, 169.6, 169.1, 76.7, 58.8, 55.1, 52.9, 51.5, 51.3, 50.6, 49.9, 38.8, 38.6, 37.7, 32.3, 32.0, 30.6, 30.1, 29.5, 29.3, 26.7, 25.5, 25.4, 23.3, 23.0, 22.9, 22.6, 19.64, 19.56, 15.2, 14.8, 14.7, 14.3, 12.0; HRMS (FAB) calcd for $C_{38}H_{70}N_7O_8$ $(M + H)^+$ 752.5286, found 752.5280.

***N*Me-β-OH-D-Leu-OEt (13).** To a stirred solution of amine **5** (657 mg, 3.75 mmol) in dry MeOH (37 mL) was added benzaldehyde (400 µl, 3.93 mmol). After the mixture was stirred for 2 h, $NaBH_3CN$ (247 mg, 3.93 mmol) was added and then stirred was continued for an additional 18 h to afford the secondary amine in situ. To this solution, paraformaldehyde (118 mg, 3.93 mmol) was added. After the mixture was stirred for 6 h, $NaBH_3CN$ (247 mg, 3.93 mmol) was added and then stirred for an additional 18 h to afford tertiary amine. After the solvent was removed in vacuo, the resulting residue was dissolved in EtOAc (25 mL). The organic layer was washed with 0.1 M HCl aq. (10 mL), saturated aqueous $NaHCO_3$ (10 mL), and brine (10 mL × 2), dried with Na_2SO_4, and then concentrated in vacuo. The resulting oil was purified by column chromatography on silica-gel (hexane/ether, 9/1→5/1→) to give tertiary amine (275 mg, 48%) as a colorless oil. To a stirred solution of tertiary amine (167 mg, 0.60 mmol) in MeOH (6.0 mL) was added 20% $Pd(OH)_2/C$ (50 mg). The reaction flask was purged with H_2 gas and stirred for 2.5 h at room temperature. The reaction mixture was then filtered on Celite, and the filtrate was concentrated *in vacuo* to give **13** (110 mg, 97%) as colorless oil. **13**: $[\alpha]_D^{23}$ −2.6 ($c = 1.5$, $CHCl_3$); IR ($CHCl_3$) 3339, 2972, 2684, 2357, 1748, 1480 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 4.30 (q, $J = 7.1$ Hz, 2 H), 4.05 (d, $J = 2.9$ Hz, 1 H), 3.53 (dd, $J = 9.3$, 2.2 Hz, 1 H), 2.68 (s, 3 H), 1.92–1.80 (m, 1 H), 1.33 (t, $J = 7.1$ Hz, 3 H), 1.03 (d, $J = 6.6$ Hz, 3 H), 1.02 (d, $J = 6.3$ Hz, 3 H); ^{13}C NMR (100 MHz, CD_3OD) δ 168.3, 76.6, 65.1, 63.4, 32.6, 32.4, 19.8, 19.3, 14.4; HRMS (FAB) calcd for $C_9H_{19}NO_3Na$ $(M + Na)^+$ 212.1263, found 212.1243.

