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Biological Activity, Structural Features, and Synthetic Studies of (−)-Ternatin, a Potent Fat-Accumulation Inhibitor of 3T3-L1 Adipocytes

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Dedicated to Professor Teruaki Mukaiyama on the occasion of his 80th birthday

Abstract: A series of studies, including preliminary screening, isolation, structure determination, synthesis, and biological evaluation, of (−)-ternatin (**1**) are described. A highly *N*-methylated cyclic heptapeptide isolated from the mushroom *Coriolus versicolor*, **1** shows an inhibitory effect on fat accumulation by 3T3-L1 murine adipocytes (EC_{50} = 0.02 μg mL^{-1}). Detailed analysis of 1D and 2D NMR spectra, as well as amino acid analysis, suggested four stereoisomers as candidates for **1**. For the complete structural elucidation of **1**, chemical syntheses were carried out by solid-phase peptide synthesis. By comparing the spectroscopic data for the natural product with the data for the synthetic stereoisomers, the structure of **1** was confirmed to be cyclo[D-*allo*-Ile^1-L-(*N*Me)Ala^2-L-(*N*Me)Leu^3-L-Leu^4-L-(*N*Me)Ala^5-D-(*N*Me)Ala^6-(2*R*,3*R*)-3-hydroxy-Leu^7].

Keywords: inhibitors · natural products · peptides · solid-phase synthesis · structure elucidation

Introduction

Obesity, which is defined as the state of having a body mass index greater than 30 kg m^{-2} and needing clinical treatment, has attracted significant attention as a serious public-health problem.[1] Generally, obesity is induced by personal habits, including food intake and preference, and physical inactivity in daily life, as well as genetic factors.[2] In particular, the developed world faces a rapid increase in the risk of hyperlipidemia, hypertension, diabetes, cardiovascular diseases, cancer, and the shortening of human life, all of which are associated with obesity.[3]

All antiobesity drugs in clinical trials have some limitations as a result of side effects.[4] To date, only two drugs, the dual serotonin–noradrenaline-reuptake inhibitor sibutramine (Meridia) and the gastric lipase inhibitor orlistat (Xenical), have been approved for the long-term treatment of obesity by the Food and Drug Administration (FDA). Additionally, the noradrenergic agent mazindol (Sanorex) has been approved in Japan, and other drugs are also used for short-term clinical treatment. Further therapeutic development and the discovery of potential alternative antiobesity agents are still required, however, because of the expected globalization of the obesity problem in the future.

In contrast to the use of clinical drugs, several low-calorie foods, including plants and mushrooms, have attracted attention worldwide. Such foods may be able to prevent bodyweight gain.[5] During our continuing studies on new biologically active compounds, we have focused much attention on wild mushrooms as potential sources of novel bioactive compounds. In the course of our screening experiments, we found that an extract in ethyl acetate of the mushroom *Cor-*

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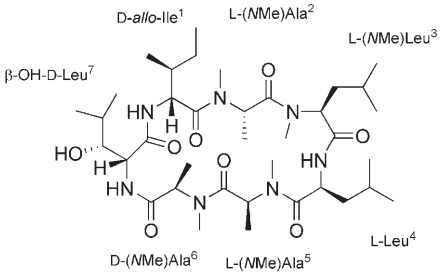
iolus versicolor significantly inhibited fat accumulation by 3T3-L1 murine adipocytes at a concentration of 100 μg mL^{-1}. Guided by this assay, we isolated the highly *N*-methylated cyclic heptapeptide (−)-ternatin (**1**).[6] We describe herein the details of our ongoing studies, including the preliminary screening of wild mushrooms, the isolation and structure elucidation of **1**, the chemical synthesis of **1** in the solid phase, and the inhibitory effect of **1** and its stereoisomers on fat accumulation by 3T3-L1 adipocytes.



(−)-Ternatin (**1**)

Results and Disucussion

Screening of Mushroom Extracts

Table 1 shows the results of the screening of 15 wild-mushroom specimens. The extracts in EtOAc and H_2O were prepared by partitioning crude mushroom extracts in EtOH between EtOAc and H_2O, and then used in the bioassay at a concentration of 100 μg mL^{-1}. We selected a fat-accumulation-inhibition assay with 3T3-L1 murine adipocytes, which were differentiated from preadipocytes by treatment with insulin.[7–9] In this bioassay, we evaluated two parameters: fat accumulation by 3T3-L1 murine adipocytes and the viability of these cells. The latter parameter is used to exclude undesired fat-accumulation inhibition arising from the toxic nature of the samples tested. Only three of the samples tested showed a significant inhibitory effect on fat accumulation (<50% fat accumulation), whereas cytotoxicity (<50% cell viability) was observed for six samples. In particular, the H_2O extract of *H. truncatum* (0.49%) and the EtOAc extract of *C. versicolor* (7.1%) strongly inhibited fat accumulation. We selected the EtOAc extract of *C. versicolor* as the most likely source of potential fat-accumulation inhibitors on the basis of its considerably greater fat-accumulation inhibitory effect relative to that of the other samples and the good cell viability of the adipocytes upon treatment with this extract.

Table 1. Screening experiments on wild mushrooms for fat accumulation and cell viability of 3T3-L1 murine adipocytes.[a]

Mushroom sample	Fat accumulation [%]		Cell viability [%]	
	EtOAc extract	H_2O extract	EtOAc extract	H_2O extract
Hypoxylon truncatum	>100	0.49	>100	50
Coriolus versicolor	7.1	>100	89	92
Daedaleopsis styracina	44	>100	68	88
Daedaleopsis tricolor	>100	>100	15	96
Schizophyllum commune	88	>100	>100	>100
Daedaleopsis purpurea	>100	>100	>100	93
Elfvingia applanata	>100	>100	>100	93
Russula rosacea	>100	>100	>100	>100
Lactarius acris	93	>100	>100	>100
Laccaria laccata	77	>100	>100	>100
Boletus reticulatus	>100	>100	78	44
Lentinus lepideus	>100	>100	15	36
Boletus sinapicolor	>100	>100	24	53
Strobilomyces confusus	86	>100	15	>100
Russula emetica	>100	>100	99	83

[a] All samples were evaluated at a concentration of 100 μg mL^{-1}.

Isolation of Ternatin, a Potent Fat-Accumulation Inhibitor

The mushroom *C. versicolor* (5 kg) was homogenized, extracted with 80% aqueous ethanol, and then partitioned between EtOAc and H_2O. The EtOAc layer, which inhibited potently fat accumulation by 3T3-L1 murine adipocytes, was then partitioned between aqueous methanol and hexane. The sample in aqueous methanol was separated by silica-gel and Sephadex LH-20 gel-permeation column chromatography. Finally, reversed-phase HPLC afforded a cyclic heptapeptide, (−)-ternatin (**1**; 11.5 mg; $[\alpha]_D^{20} = -23.5$ ($c = 0.13$, EtOH)). Compound **1** inhibited significantly fat accumulation by 3T3-L1 murine adipocytes ($EC_{50} = 0.02$ μg mL^{-1}). Cell viability was not affected greatly at the concentration corresponding to the EC_{50} value.

Structure Elucidation

The NMR spectroscopic data for **1** in [D_6]benzene (Table 2) and the HRMS (FAB) data (*m/z* 760.4945 $[M+Na]^+$, $\Delta = -0.4$ mmu) for **1** suggested the molecular formula $C_{37}H_{67}N_7O_8$. In the ^{13}C NMR spectrum, seven signals were observed in the range $\delta = 169$–175 ppm, and were assigned to carbonyl carbon atoms. Eight signals between $\delta = 50.0$ and 76.0 ppm were assigned to α carbon atoms of amino acid residues and a carbon atom bonded to a hydroxy group. Notably, signals for four *N*-methyl groups ($\delta = 3.28$, 2.78, 2.68, 2.59 ppm) were observed as singlets. A detailed analysis of ^1H and ^{13}C NMR, HMBC, and COSY spectra revealed that **1** contained seven amino acids: three *N*-methyl-

Abstract in Japanese:
肥満は生活習慣病を引き起こす要因の一つであると考えられている。このような背景から、マウス胎仔由来 3T3-L1 白色脂肪細胞に対する脂肪蓄積阻害活性を指標として、有用な脂肪蓄積阻害物質の探索研究を行った。16種類のキノコに対しスクリーニングを行った結果、サルノコシカケ科カワラタケ*Coriolus versicolor* に顕著な活性が見られた。本活性を指標にし、さらに精製を行うことで強力な阻害活性を有する環状ヘプタペプチド (−)-ternatinを単離した。各種 NMR スペクトルの解析、Marfey 法、さらに固相合成を用いることで天然物の構造を決定した。

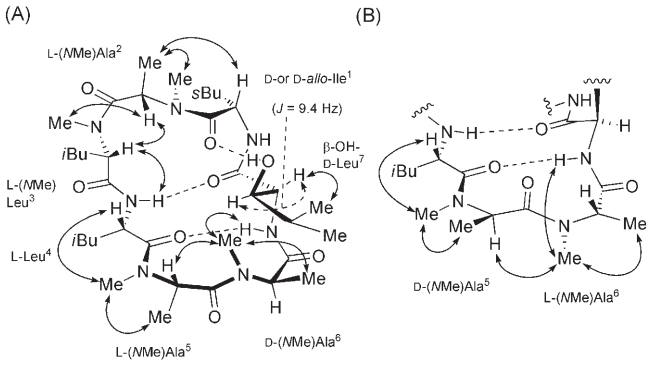
Table 2. NMR spectroscopic data for (−)-ternatin (**1**) in C_6D_6.

