Synthesis and inhibitory effect on fat accumulation of (−)-ternatin derivatives modified in the β-OH-D-Leu[7] moiety†

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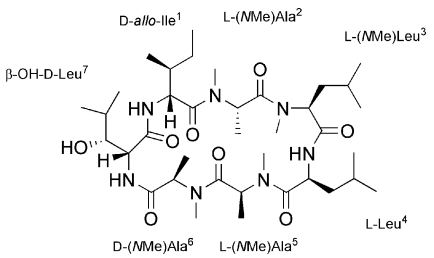
An efficient synthesis of (−)-ternatin derivatives directed toward their SAR at the β-OH-D-Leu[7] moiety and their biological activities against 3T3-L1 murine adipocytes are described.

(−)-Ternatin (**1**) is a highly *N*-methylated cyclic heptapeptide that was isolated from the mushroom *Coriolus versicolor* during our continuing search for potential anti-obesity agents from natural resources such as mushrooms. In our previous paper, we described the isolation, structure elucidation and synthesis of **1**, which potently inhibited fat accumulation against 3T3-L1 murine adipocytes.[1] Additionally, we also reported a concise synthesis of **1** in solution and its *in vivo* biological activity.[2] Treatment with **1** at 5 mg kg^{-1} per day was found to suppress the increase in body weight and fat accumulation in diet-induced obese mice.

To further evaluate **1** as a new lead compound for therapeutic development, we commenced on new research to clarify the detailed mode of action of **1** with regard to fat-accumulation inhibition in adipocytes. Moreover, a structure–activity relationship (SAR) study of **1** was first investigated in parallel, aimed at the recognition of the importance of side chain functionalities as well as a suitable site for advanced functionalization in its structure, *e.g.*, biotinylation and introduction of a fluorescent unit.



(−)-Ternatin (**1**)

Structurally, the existence of non-coded (D- and *N*-methylated) amino acids is a novel feature in **1**. Of particular interest is the effect on biological activity of modifying the unusual β-OH-D-Leu [(2*R*,3*R*)-3-hydroxyleucine], which is a key constituent of **1**. Based on an analysis of the X-ray crystal structure of (−)-ternatin (**1**),[3] it is proposed that **1** adopts type II β-turn structure in the region of L-Leu[4] to β-OH-D-Leu[7] moieties with the assistance of a novel intramolecular H-bond network (Fig. 1A). In this network, the OH group in the β-OH-D-Leu[7] moiety is supposed to contribute toward the stabilization of the macrocyclic conformation of **1** by forming a H-bond between the OH proton (H_C) in that moiety and the C=O in the Ile[1] moiety, whereas two H-bonds of NH protons (H_A, H_B) act strongly on stabilizing β-turn structure. Presumably, these intramolecular H-bonds are key interactions in **1**, since they build the β-turn structure, which is one of the major motifs of peptide and protein secondary structure playing a key role in many biological processes.[4,5]

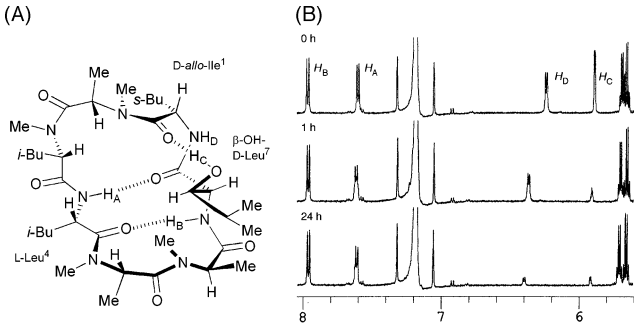


Fig. 1 (A) Stereostructure of **1** fixed by intramolecular H-bond networks (indicated with dotted lines); (B) H–D exchange experiment evaluated in ^{1}H NMR spectrum (600 MHz) in C_6D_6 with addition of D_2O.

In order to demonstrate the intramolecular H-bonds of compound **1** in solution, we evaluated hydrogen–deuterium (H–D) exchange properties of NH and OH protons (Fig. 1B). The experiment was conducted by adding 20 μL of D_2O in a C_6D_6 solution. As a result, NH protons H_A and H_B remained over 24 h, expectedly. Meanwhile, a OH proton H_C and a NH proton H_D smoothly exchanged within 24 h. These results strongly suggested the existence of intramolecular H-bonds of two NH protons, H_A and H_B. However, the possibility of a H-bond of H_C was unclear due to the flexible nature of the OH proton.