Boc-*N*Me-L-Ala-*N*Me-D-Ala-*N*Me-β-OH-D-Leu-OEt (14). To a stirred solution of **13** (110 mg, 0.58 mmol) in dry CH_2Cl_2 (1 mL) and DMF (0.1 mL) was added Boc-*N*Me-D-Ala-OH (118 mg, 0.58 mmol) and HATU (243 mg, 0.64 mmol). After the solution was cooled to 0 °C, DIPEA (304 µL, 1.74 mmol) was added dropwise. The solution was warmed to room temperature and allowed to stir for 2 h. The reaction was quenched with 0.1 M HCl aq., and extracted with EtOAc (5 mL × 2). The extracts were washed with saturated aqueous $NaHCO_3$ (5 mL), water (5 mL), and brine (5 mL × 2), dried with Na_2SO_4, and then concentrated in vacuo. The resulting oil was purified by column chromatography on silica-gel (hexane/ether, 2/1→1/1→) to give Boc-*N*Me-D-Ala-*N*Me-β-OH-D-Leu-OEt (219 mg, 100%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$, a major rotamer) δ 5.08 (q, $J = 6.8$ Hz, 1 H), 4.71 (d, $J = 7.6$ Hz, 1 H), 4.20 (q, $J = 7.2$ Hz, 2 H), 3.95–3.86 (m, 1 H), 3.01 (s, 3 H), 2.75 (s, 3 H), 2.63 (d, $J = 4.8$ Hz, 1 H), 1.74–1.68 (s, 1 H), 1.46 (s, 9 H), 1.27 (d, $J = 7.2$ Hz, 3 H), 1.26 (t, $J = 7.6$ Hz, 3 H), 1.02 (d, $J = 6.8$ Hz, 3 H), 0.90 (d, $J = 6.8$ Hz, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.2, 171.8, 155.4, 80.2, 74.3, 61.5, 61.1, 50.5, 38.6, 34.0, 29.6, 28.3 (3 C), 20.1, 15.7, 14.4, 14.1; HRMS (FAB) calcd for $C_{18}H_{35}N_2O_6$ $(M + H)^+$ 375.2495, found 375.2511.

To a stirred solution of Boc-*N*Me-D-Ala-*N*Me-β-OH-D-Leu-OEt (173 mg, 0.46 mmol) in dry CH_2Cl_2 (1 mL) cooled to 0 °C was added TFA (1 mL). After s.m. consumption was indicated by TLC, the solution was concentrated in vacuo. The resulting oil was lyophilized three times with toluene to afford amine as a colorless paste. To a stirred solution of amine in dry CH_2Cl_2 (1 mL) and DMF (0.1 mL) was added Boc-*N*Me-L-Ala-OH (94 mg, 0.46 mmol) and HATU (193 mg, 0.51 mmol). After the solution was cooled to 0 °C, DIPEA (241 µL, 1.39 mmol) was added dropwise. The solution was warmed to room temperature and allowed to stir for 2 h. The reaction was quenched with 0.1 M HCl aq., and extracted with EtOAc (5 mL × 2). The extracts were washed with saturated aqueous $NaHCO_3$ (5 mL), water (5 mL), and brine (5 mL × 2), dried with Na_2SO_4, and then concentrated in vacuo. The resulting oil was purified by column chromatography on silica-gel (hexane/ether, 2/1→1/1→0/1) to give **14** (164 mg, 77%) as a colorless oil. **14**: $[\alpha]_D^{24}$ +28.7 ($c = 2.6$, $CHCl_3$); IR ($CHCl_3$) 3496, 3011, 2972, 2350, 1649, 1473, 1395 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, a major rotamer) δ 5.43 (q, $J = 7.2$ Hz, 1 H), 5.04 (q, $J = 6.8$ Hz, 1 H), 4.74 (d, $J = 8.4$ Hz, 1 H), 4.19 (q, $J = 7.2$ Hz, 2 H), 3.96–3.90 (m,1 H), 3.35–3.20 (br s, 1 H), 2.96 (s, 3 H), 2.95 (s, 3 H), 2.73 (s, 3 H), 1.73–1.62 (m, 1 H), 1.43 (s, 9 H), 1.28 (d, $J = 7.2$ Hz, 3 H), 1.27 (d, $J = 6.8$ Hz, 3 H), 1.22 (t, $J = 6.8$ Hz, 3 H), 1.01 (d, $J = 6.8$ Hz, 3 H) 0.87 (d, $J = 6.8$ Hz, 3 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.9, 171.8, 171.7, 155.5, 128.9, 80.1, 73.7, 61.5, 60.6, 50.5, 49.2, 38.6, 34.0, 29.8, 28.3 (3 C), 20.2, 15.3, 14.4, 14.3, 14.1; HRMS (FAB) calcd for $C_{22}H_{42}N_3O_7$ $(M + H)^+$460.3023, found 460.3012.