Residue	Position	$\delta(^{13}C)$[a] [ppm]	$\delta(^{1}H)$[b] [ppm]	Residue	Position	$\delta(^{13}C)$[a] [ppm]	$\delta(^{1}H)$[b] [ppm]
D-or D-*allo*-Ile[1]	CO	173.0 (s)		L-Leu[4]	CO	174.4 (s)	
	α	55.0 (d)	4.48 (dd, J = 7.2, 3.0 Hz)		α	50.0 (d)	5.20 (td, J = 8.0, 2.0 Hz)
	β	33.5 (d)	1.21–1.26 (m)		β	38.0 (t)	1.62–1.65 (m)
	γ	27.0 (t)	0.77–0.81 (m)				2.09–2.14 (m)
			0.88–0.92 (m)		γ	26.0 (d)	1.73–1.79 (m)
	γ′	13.8 (q)	0.60 (d, J = 7.3 Hz, 3H)		δ	21.7 (q)	0.97 (d, J = 6.7 Hz, 3H)
	δ	11.5 (q)	0.57 (t, J = 7.3 Hz, 3H)		δ′	24.2 (q)	0.90 (d, J = 6.7 Hz, 3H)
	NH		6.22 (br d, J = 7.2 Hz)		NH		7.57 (d, J = 8.0 Hz)
L-(*N*Me)Ala[2]	CO	169.0 (s)		L(D)-(*N*Me)Ala[5]	CO	174.8 (s)	
	α	50.0 (d)	5.68 (q, J = 6.5 Hz)		α	51.5 (d)	4.34 (q, J = 7.3 Hz)
	β	16.2 (q)	1.24 (d, J = 6.5 Hz, 3H)		β	13.2 (q)	0.75 (d, J = 7.3 Hz, 3H)
	NCH_3	29.5 (q)	2.78 (s, 3H)		NCH_3	30.5 (q)	2.68 (s, 3H)
L-(*N*Me)Leu[3]	CO	169.0 (s)		D(L)-(*N*Me)Ala[6]	CO	170.4 (s)	
	α	59.5 (d)	4.31 (dd, J = 10.1, 4.0 Hz)		α	52.5 (d)	5.64 (q, J = 6.8 Hz)
	β	40.7 (t)	1.01 (ddd, J = 12.6, 10.1, 3.8 Hz)		β	14.4 (q)	1.26 (d, J = 6.8 Hz, 3H)
			2.36 (ddd, J = 12.6, 11.6, 4.0 Hz)		NCH_3	30.5 (q)	2.59 (s, 3H)
	γ	25.0 (d)	1.77–1.81 (m)	β-OH-D-Leu[7]	CO	174.8 (s)	
	δ	22.6 (q)	1.17 (d, J = 6.3 Hz, 3H)		α	56.3 (d)	5.17 (dd, J = 8.0, 9.4 Hz)
	δ′	23.4 (q)	0.93 (d, J = 6.7 Hz, 3H)		β	76.0 (d)	3.96 (dd, J = 9.4, 2.1 Hz)
	NCH_3	30.0 (q)	3.28 (s, 3H)		OH		5.87 (s)
					γ	29.5 (d)	2.21–2.25 (m)
					δ	21.0 (q)	1.44 (d, J = 6.8 Hz, 3H)
					δ′	15.0 (q)	1.52 (d, J = 6.8 Hz, 3H)
					NH		7.92 (d, J = 8.0 Hz)

[a] Corresponding signal in the ^{13}C NMR spectrum recorded at 201 MHz. The multiplicity of the signal is derived from the HMQC spectrum. [b] Corresponding signal in the ^{1}H NMR spectrum recorded at 800 MHz.

alanine units ((*N*Me)Ala[2,5,6]), isoleucine (Ile[1]), *N*-methylleucine ((*N*Me)Leu[3]), leucine (Leu[4]), and β-OH-leucine (β-OH-Leu[7]). Finally, the planar structure of **1** was deduced by further analysis of the HMBC correlations.

Compound **1** was observed as a single conformer, even in CD_3OD. Thus, (−)-ternatin (**1**) appears to have a single conformation (Scheme 1). Such a rigid conformation was thought to be fixed by three intramolecular hydrogen bonds between two amide NH hydrogen atoms (δ = 7.92, 7.57 ppm) in the Leu[4] and β-OH-Leu[7] moieties and the β-OH-Leu[7] hydroxy hydrogen atom, which gave rise to a sharp signal at quite low field (δ = 5.87 ppm).[10]



Scheme 1. Detailed analysis of the stereostructure of (−)-ternatin (**1**) on the basis of 2D NMR spectra. The structures in A and B are both plausible on the basis of ROESY correlations. Arrows indicate ROESY correlations. Possible intramolecular hydrogen bonds between OH and NH hydrogen atoms and carbonyl oxygen atoms are indicated with dotted lines.

The absolute configuration of the amino acid constituents of **1** was determined by analysis of 2D NMR spectra and degradation reactions. The acid hydrolysis of **1** by treatment with 6 M HCl at 100 °C for 3 h gave its hydrolysates. Amino acid analysis by the Marfey method[11] revealed the presence of L-(*N*Me)Leu[3], L-Leu[4], and both L- and D-(*N*Me)Ala[2,5,6].

Detailed analysis of ROESY correlations and coupling constants led to the elucidation of plausible stereostructures (Scheme 1). The absolute configuration of (*N*Me)Ala[2] was confirmed to be L. The coupling constant of 9.4 Hz determined for the two methine protons 2-H and 3-H in β-OH-Leu[7] suggested an *anti* arrangement. Thus, the absolute configuration of β-OH-Leu[7] was confirmed to be 2*R*,3*R*. As for the (*N*Me)Ala[5]-(*N*Me)Ala[6] fragment, L-(*N*Me)Ala[5]-D-(*N*Me)Ala[6] (Scheme 1A) and D-(*N*Me)Ala[5]-L-(*N*Me)Ala[6] (Scheme 1B) were considered as plausible stereostructures. The true configuration of this fragment could not be identified solely on the basis of ROESY correlations. Moreover, both D- and D-*allo*-Ile[1] were found to be possible components of **1**. Therefore, four stereoisomers, **1a**–**1d** (**1a**: D-Ile[1], L-(*N*Me)Ala[5], D-(*N*Me)Ala[6]; **1b**: D-Ile[1], D-(*N*Me)Ala[5], L-(*N*Me)Ala[6]; **1c**: D-*allo*-Ile[1], L-(*N*Me)Ala[5], D-(*N*Me)Ala[6]; **1d**: D-*allo*-Ile[1], D-(*N*Me)Ala[5], L-(*N*Me)Ala[6]), were considered as candidates for the true stereostructure of **1** (Scheme 2). To confirm the stereostructure of the natural product, the plausible stereoisomers **1a**–**1c** were synthesized and their biological activities evaluated.