To confirm directly whether the β-OH-D-Leu[7] moiety is important for the bioactivity of **1**, chemical modification at this position was first investigated. We describe here the first and efficient synthesis of ternatin derivatives and the SAR at the β-OH-D-Leu[7] moiety with regard to the inhibitory activity of fat accumulation against 3T3-L1 adipocytes.

For this purpose, we designed two types of derivatives, a non-OH series [D-Ala[7]-**1** (**1a**), D-Leu[7]-**1** (**1b**), D-Ser(OBn)[7]-**1** (**1c**)] and a OH series [D-Thr[7]-**1** (**1d**) and D-Ser[7]-**1** (**1e**)], as final targets. The latter compounds were used to realize whether β-OH-D-Leu[7] could be replaced with normal β-hydroxy-α-amino acids such as serine and threonine.

Synthesis of the derivatives **1a–e** was performed in solution by exploiting our efficient synthetic route to **1**, which is amenable to

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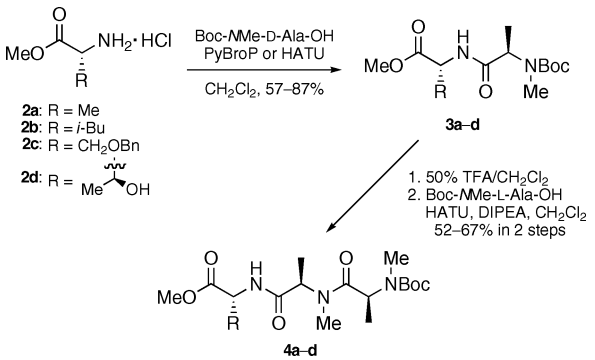
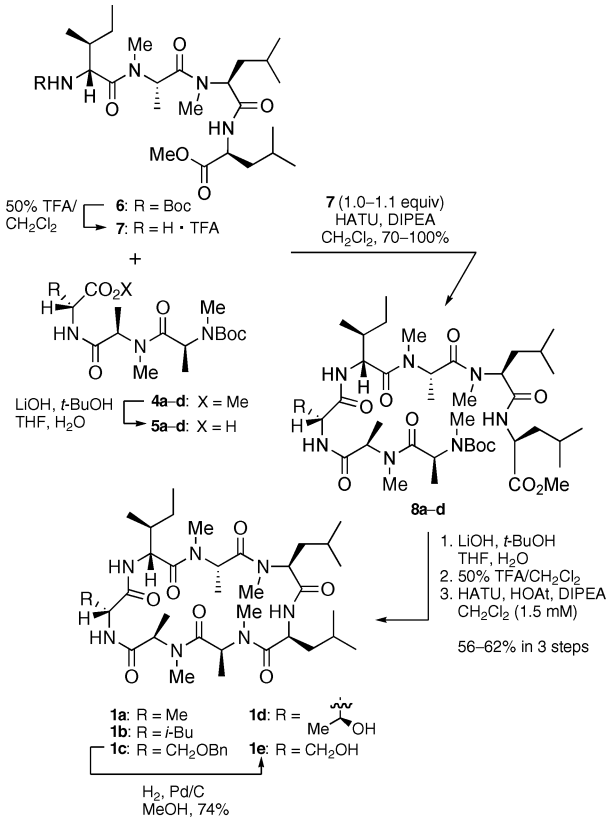
large-scale synthesis for extensive biological evaluations. Beginning with four D-amino acid methyl esters **2a–d**, we assembled tripeptides **5a–d** in the C to N direction (Scheme 1). Couplings of **2a–d** with Boc-*N*Me-D-Ala-OH provided dipeptides **3a–d**. The Boc deprotections followed by second HATU-mediated condensations with Boc-*N*Me-L-Ala-OH gave tripeptides **4a–d**.



Scheme 1 Synthesis of the carboxylic acid fragments **4a–d**.