Boc-*N*Me-L-Ala-*N*Me-D-Ala-*N*Me-β-OH-D-Leu-D-*allo*-Ile-*N*Me-L-Ala-*N*Me-L-Leu-L-Leu-OMe (16). To a stirred solution of **14** (52 mg, 0.11 mmol) in 1,4-dioxane (0.5 mL) cooled to 0 °C was added 1.0 M NaOH aq. (0.5 mL). The solution was stirred at the same temperature for 2 h (until TLC indicated s.m. consumption). The reaction was quenched with 0.1 M HCl aq. and extracted with EtOAc (5 mL × 2). The extracts were dried with Na_2SO_4 and concentrated *in vacuo* to afford **15** as a colorless oil.

To a stirred solution of **15** and **8** (0.11 mmol) in dry CH_2Cl_2 (1 mL) and DMF (0.1 mL) was added HATU (48 mg, 0.13 mmol). After the solution was cooled to 0 °C, DIPEA (60 µL, 0.34 mmol) was added dropwise. The solution was warmed to room temperature and allowed to stir for 2 h. The reaction was quenched with 0.1 M HCl aq. and extracted with EtOAc (5 mL × 2). The extracts were washed with saturated aqueous $NaHCO_3$ (5 mL), water (5 mL), and brine (5 mL × 2), dried with Na_2SO_4, and then concentrated in vacuo. The resulting oil was purified by column chromatography on silica-gel (hexane/ether, 1/2→0/1) to give **16** (71 mg, 70%) as a colorless paste. 16: $[\alpha]_D^{23}$ −48.2 ($c = 1.5$, $CHCl_3$); IR ($CHCl_3$) 3365, 2959, 2881, 2344, 1682, 1644, 1512, 1466 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$, a major rotamer) δ 6.90 (d, $J = 8.4$ Hz, 1 H), 6.46 (d, $J = 8.5$ Hz, 1 H), 5.47 (q, $J = 6.6$ Hz, 1 H), 5.44 (d, $J = 6.5$ Hz, 1 H), 5.10–4.89 (m, 4 H), 4.78 (dd, $J = 8.4$, 5.1 Hz, 1 H), 4.54–4.46 (m, 1 H), 3.92–3.85 (m, 1 H), 3.71(s, 3 H), 2.97, (s, 3 H), 2.95 (s, 3 H), 2.93 (s, 3 H), 2.82 (s, 3 H), 2.75

(s, 3 H), 1.76–0.83 (m, 18 H), 1.44 (s, 9 H), 1.27 (d, $J = 6.6$ Hz, 3 H), 1.26 (d, $J = 6.6$ Hz, 3 H), 0.95 (d, $J = 6.6$ Hz, 3 H), 0.90 (d, $J = 6.2$ Hz, 3 H), 0.88 (d, $J = 6.6$ Hz, 3 H), 0.86 (d, $J = 7.7$ Hz, 3 H), 0.82 (d, $J = 6.2$ Hz, 3 H), 0.80 (d, $J = 7.0$ Hz, 3 H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 173.0, 172.4, 171.3, 170.6, 170.1, 168.9, 168.7, 154.9, 80.1, 58.0, 55.1, 52.5, 52.2, 50.6, 50.4, 50.3, 49.4, 41.1, 38.7, 36.8, 36.7, 36.1, 29.8, 29.1, 28.3 (3 C), 26.8, 26.7, 25.0, 24.7, 23.1, 22.8, 21.8, 21.7, 21.5, 20.1, 15.1, 14.7, 14.5, 14.4, 14.2, 11.7, 11.6; HRMS (FAB) calcd for $C_{44}H_{81}N_7O_{11}Na$ $(M + Na)^+$ 906.5892, found 906.5885.

cyclo[D-*allo*-Ile-*N*Me-L-Ala-*N*Me-L-Leu-L-Leu-*N*Me-L-Ala-*N*Me-D-Ala-*N*Me-β-OH-D-Leu] (3)