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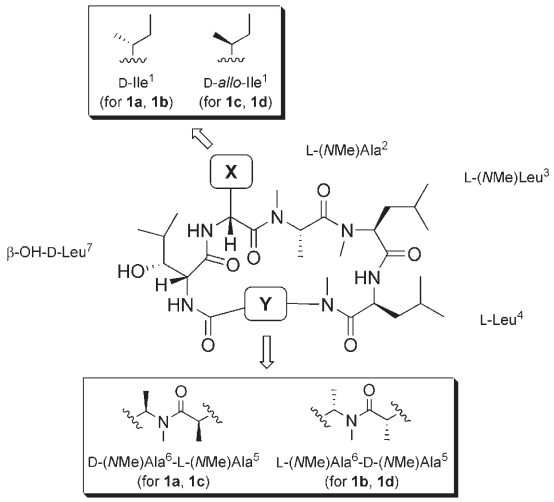
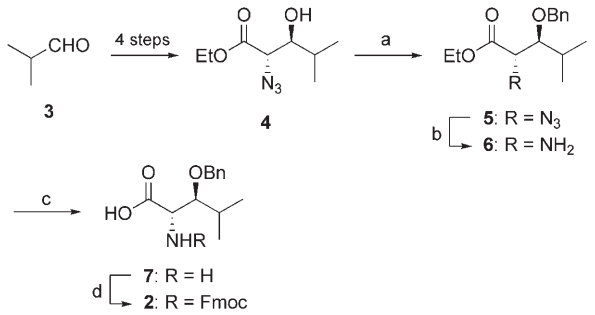


Scheme 2. Structures of four plausible stereoisomers: **1a–d**.

Synthesis of Plausible Stereoisomers

First, we prepared Fmoc-β-OBn-D-Leu-OH (**2**) as an essential building block for solid-phase peptide synthesis (SPPS; Scheme 3). Isobutyraldehyde (**3**) was converted into the



Scheme 3. Synthesis of Fmoc-β-OBn-D-Leu (**2**). Reagents and conditions: a) Benzyl-2,2,2-trichloroacetimidate, TfOH, CH_2Cl_2, room temperature, 57% (recovered **4**: 42%); b) H_2, Pd/C(en), MeOH, room temperature, 70%; c) aqueous NaOH (1 M), THF, room temperature, 89%; d) Fmoc–ONSu, 9% aqueous Na_2CO_3, DMF, 0 °C→room temperature, 89%. Bn = benzyl, DMF = *N*,*N*-dimethylformamide, Fmoc = 9-fluorenylmethoxycarbonyl, Pd/C(en) = Pd/C–ethylenediamine complex, Su = succinimidyl, TfOH = trifluoromethanesulfonic acid.

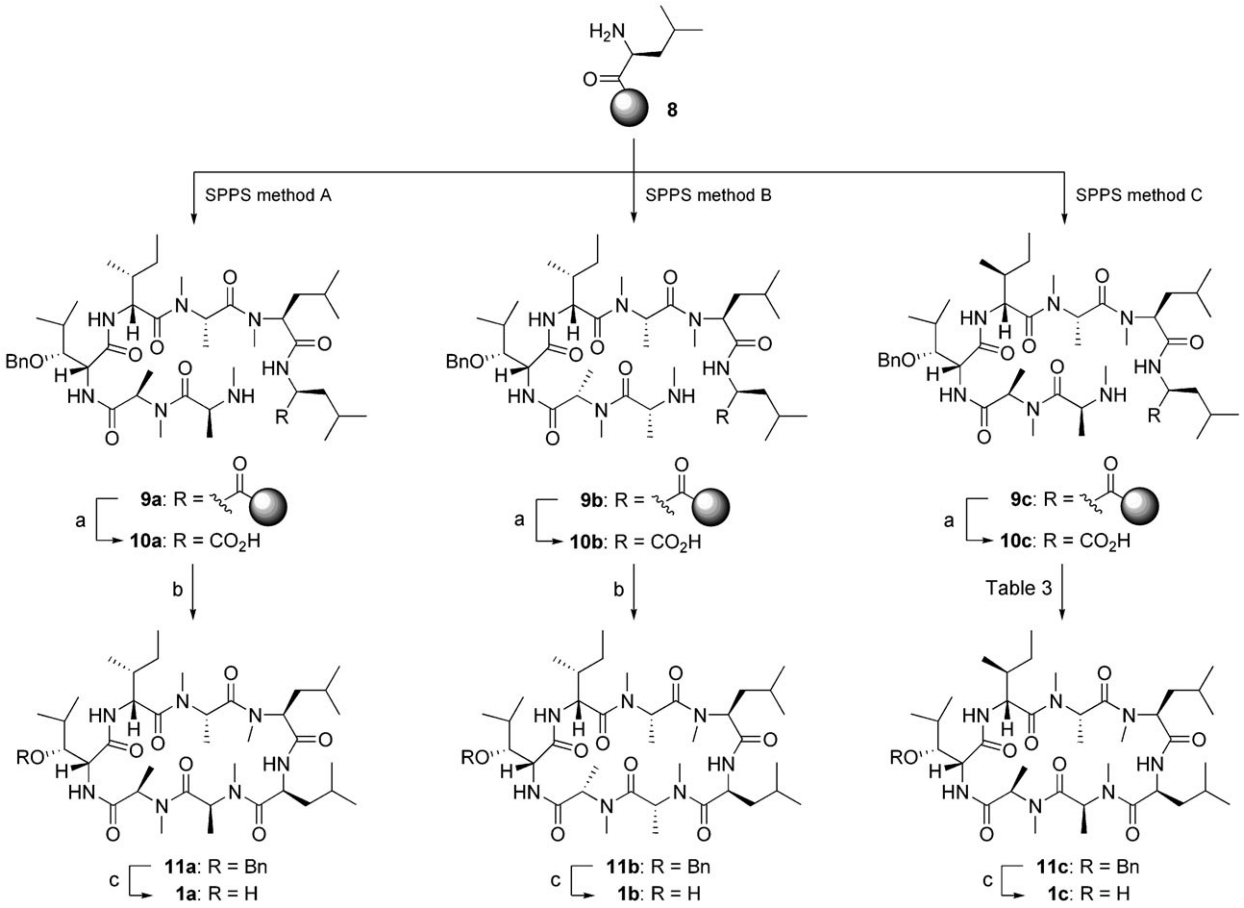
chiral secondary alcohol **4** as described previously.[12] The hydroxy group in **4** was protected with a benzyl group to give the benzyl ether **5** in 57% yield (compound **4** was recovered in 42% yield). Subsequent Pd/C(en)-mediated selective hydrogenation of the azide group[13] in **5** afforded the amine **6** (70%), which was converted into **2** through hydrolysis of the ethyl ester group and Fmoc protection of the amino group (79% over two steps).

The required stereoisomers of **1** were then synthesized on the solid phase (Scheme 4). In all cases a Trt(2-Cl) (2-chlorotrityl) resin preloaded with L-Leu[4] (H-L-Leu-Trt(2-Cl) resin (**8**)) was used as the starting material. The stereoisomer **1a** was elaborated in the C-to-N direction by sequential Fmoc deprotection with 20% piperidine followed by HATU-mediated coupling with the synthesized leucine derivative **2** and the appropriate Fmoc-protected amino acids according to SPPS method A (see Experimental Section). Subsequent Fmoc deprotection gave the linear peptide **9a**, which was cleaved from the resin by treatment with 0.5% TFA/CH_2Cl_2 to afford the linear heptapeptide **10a** in 46% yield from compound **8**.

As a key construction step in our synthesis, we carried out the macrolactamization of **10a** in the presence of HATU (2.0 equiv) and iPr$_2$NEt (4.5 equiv) at high dilution (0.77 mM). After purification by HPLC, the desired cyclic peptide **11a** was obtained in moderate yield (45%). Finally, the removal of the benzyl group in **11a** afforded the cyclic heptapeptide **1a**. The stereoisomer **1b** was synthesized in a similar manner: The linear heptapeptide **9b** was prepared by SPPS method B and cleaved from the resin to give **10b**. The macrolactamization of **10b** provided the cyclic peptide **11b** in particularly low yield. Subsequent removal of the benzyl group gave **1b** (40%). The ^{1}H NMR spectra of the two synthetic compounds **1a** and **1b** were not identical to that of the natural compound (Figure 1). However, major differences between **1a** and natural **1** in terms of the signals in their ^{1}H NMR spectra were only found for the Ile[1] moiety. Therefore, we believed that the D-*allo*-Ile[1] analogue **1c** would prove to be the true natural compound.

In the SPPS of the linear peptides **9a** and **9b**, incomplete amide-bond formation was a significant problem, mainly as a result of the low reactivity of *N*-methylamine moieties.[14] To overcome this problem, we modified the SPPS reaction conditions as follows (SPPS method C): The initial coupling reaction between the sterically hindered L-Leu-bound resin **8** and Fmoc-*N*Me-L-Leu was performed twice without the removal of the Fmoc group after the first coupling reaction (double coupling). Furthermore, the reaction time was prolonged to 3 h for each coupling step to enable complete conversion. In this way, the desired linear heptapeptide **10c** was obtained in 71% yield from **8**.