The final conversions to the desired **1a–d** were elaborated by key fragment couplings and cyclization reactions (Scheme 2). The alanine methyl esters of **4a–d** were cleaved by treatment with LiOH to yield the carboxylic acid fragments **5a–d** as key intermediates. Attempts to couple **5a–d** with amine **7**, prepared by the Boc deprotection of tetrapeptide **6**, proceeded smoothly to provide the linear peptides **8a–d** in 65–100% yields from **5a–d**. The leucine methyl esters were then saponified, and the resulting carboxylic acids were subjected to Boc deprotection. Finally, the key macrolactamizations of the linear peptides were accomplished in the presence of HATU, HOAt and DIPEA under dilute conditions to generate the cyclized products **1a–d** in successful yields (56–62% yields from **8a–d**). Furthermore, modification of compound **1c** was performed. The Bn protecting group of **1c** was removed by hydrogenolysis to give **1e** in 74% yield.

The bioactivities of synthetic derivatives were then evaluated with regard to their inhibitory effect on fat accumulation and cytotoxicity against 3T3-L1 murine adipocytes. Table 1 summarizes the biological activities of derivatives **1a–e** with two controls, (−)-ternatin (**1**) and (−)-noradrenaline (+)-bitartrate salt, respectively. Based on the results, all synthetic derivatives were found to exhibit an inhibitory effect. The potency of inhibition tended to decrease in the order **1b** > **1d** > **1c** > **1a** > **1e**. Interestingly,



Scheme 2 Synthesis of ternatin derivatives **1a–e**.

the simplified D-Leu[7]-derivative **1b** displayed 8-fold lower activity compared to compound **1** which differs only in the presence of the hydroxy group in the β-position of the Leu[7] moiety. In contrast, other derivatives showed less activity (120–520 fold) than **1**, though they still remained active. It is interesting to note that synthetic derivatives possessed almost the same IC_{50} values of fat-accumulation inhibition independent of the presence of the hydroxy group in their structures [a non-OH series (**1a** and **1c**) *vs.* a OH series (**1d** and **1e**)].

With regard to the amino acid in the 7-position in **1**, the results indicated that the specific side chain, *i.e.*, isobutyl group, is strictly required for potent bioactivity. On the other hand, the presence of the hydroxy group in the β-position of the amino acid residue is not absolutely important. Therefore, it may be possible to install

Table 1 Fat-accumulation inhibitory effect of derivatives **1a–e** and cell viability of 3T3-L1 murine adipocytes[a]

Compound	Fat-accumulation inhibitory effect: IC_{50}/μM	Cell viability [b]: IC_{50}/μM
[D-Ala[7]]ternatin (**1a**)	14 ± 4	>150
[D-Leu[7]]ternatin (**1b**)	0.22 ± 0.02	3.3 ± 0.2
[D-Ser(OBn)[7]]ternatin (**1c**)	5.9 ± 1	89 ± 10
[D-Thr[7]]ternatin (**1d**)	3.1 ± 0.4	51 ± 9
[D-Ser[7]]ternatin (**1e**)	12 ± 3	>150
(−)-Ternatin (**1**)	0.027 ± 0.003	0.28 ± 0.03
(−)-Noradrenaline (+)-bitartrate salt	260 ± 6	>300

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

new functional groups at this position, since all derivatives still possess considerable activity.

At 10–15 fold higher concentrations compared to those of IC_{50} values of the fat-accumulation inhibitory effect, cytotoxicity was observed for all derivatives. This may suggest that fat-accumulation inhibition is the direct cause of toxicity at higher concentration. In addition, the fat-accumulation inhibitory effect of compounds may be caused by the prevention of adipogenesis. Further investigation on the mechanism of inhibition is urgently needed.

In conclusion, we have constructed a series of ternatin derivatives modified in the β-OH-D-Leu[7] moiety based on our convergent synthetic route. Actually, unusual β-OH-leucine is a novel class of amino acid that is commonly found in pharmacologically important cyclic peptides such as lysobactin,[6] YM-254890,[7] GE3,[8] and cyclosporin A[9,10] (as MeBmt; (4*R*)-4-[(*E*)-butenyl]-4,*N*-dimethyl-L-threonine). On the other hand, we found that the naturally occurring β-OH-D-Leu[7] in **1** is not absolutely required but is important for the potent inhibitory effect on fat accumulation against 3T3-L1 adipocytes. Further studies on this bioactive molecule are ongoing.

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