To a stirred solution of **16** (53 mg, 60 μmol) in *t*-BuOH (1 mL), THF (0.25 mL), and H_2O (0.25 mL) was added $LiOH·H_2O$ (13 mg, 0.30 mmol). The solution was stirred at r.t. until TLC indicated s.m. consumption. The reaction was quenched with 0.1 M HCl aq. and extracted with EtOAc (5 mL × 2). The extracts were dried with Na_2SO_4 and concentrated in vacuo. The resulting residue was dissolved in dry CH_2Cl_2 (0.5 mL) and then cooled to 0 °C. To this mixture was added TFA (0.5 mL). After s.m. consumption was indicated by TLC, the solution was concentrated in vacuo. The resulting oil was lyophilized three times with toluene to afford cyclic precursor. To a stirred solution of this compound in dry CH_2Cl_2 (40 mL) and DMF (0.5 mL) was added a solution of HATU (46 mg, 0.12 mmol) and HOAt (16 mg, 0.12 mmol). Afer the solution was cooled to 0 °C, DIPEA (47 μL, 0.27 mmol) was added. The reaction mixture was stirred for 2 d at room temperature. The reaction mixture was quenched with 0.1 M HCl aq. (10 mL) and diluted with EtOAc (100 mL). The extracts were washed with saturated aqueous $NaHCO_3$ (30 mL) and brine (30 mL × 2), dried with Na_2SO_4, and then concentrated in vacuo. The resulting paste was purified by column chromatography on silica-gel ($CHCl_3$/MeOH, 1/0→39/1→) to give crude product. Further purification was conducted by HPLC using Develosil ODS HG-5 column (ϕ 20 × 250 mm) eluting with 55% CH_3CN aq., at a flow rate of 5 mL/min monitoring at 215 nm, to give pure **3** (16 mg, 33%, $t_R = 49.9$ min) as an amorphous solid. **3** exists as the mixture of conformers in the ^{1}H NMR spectrum as shown in the ESI†; IR ($CHCl_3$) 3292, 2959, 2363, 1617, 1466 cm^{-1}; HRMS (FAB) calcd for $C_{38}H_{70}N_7O_8$ $(M + H)^+$ 752.5286, found 752.5302.

Bioassay

The murine preadipocyte cell line 3T3-L1 was purchased from the Human Science Research Resources Bank, Japan Health Sciences Foundation (Osaka, Japan). Fetal calf serum (FCS) was purchased from ICN Biomedicals Inc. (Aurora, Ohio). Penicillin and streptomycin were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Insulin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO) unless otherwise stated.

The preadipocyte cell line 3T3-L1 was cultured in Dulbecco's modified Eagle medium (DMEM) with 10% FCS in two 96-well plates at 37 °C, 5% CO_2 for 4–7 days. After the cells reached 100% confluence, the culture buffer was changed to a differentiation buffer (150 μL per well) and samples that were dissolved in MeOH or water (7.5 μL) were added. The differentiation buffer was composed of DMEM containing 10% FCS, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 90 U/mL penicillin, 90 μg/mL streptomycin, and 10 μg/mL insulin. As a control, MeOH or water (7.5 μL) was added in place of samples. After 7 days, the differentiated 3T3-L1 adipocytes in the 96-well plate were treated with 2% Triton-X 100 (10 μL/well) for 30 min at room temperature followed by sonication for 1 min. Fat accumulation was determined by measuring the liberated triglyceride using a Triglyceride E-test Kit (Wako), and the absorbance at 630/690 nm (Microplate Reader EL-800, BIO-TEK Instruments, Inc) was measured according to manufacture's instructions. The fat-accumulation rate was calculated as a percentage of the control. To determine the cell viability of the differentiated 3T3-L1 adipocytes, another 96-well plate was treated with a Cell Counting Kit-8 Test (Wako), and the absorbance at 450 nm was measured according to manufacture's instructions. The viability was calculated as a percentage of the control.

Acknowledgements

This study was supported in part by Grants-in-Aid for Scientific Research for Creative Scientific Research (Grant No. 16GS0206) and the Global COE program in Chemistry at Nagoya University (Grant No. B-021), from the Ministry of Education, Culture, Sports, Science and Technology, Japan. We are indebted to Banyu Pharmaceutical Co., Ltd. for their financial support.

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