Next, the key cyclization was investigated (Table 3).[15] We tested various coupling reagents: a combination of EDC and HOAt, phosphonium-based BOPCl and PyBroP, and uronium-salt-based HATU, all of which are known to be advantageous with regard to the speed of the reaction, the yield of the product, and the degree of racemization that occurs.[16] Each reaction was conducted at high dilution (0.77 mM) and continued until analytical HPLC indicated the consumption of the linear heptapeptide **10c**. The use of BOPCl resulted in the formation of the desired cyclized product **11c** in 73% yield (Table 3, entry 3) along with some impurities that remained after purification by HPLC, whereas EDC/HOAt and PyBroP provided **11c** in low yield (Table 3, entries 1 and 2). With HATU, the cyclization proceeded smoothly to give the product in moderate yield (53%; Table 3, entry 4). However, the addition of HOAt (2.0 equiv) improved the cyclization with HATU to give **11c** in 73% yield and the best overall result (Table 3, entry 5).



Scheme 4. Synthesis of stereoisomers **1a–c**. SPPS method A/B: HATU, DMF, room temperature, 1 h for each coupling step; deprotection with 20% piperidine in DMF. SPPS method C: 1) Fmoc-L-(*N*Me)Leu, HATU, *i*Pr$_2$NEt, DMF, room temperature, 3 h; Fmoc-L-(*N*Me)Leu, HATU, *i*Pr$_2$NEt, DMF, room temperature, 3 h (double coupling); 2) 20% piperidine/DMF; Fmoc-L-(*N*Me)Ala, HATU, *i*Pr$_2$NEt, DMF, room temperature, 3 h; 3) 20% piperidine/DMF; Fmoc-D-Ile, HATU, *i*Pr$_2$NEt, DMF, room temperature, 3 h; 4) 20% piperidine/DMF; **2**, HATU, *i*Pr$_2$NEt, DMF, room temperature, 3 h; 5) 20% piperidine/DMF; Fmoc-D-(*N*Me)Ala, HATU, *i*Pr$_2$NEt, DMF, room temperature, 3 h; 6) 20% piperidine/DMF; Fmoc-L-(*N*Me)Ala, HATU, *i*Pr$_2$NEt, DMF, room temperature, 3 h; 7) 20% piperidine/DMF. Other reagents and conditions: a) 0.5% TFA/CH_2Cl_2, room temperature, 46% for **10a**, 29% for **10b**, 71% for **10c** (from **8**); b) HATU, *i*Pr$_2$NEt, CH_2Cl_2, 0 °C→room temperature, 45% for **11a**, 14% for **11b**; c) H_2, Pd/C(en), MeOH, room temperature, 56% for **1a**, 40% for **1b**, 81% for **1c**. HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, TFA = trifluoroacetic acid.

Finally, the removal of the benzyl group in **11c** gave **1c** (81%).

As expected, the ^{1}H NMR spectroscopic data for the synthetic stereoisomer **1c** were completely identical to the data for natural (−)-**1** (Figure 1). The optical rotation of **1c** ($[\alpha]_D^{24.5} = -35.5$ ($c = 0.40$, EtOH)) agreed with that of the natural compound within the limits of experimental error. The experimental error was thought to be due to the limited quantity of the natural sample. Thus, the structure of **1** was confirmed to be cyclo[D-*allo*-Ile^1-L-(*N*Me)Ala^2-L-(*N*Me)Leu^3-L-Leu^4-L-(*N*Me)Ala^5-D-(*N*Me)Ala^6-(2*R*,3*R*)-3-hydroxy-Leu^7].

Ternatin was isolated previously from the fungi *Didymocladium ternatum*[17] and *Cladobotryum* sp.[18] and identified as an antibacterial or antimicrobial compound. Its X-ray crystal structure was also reported.[19] However, the ^{1}H and ^{13}C NMR spectra of ternatin were not described previously, and the reported optical rotation ($[\alpha]_D^{20} = 39.9$ ($c = 0.40$, EtOH)) was opposite in sign to that measured in our study. This discrepancy prompted us to carry out the synthesis of its stereoisomers. Furthermore, the configuration of the Ile^1 moiety in ternatin was described mistakenly as the D form in reference [19]. In fact, the X-ray crystal data in reference [19] and the amino acid analysis of authentic ternatin in reference [17] showed clearly the presence of the D-*allo* form, as in our compound **1**. Therefore, the structure of authentic ternatin could be revised as **1c**, although the significance of the difference in the sign of optical rotation is unclear.

Biological Evaluation

We carried out a biological evaluation of synthetic ternatin and its stereoisomers (Table 4). Synthetic (−)-ternatin (**1c**) was found to inhibit potently fat accumulation by 3T3-L1 cells with an EC_{50} value of 0.02 μg mL^{-1}. Stereoisomer **1a**, a

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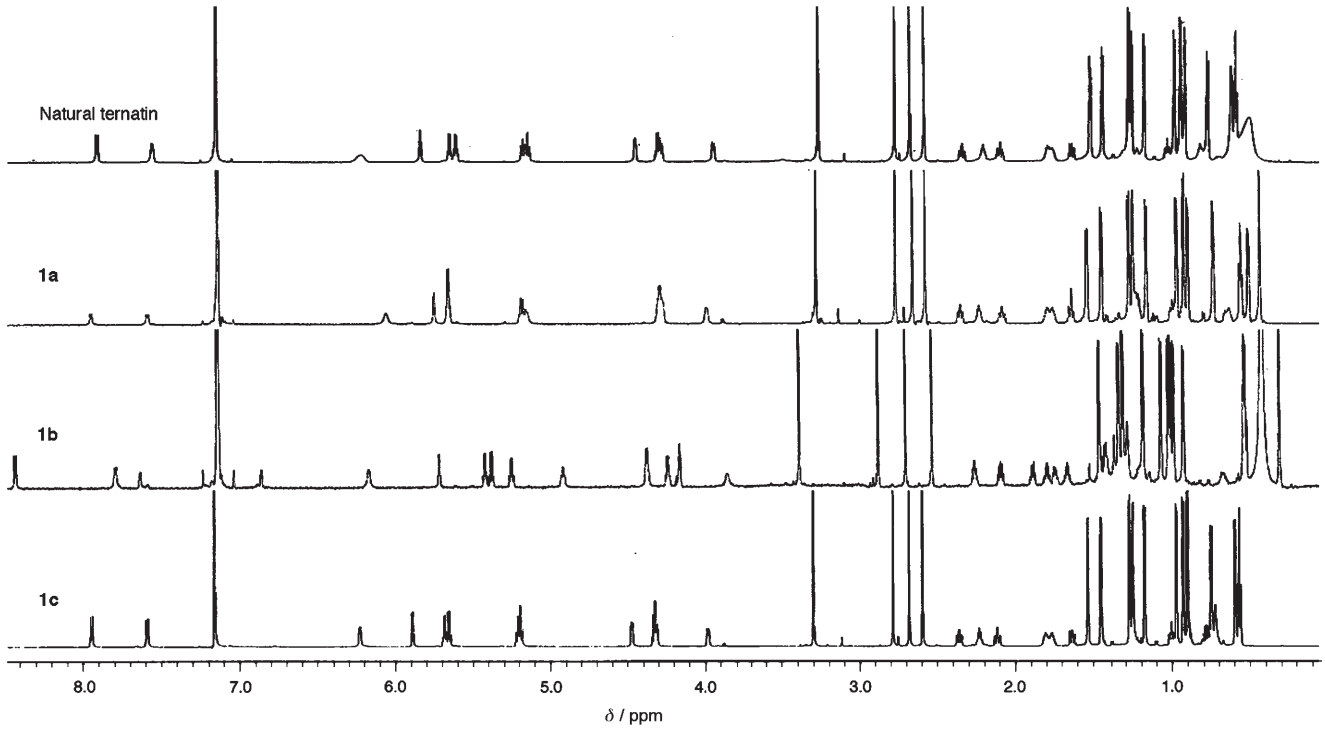
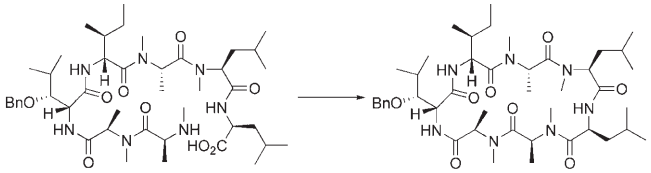


Figure 1. ^{1}H NMR spectra of natural (−)-ternatin and **1a–c** in C_6D_6 (800 MHz).

Table 3. Macrocyclization of the linear heptapeptide **10c**.



Entry	Conditions[a]	Yield [%]
1	EDC (2.0 equiv), HOAt (2.0 equiv), *i*Pr$_2$NEt (4.5 equiv)	41
2	PyBroP (5.0 equiv), *i*Pr$_2$NEt (4.5 equiv)	40
3	BOPCl (5.0 equiv), *i*Pr$_2$NEt (4.0 equiv)	73[b]
4	HATU (2.0 equiv), *i*Pr$_2$NEt (4.5 equiv)	53
5	HATU (2.0 equiv), HOAt (2.0 equiv), *i*Pr$_2$NEt (4.5 equiv)	73

[a] Reactions were carried out in CH_2Cl_2 at high dilution (0.77 mM) for 1–3 days until analytical HPLC analysis (RP-aqueous; 50% aqueous CH_3CN/0.1% TFA) indicated complete consumption of the starting material. [b] Some impurities were inseparable from the desired cyclic peptide by HPLC. HOAt = 1-hydroxy-7-azabenzotriazole, EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, PyBroP = bromotrispyrrolidinophosphonium hexafluorophosphate, BOPCl = bis(2-oxo-3-oxazolidinyl)phosphine chloride.

diastereomer with a D-Ile[1] moiety, also had an inhibitory effect of the same order of magnitude as that of the natural compound. Interestingly, however, stereoisomer **1b**, which contains two *N*Me(Ala) residues of opposite absolute configuration to that of the equivalent residues in **1a**, did not show any activity at a concentration of 100 μg mL^{-1}. Therefore, the conformation of the cyclic peptide in **1**, which is partially fixed by intramolecular hydrogen bonding, seems to be important for the potent inhibitory effect of **1** on fat accumulation.

The in vivo inhibitory effect of **1** on fat accumulation was also investigated as part of extensive biological evaluations. Upon treatment with **1** at 5 mg kg^{-1} day^{-1}, increases in body weight and fat accumulation in mice fed a high-fat diet were both suppressed significantly.[20]

Conclusions

In summary, the highly N-methylated cyclic heptapeptide (−)-ternatin (**1**) was isolated from the mushroom *Coriolus versicolor*. Compound **1** suppressed significantly fat accumulation by 3T3-L1 murine adipocytes. Except for the configu-

Table 4. Inhibitory effect of synthetic compounds **1a–c** on fat accumulation, and cell viability of the 3T3-L1 adipocytes.

Compound	Fat-accumulation inhibitory effect: EC_{50} [μg mL^{-1}]	Cell viability: IC_{50} [μg mL^{-1}]
1a	0.045	0.074
1b	>100	>100
1c	0.02[a]	0.21[a]
(−)-noradrenaline (+)-bitartrate[b]	88[a]	>100[a]

[a] See reference [21]. [b] (+)-Bitartrate was used as a standard in this assay.

ration of three stereogenic centers, the planar and absolute stereostructure of **1** was determined by spectroscopic analysis and amino acid analysis of hydrolysis products by the Marfey method. To elucidate the complete stereostructure of **1**, three possible stereoisomers were synthesized by SPPS. Comparison of the ^{1}H NMR spectroscopic data of natural and synthetic ternatin stereoisomers confirmed the structure of **1** as cyclo[D-*allo*-Ile^1-L-(*N*Me)Ala^2-L-(*N*Me)Leu^3-L-Leu^4-L-(*N*Me)Ala^5-D-(*N*Me)Ala^6-(2*R*,3*R*)-3-hydroxy-Leu^7].

To date, several bioactive cyclic peptides containing *N*-methylated amino acids have been reported, such as cyclosporin A, a potent immunosuppressant.[22] (−)-Ternatin (**1**) is a potent candidate for an antiobesity drug. Although the inhibitory effect of **1** may be caused by the prevention of adipogenesis, the mechanism of action is unknown. Further studies on the different aspects of the biological activity of (−)-ternatin and the corresponding modes of action, along with structure–activity-relationship (SAR) studies, are in progress.

Experimental Section

General

Reactions were performed under nitrogen atmosphere unless otherwise noted. Fuji Silysia silica gel FL-60D (Aichi, Japan), Nacalai Tesque cosmosil 75C_{18}-OPN gel (Kyoto, Japan), and sephadex LH-20 gel (GE Healthcare, UK) were used for column chromatography. High-pressure liquid chromatography (HPLC) was performed with Develosil ODS HG-5 and RP-aqueous reversed-phase columns (Nomura Chemical Co., Aichi, Japan). Optical-rotation measurements were made with a Jasco DIP-1000 polarimeter. IR spectra were recorded on a Jasco FT/IR-230 spectrometer. ^{1}H, ^{13}C, and 2D NMR spectra were recorded on a JEOL JNM-A400, JNM-A600, or JNM-ECA800 spectrometer. Chemical shifts are referenced to the solvent peak: δ_H = 7.26 ppm, δ_C = 77.0 ppm for $CDCl_3$; δ_H = 3.30 ppm, δ_C = 49.0 ppm for [D_4]MeOH; δ_H = 7.16 ppm, δ_C = 128.0 ppm for [D_6]benzene. The following abbreviations are used to describe the signals: br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Mass spectra were determined on a JEOL JMS-700 spectrometer operating in the positive FAB mode (with *m*-nitrobenzyl alcohol as the matrix).

Mushroom Materials

Fifteen types of mushroom specimens were collected by hand in Aichi, Japan. Their fruit bodies were homogenized and extracted with 80% aqueous EtOH for two days. The extracts were then partitioned between EtOAc and H_2O. Each layer was concentrated in vacuo to afford a crude extract. These extracts were used directly for bioassays.

Fat-Accumulation Inhibitory Assay

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 (Aurora, Ohio). Penicillin and streptomycin were purchased from Nacalai Tesque (Kyoto, Japan). Insulin was purchased from Wako Pure Chemical Industries (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 the Dulbecco 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 exchanged for a differentiation buffer (150 μL per well), and samples (mushroom extracts, purified ternatin, or (−)-noradrenaline bitartrate hydrate as a standard) dissolved in MeOH or water (7.5 μL) were added. The differentiation buffer was composed of DMEM containing FCS (10%), dexamethasone (1 μM), 3-isobutyl-1-methylxanthine (IBMX; 0.5 mM), penicillin (90 U mL^{-1}), streptomycin (90 μg mL^{-1}), and insulin (10 μg mL^{-1}). As a control, MeOH or water (7.5 μL) was added in place of samples. After 7 days, the differentiated 3T3-L1 adipocytes in a 96-well plate were treated with 2% Triton-X 100 (10 μL/well) for 30 min at room temperature and then subjected to sonication for 1 min. Fat accumulation was determined by measuring liberated triglyceride by using a Triglyceride E-Test kit (Wako) according to the instructions of the manufacturer. To determine the cell viability of differentiated 3T3-L1 adipocytes, the other 96-well plate was treated with a Cell Counting Kit-8 test (Wako), and the absorption at 450 nm was measured according to the instructions of the manufacturer.

Extraction and Isolation

The wild mushroom *Coriolus versicolor* was purchased from Shinwabussan Co. (Osaka, Japan). It had been collected in Iwate Prefecture, Japan. The fruit bodies of *C. versicolor* (5 kg, dry weight) were crushed and extracted with 80% aqueous ethanol for a month. The concentrated extract was partitioned between EtOAc (4 × 5 L) and H_2O (5 L). The concentrated EtOAc extract (39.3 g) was partitioned between hexane (2 L) and 90% aqueous MeOH (3 × 2 L). The concentrated sample from the aqueous MeOH layer (21.6 g) was divided into four portions. Each portion was applied to a column of 75C_{18} gel (50 g) and eluted with 70, 80, 90% aqueous MeOH, and finally with MeOH. The combined fractions in 90% aqueous MeOH (2.57 g) were divided into four portions. Each portion was eluted through a sephadex LH-20 gel column (ϕ 10 × 830 mm) with MeOH. The active fraction was purified further by elution twice with MeOH through the same column. Finally, 10/11 of the active fraction (360 mg) was loaded on a Develosil ODS HG-5 HPLC column and eluted with 55% aqueous CH_3CN at a flow rate of 5 mL min^{-1}, with monitoring at 215 nm. The combined fraction was concentrated in vacuo to give (−)-ternatin as a colorless amorphous solid (11.5 mg, 2.3×10^{-4}%, t_R = 55.0 min). $[\alpha]_D^{20} = -23.5$ (c = 0.13, EtOH); IR ($CHCl_3$): $\tilde{\nu}$ = 3426, 3344, 3313, 2960, 2933, 2871, 1636, 1526, 1506, 1466, 1409 cm^{-1}; for ^{1}H and ^{13}C NMR spectroscopic data, see Table 1; HRMS (FAB): m/z calcd for $C_{37}H_{67}N_7O_8Na$: 760.4949 $[M+Na]^+$; found: 760.4945 (Δ = −0.4 mmu).

Hydrolysis and Amino Acid Analysis

(−)-Ternatin (**1**; 0.20 mg, 0.27 μmol) was hydrolyzed with 6 M HCl (0.1 mL) at 110 °C for 3 h. The resulting mixture was lyophilized and treated with a 1% solution of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Marfey reagent[11]) in acetone (50 μL) and 0.38 M aqueous $NaHCO_3$ (80 μL) at 37 °C for 2 h. The reaction mixture was then cooled to room temperature, neutralized with 1 M HCl (20 μL), diluted with CH_3CN (0.5 mL), and subjected to HPLC analysis. DAA derivatives of standard amino acids were prepared by the same procedure and subjected to HPLC analysis (Develosil ODS HG-5 (ϕ 4.6 × 250 mm); 40–50% (linear gradient) aqueous CH_3CN/0.1% trifluoroacetic acid (TFA), 60 min, 1 mL min^{-1}, 254 nm): t_R(DAA-L- and D-(*N*Me)Ala) = 6.4 min, t_R-(DAA-L-(*N*Me)Leu) = 14.2 min, t_R(DAA-L-Leu) = 15.6 min. Under different conditions (MeOH/0.02 M sodium acetate 4:1, 0.8 mL min^{-1}), the DAA derivatives of L- and D-(*N*Me)Ala in the hydrolyzed products were eluted separately (t_R = 10.8 and 13.0 min, respectively). Unfortunately, however, DAA derivatives of β-OH-D-Leu and D-*allo*-Ile were not detected clearly by HPLC analysis owing to the presence of other contaminants.

Syntheses

5: Azide **4** ($[\alpha]_D^{27.1} = +40.0$ (c = 2.0, $CHCl_3$); lit.:[12] $[\alpha]_D^{27.1} = +39.8$° ($c$ = 2, $CHCl_3$)) was synthesized according to reference [12]. Benzyl-2,2,2-trichloroacetimidate (2.1 mL, 11.3 mmol) and trifluoromethanesulfonic acid (125 μL, 1.42 μmol) were added dropwise to a stirred solution of **4** (1.9 g, 9.44 mmol) in dry CH_2Cl_2 (19 mL) at 0 °C. The resulting mixture was warmed to room temperature and allowed to stand for 24 h. The reaction was then quenched with saturated aqueous $NaHCO_3$ (5 mL), and the mixture was diluted with water (15 mL) and extracted with CH_2Cl_2 (4 × 20 mL). The combined extracts were washed with water (50 mL) and brine (50 mL), dried with Na_2SO_4, and concentrated. The residual solid

was suspended in hexane and filtered through cotton wool. The filtrate was concentrated and purified by column chromatography on silica gel (80 g, hexane/acetone 199:1→120:1→80:3→48:1→40:1→35:1) to give **5** (1.6 g, 57%) as a colorless oil, along with recovered **4** (0.82 g, 42%). ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.99 (d, J=6.8 Hz, 3H), 1.01 (d, J=6.8 Hz, 3H), 1.28 (t, J=7.0 Hz, 3H), 2.02–2.10 (m, 1H), 3.61 (dd, J=6.0, 2.8 Hz, 1H), 4.03 (d, J=6.0 Hz, 1H), 4.17–4.26 (m, 2H), 4.58 (d, J=11.0 Hz, 1H), 4.65 (d, J=11.0 Hz, 1H), 7.05–7.45 ppm (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ=169.2, 137.9, 128.3 (2C), 127.7 (3C), 84.3, 74.2, 63.2, 61.8, 30.2, 19.3, 17.5, 14.1 ppm; HRMS (FAB): m/z calcd for $C_{15}H_{22}N_3O_3$: 292.1661 $[M+H]^+$; found: 292.1639.

6: Pd/C(en)[13] (30 mg) was added to a stirred solution of **5** (306 mg, 1.05 mmol) in MeOH (15 mL). The reaction flask was purged with H_2 gas, and the mixture was stirred for 1 h at room temperature. The reaction mixture was then filtered through celite, and the filtrate was concentrated. The resulting oil was purified by column chromatography on silica gel (3.0 g, hexane/EtOAc 1:0→9:1→0:1) to give **6** (194 mg, 70%) as a yellow oil. $[\alpha]_D^{24.4}=-7.92$ (c=1.04, EtOH); IR ($CHCl_3$): $\tilde{\nu}$=2965, 2872, 1730, 1455, 1378 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ=0.96 (d, J=7.2 Hz, 3H), 1.00 (d, J=6.8 Hz, 3H), 1.25 (t, J=7.2 Hz, 3H), 2.06 (m, 1H), 3.40 (dd, J=5.8, 5.2 Hz, 1H), 3.69 (d, J=5.2 Hz, 1H), 4.10–4.25 (m, 2H), 4.58 (d, J=11.0 Hz, 1H), 4.65 (d, J=11.0 Hz, 1H), 7.25–7.35 ppm (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=174.5, 138.5, 128.3 (2C), 127.5 (3C), 87.0, 74.1, 60.9, 56.4, 29.8, 19.8, 18.0, 14.1 ppm; HRMS (FAB): m/z calcd for $C_{15}H_{24}NO_3$: 266.1756 $[M+H]^+$; found: 266.1754.

7: Aqueous NaOH (1M, 5 mL) was added to a stirred solution of **6** (1.11 g, 4.18 mmol) in THF (5 mL) at 0°C. The reaction mixture was allowed to warm to room temperature and was stirred for 11 h at this temperature. It was then cooled to 0°C, aqueous HCl (3M) was added, and the mixture was concentrated. The resulting solid was purified by ODS (octadecasilyl silica) column chromatography (ODS silica gel: 15 g, MeOH/H_2O 10:90→15:85→20:80→40:60→0:100) to give **7** (881 mg, 89%) as a colorless solid. $[\alpha]_D^{27.2}=+14.1$ (c=0.92, MeOH); IR (KBr): $\tilde{\nu}$=3422, 3286, 3061, 3033, 2958, 2870, 1611, 1583 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD): δ=0.95 (d, J=6.8 Hz, 3H), 0.99 (d, J=6.4 Hz, 3H), 1.88–1.95 (m, 1H), 3.67 (dd, J=7.2, 4.0 Hz, 1H), 3.85 (d, J=4.0 Hz, 1H), 4.50 (d, J=11.2 Hz, 1H), 4.71 (d, J=11.2 Hz, 1H), 7.24–7.42 ppm (m, 5H); ^{13}C NMR (100 MHz, CD_3OD): δ=174.0, 139.6, 129.3 (3C), 128.7 (2C), 84.7, 73.6, 57.6, 30.8, 20.4, 19.5 ppm; HRMS (FAB): m/z calcd for $C_{13}H_{18}NO_3Na_2$: 282.1082 $[M-H+2Na]^+$; found: 282.1095.

2: *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc−ONSu; 798 mg, 2.37 mmol) in DMF (5 mL) was added dropwise to a stirred solution of **7** (510 mg, 2.15 mmol) in 9% aqueous Na_2CO_3 (5 mL, 4.30 mmol) at 0°C, and the resulting mixture was stirred for 1 h. The reaction mixture was then neutralized with 1M HCl, diluted with water, and extracted with EtOAc (3×10 mL). The extracts were washed with water (20 mL) and brine (10 mL), dried with Na_2SO_4, and concentrated. The resulting oil was purified by column chromatography on silica gel (15 g, hexane/EtOAc 19:1→9:1→5:1→3:1→1:1→0:1, then EtOAc/MeOH 1:1) to give Fmoc-β-(OBn)-D-Leu-OH (**2**; 885 mg, 89%). $[\alpha]_D^{24.6}=-7.7$ (c=0.89, $CHCl_3$); IR ($CHCl_3$): $\tilde{\nu}$=3433, 2955, 1717, 1511, 1450 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ=1.05 (d, J=4.0 Hz, 3H), 1.06 (d, J=4.0 Hz, 3H), 2.04–2.10 (m, 1H), 3.44 (dd, J=5.2, 2.0 Hz, 1H), 4.20 (t, J=4.8 Hz, 1H), 4.40 (d, J=4.8 Hz, 2H), 4.58 (d, J=7.4 Hz, 1H), 4.63 (d, J=7.4 Hz, 1H), 4.68 (dd, J=5.2, 2.4 Hz, 1H), 5.40 (d, J=5.2 Hz, 1H), 7.20–7.80 ppm (m, 13H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=174.0, 155.8, 144.3, 143.6, 141.5, 141.3, 137.8, 128.5, 128.0, 127.9, 127.7, 127.6, 127.1, 125.0, 124.7, 120.0 (2C), 86.3, 77.2, 74.9, 67.0, 65.1, 55.7, 50.3, 40.2, 30.7, 19.5, 19.0 ppm; HRMS (FAB): m/z calcd for $C_{28}H_{29}NO_5Na$: 482.1944 $[M+Na]^+$; found: 482.1927.

10c: Solid-phase peptide synthesis (SPPS) was carried out with an Applied Biosystems peptide synthesizer (model ABI Pioneer). Starting from H-L-Leu-Trt(2-Cl) resin (**8**; 512 mg, 0.48 mmol g^{-1}, 0.25 mmol) each Fmoc-protected amino acid (1.0 mmol) was coupled after removal of the Fmoc protecting group on the previously coupled amino acid. Coupling reactions were mediated by HATU (1.0 mmol) and iPr$_2$NEt (2.0 mmol), and deprotection was performed by treatment with 20% piperidine in DMF. The coupling reactions were each carried out for 3 h and incorporated Fmoc-L-(*N*Me)Leu-OH (the coupling reaction was carried out twice), Fmoc-L-(*N*Me)Ala-OH, Fmoc-D-*allo*-Ile-OH, Fmoc-β-(OBn)-D-Leu-OH (**2**), Fmoc-D-(*N*Me)Ala-OH, and Fmoc-L-(*N*Me)Ala-OH. Following the removal of the final Fmoc protecting group, the resin containing the resulting linear peptide was washed with DMF (10 mL), MeOH (10 mL), and CH_2Cl_2 (2×10 mL). The resin was then treated with 0.5% TFA in CH_2Cl_2 for 10 min with vibromixing, and the resulting mixture was filtered through cotton wool. The filtrate was concentrated, and the residual paste was purified by column chromatography on silica gel (4 g, $CHCl_3$/MeOH 49:1→9:1→3:1→1:1→0:1) to give the linear peptide **10c** (149 mg, 71%) as a colorless amorphous solid. $[\alpha]_D^{26.4}=-37.8$ (c=0.86, $CHCl_3$); IR ($CHCl_3$): $\tilde{\nu}$=3322, 2963, 2939, 2872, 1722, 1669, 1511, 1461, 1411, 1384 cm^{-1}; ^{1}H NMR (major conformer; 600 MHz, CD_3OD): δ=0.80–1.75 (m, 33H), 1.23 (d, J=7.2 Hz, 3H), 1.43 (d, J=7.2 Hz, 3H), 1.47 (d, J=7.2 Hz, 3H), 1.78–1.83 (m, 1H), 1.85–1.91 (m, 1H), 2.61 (s, 3H), 2.71 (s, 3H), 3.00 (s, 3H), 3.07 (s, 3H), 3.64–3.67 (m, 1H), 4.35–4.40 (m, 2H), 4.44 (d, J=11.4 Hz, 1H), 4.66–4.72 (m, 1H), 4.82–4.85 (m, 1H), 4.90 (q, J=7.2 Hz, 1H), 5.11 (dd, J=5.4, 10.2 Hz, 1H), 5.44 (q, J=7.2 Hz, 1H), 7.21–7.38 ppm (m, 5H); ^{13}C NMR (major conformer; 150 MHz, CD_3OD): δ=175.7, 173.4, 173.3 (2C), 172.9, 172.3, 170.9, 139.8, 129.4 (2C), 129.1 (2C), 128.8, 102.0, 84.5, 75.7, 56.8, 56.3, 55.2, 54.2, 54.1, 52.1, 52.0, 41.2, 38.4, 38.1, 32.1, 31.8, 31.6, 31.2, 27.5, 26.1 (2C), 23.6, 23.4, 21.9, 21.7, 20.4, 17.5, 15.3, 15.1, 14.8, 14.7, 12.1 ppm; HRMS (FAB): m/z calcd for $C_{44}H_{74}N_7O_9Na_2$: 890.5344 $[M-H+2Na]^+$; found: 890.5327.

11c: A solution of HATU (9.2 mg, 24 μmol) and HOAt (3.3 mg, 24 μmol) in DMF (0.1 mL) and a solution of iPr$_2$NEt (6.6 mg, 55 μmol) in CH_2Cl_2 (0.1 mL) were added to a stirred solution of **10c** (10.2 mg, 12.1 μmol) in dry CH_2Cl_2 (15.6 mL) at 0°C. The reaction mixture was stirred for 2 h at 0°C and then for 3 days at room temperature. It was then concentrated, and the resulting paste was purified by HPLC (Develosil RP-aqueous (ϕ 20×250 mm); 70% aqueous CH_3CN/0.1% TFA; 5 mL min^{-1}) to give the cyclic peptide **11c** (7.3 mg, 73%; t_R=69.2 min). $[\alpha]_D^{23.5}=+23.6$ (c=0.50, EtOH); IR ($CHCl_3$): $\tilde{\nu}$=3311, 2962, 2933, 2872, 1778, 1638, 1505, 1461, 1406 cm^{-1}; ^{1}H NMR (major conformer; 600 MHz, CD_3OD): δ=0.67–1.60 (m, 5H), 0.73 (d, J=7.3 Hz, 3H), 0.77 (d, J=6.6 Hz, 3H), 0.80 (t, J=7.3 Hz, 3H), 0.88 (d, J=6.2 Hz, 3H), 0.92 (d, J=6.6 Hz, 3H), 0.95 (d, J=6.2 Hz, 3H), 0.99 (d, J=6.6 Hz, 3H), 1.05 (d, J=6.6 Hz, 3H), 1.06 (d, J=7.0 Hz, 3H), 1.37 (d, J=7.3 Hz, 3H), 1.41 (d, J=7.3 Hz, 3H), 1.72–1.84 (m, 2H), 1.88–1.93 (m, 1H), 2.07–2.11 (m, 1H), 2.35–2.39 (m, 1H), 2.73 (s, 3H), 2.86 (s, 3H), 3.03 (s, 3H), 3.13 (s, 3H), 3.80 (dd, J=9.9, 1.5 Hz, 1H), 4.12 (t, J=9.9 Hz, 1H), 4.55 (d, J=11.7 Hz, 1H), 4.62 (dd, J=11.7, 2.2 Hz, 1H), 4.66 (d, J=11.7 Hz, 1H), 4.90–5.28 (m, 3H), 5.29 (q, J=7.3 Hz, 1H), 5.96 (q, J=7.3 Hz, 1H), 7.15–7.50 (m, 5H), 8.04 (d, J=9.2 Hz, 1H), 8.76 (d, J=8.5 Hz, 1H), 9.34 ppm (d, J=9.3 Hz, 1H); ^{13}C NMR (major conformer; 150 MHz, CD_3OD): δ=176.3, 174.0, 173.6, 172.6, 172.4, 172.2, 169.7, 140.3, 129.5 (2C), 129.2, 127.4 (2C), 85.1, 75.7, 59.2, 56.6, 56.3, 53.7, 53.6, (2C), 53.4, 43.3, 39.9, 37.8, 31.9, 31.4, 30.7, 30.2, 30.0, 27.3, 26.6, 26.0, 24.0, 23.9, 22.0, 21.8, 21.5, 16.2, 15.8, 15.5, 14.6, 13.4, 11.5 ppm; HRMS (FAB): m/z calcd for $C_{44}H_{73}N_7O_8Na$: 850.5419 $[M+Na]^+$; found: 850.5430.

1c: Pd/C (10%, 1.0 mg) was added to a stirred solution of **11c** (2.5 mg, 3.0 μmol) in MeOH (1.5 mL). The reaction flask was purged with H_2 gas, and the mixture was stirred for 24 h at room temperature. The reaction mixture was then filtered through celite, the filtrate was concentrated, and the residual oil was purified by HPLC (Develosil ODS HG-5 (ϕ 10×250 mm); 55% aqueous CH_3CN; 2 mL min^{-1}; 60 min; 215 nm) to give **1c** (1.8 mg, 81%; t_R=33.3 min) as a colorless amorphous solid. $[\alpha]_D^{24.5}=-35.5$ (c=0.40, EtOH); IR ($CHCl_3$): $\tilde{\nu}$=3426, 3344, 3313, 2960, 2933, 2871, 1636, 1526, 1506, 1466, 1409 cm^{-1}; ^{1}H NMR (800 MHz, C_6D_6): δ=0.57 (t, J=7.4 Hz, 3H), 0.60 (d, J=6.9 Hz, 3H), 0.75 (d, J=7.3 Hz, 3H), 0.77–0.81 (m, 1H), 0.90 (m, 1H), 0.90 (d, J=6.9 Hz, 3H), 0.93 (d, J=6.9 Hz, 3H), 0.97 (d, J=6.4 Hz, 3H), 1.01 (ddd, J=13.3, 9.4, 4.1 Hz, 1H), 1.18 (d, J=6.9 Hz, 3H), 1.25 (d, J=6.9 Hz, 3H), 1.24–1.27 (m, 1H), 1.27 (d, J=7.3 Hz, 3H), 1.45 (d, J=6.9 Hz, 3H), 1.54 (d, J=6.4 Hz, 3H), 1.62–1.66 (m, 1H), 1.73–1.84 (m, 2H), 2.09–2.13 (m, 1H), 2.21–2.25 (m, 1H), 2.34–2.38 (m, 1H), 2.60 (s, 3H), 2.68 (s, 3H), 2.79 (s, 3H), 3.30 (s, 3H), 3.98 (dd, J=9.4, 2.1 Hz, 1H), 4.31 (dd, J=10.1, 4.0 Hz, 1H), 4.34 (q, J=7.4 Hz, 1H), 4.48 (dd, J=6.9, 3.2 Hz, 1H), 5.17 (dd, J=8.0, 9.4 Hz,

1H), 5.20 (td, J=8.0, 2.0 Hz, 1H), 5.66 (q, J=6.9 Hz, 1H), 5.69 (q, J=6.9 Hz, 1H), 5.89 (d, J=2.7 Hz, 1H), 6.23 (d, J=6.4 Hz, 1H), 7.59 (d, J=8.7 Hz, 1H), 7.95 ppm (d, J=9.2 Hz, 1H); ^{13}C NMR (201 MHz, C_6D_6): δ=174.6, 174.5, 174.2, 172.7, 169.9, 168.8 (2C), 76.0, 59.2, 56.3, 55.1, 52.3, 51.3, 49.8 (2C), 40.5, 37.9, 33.7, 30.7, 30.2, 29.7, 29.4 (2C), 26.7, 26.0, 25.3, 23.9, 23.3, 22.6, 21.3 (2C), 15.9, 14.8, 14.2, 13.6, 13.1,11.6 ppm; HRMS (FAB): m/z calcd for $C_{37}H_{67}N_7O_8Na$: 760.4949 $[M+Na]^+$; found: 760.4922.

Methods A and B for SPPS (for **1a** and **1b**): SPPS was carried out with an Applied Biosystems peptide synthesizer (model ABI Pioneer). Starting from H-L-Leu-Trt(2-Cl) resin (**8**; 156 mg, 0.32 mmol g^{-1}, 0.05 mmol), each Fmoc-protected amino acid (0.20 mmol) was coupled to the peptide after removal of the Fmoc protecting group on the previously coupled amino acid. Coupling reactions (1 h) were mediated by HATU (0.20 mmol) and iPr$_2$NEt (0.40 mmol), and deprotection was performed by treatment with 20% piperidine in DMF. Amino acids were incorporated in the following order: Fmoc-L-(*N*Me)Leu-OH, Fmoc-L-(*N*Me)Ala-OH, Fmoc-D-Ile-OH, Fmoc-β-(OBn)-D-Leu-OH (**2**), Fmoc-D-(*N*Me)Ala-OH, and Fmoc-L-(*N*Me)Ala-OH for **1a**; Fmoc-L-(*N*Me)Leu-OH, Fmoc-L-(*N*Me)Ala-OH, Fmoc-D-Ile-OH, Fmoc-β-(OBn)-D-Leu-OH (**2**), Fmoc-L-(*N*Me)Ala-OH, and Fmoc-D-(*N*Me)Ala-OH for **1b**. Following the removal of the final Fmoc protecting group, the resin containing the resulting linear peptide was washed with DMF (10 mL), MeOH (10 mL), and CH_2Cl_2 (2×10 mL). The resin was treated with 0.5% TFA in CH_2Cl_2 for 10 min with vibromixing, and the resulting mixture was filtered through cotton wool. The filtrate was concentrated, and the residual paste was purified by column chromatography on silica gel and HPLC.

1a (colorless amorphous solid): ^{1}H NMR (800 MHz, C_6D_6): δ=0.51 (d, J=6.9 Hz, 3H), 0.56 (t, J=6.9 Hz, 3H), 0.62–0.67 (m, 1H), 0.74 (d, J=7.3 Hz, 3H), 0.90 (d, J=6.9 Hz, 3H), 0.93 (d, J=6.9 Hz, 3H), 0.97 (d, J=6.4 Hz, 3H), 0.97–1.01 (m, 1H), 1.17 (d, J=6.4 Hz, 3H), 1.19–1.25 (m, 2H), 1.25 (d, J=6.4 Hz, 3H), 1.28 (d, J=6.9 Hz, 3H), 1.45 (d, J=6.9 Hz, 3H), 1.55 (d, J=5.9 Hz, 3H), 1.61–1.65 (m, 1H), 1.74–1.80 (m, 1H), 1.78–1.82 (m, 1H), 2.07–2.11 (m, 1H), 2.22–2.26 (m, 1H), 2.34–2.38 (m, 1H), 2.59 (s, 3H), 2.67 (s, 3H), 2.78 (s, 3H), 3.29 (s, 3H), 3.98–4.01 (m, 1H), 4.27–4.34 (m, 3H), 5.15–5.19 (m, 1H), 5.17–5.22 (m, 1H), 5.67 (q, J=6.9 Hz, 2H), 5.76 (s, 1H), 6.07 (br s, 1H), 7.61 (d, J=8.2 Hz, 1H), 7.97 ppm (d, J=9.2 Hz, 1H); HRMS (FAB): m/z calcd for $C_{37}H_{67}N_7O_8Na$: 760.4949 $[M+Na]^+$; found: 760.4946.

1b (colorless amorphous solid): ^{1}H NMR (800 MHz, C_6D_6): δ= 0.52 (t, J=6.9 Hz, 3H), 0.53 (d, J=5.9 Hz, 3H), 0.63–0.69 (m, 1H), 0.92 (d, J=7.3 Hz, 3H), 0.98 (d, J=6.5 Hz, 3H), 1.00 (d, J=6.9 Hz, 3H), 1.02 (d, J=6.5 Hz, 3H), 1.06 (d, J=6.5 Hz, 3H), 1.18 (d, J=7.3 Hz, 3H), 1.26–1.30 (m, 1H), 1.31 (d, J=6.9 Hz, 3H), 1.33 (d, J=6.4 Hz, 3H), 1.35–1.38 (m, 1H), 1.46 (d, J=6.9 Hz, 3H), 1.40–1.47 (m, 1H), 1.65–1.68 (m, 1H), 1.73–1.76 (m, 1H), 1.77–1.82 (m, 1H), 1.87–1.91 (m, 1H), 2.07–2.11 (m, 1H), 2.24–2.28 (m, 1H), 2.54 (s, 3H), 2.71 (s, 3H), 2.89 (s, 3H), 3.39 (s, 3H), 3.84–3.89 (m, 1H), 4.17 (t, J=4.8 Hz, 1H), 4.25 (q, J=6.8 Hz, 1H), 4.36–4.40 (m, 1H), 4.91–4.94 (m, 1H), 5.27 (q, J=7.3 Hz, 1H), 5.39 (q, J=6.9 Hz, 1H), 5.44 (q, J=6.9 Hz, 1H), 5.73 (s, 1H), 6.19 (br s, 1H), 7.82 (d, J=7.8 Hz, 1H), 8.46 ppm (d, J=9.2 Hz, 1H); HRMS (FAB): m/z calcd for $C_{37}H_{67}N_7O_8Na$: 760.4949 $[M+Na]^+$; found: 760.4935.

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