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Design, synthesis, and biological evaluation of biotin-labeled (−)-ternatin, a potent fat-accumulation inhibitor against 3T3-L1 adipocytes

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ABSTRACT

The design, synthesis, and biological activity of biotin-labeled (−)-ternatin are reported. Chemical modification, that is, biotinylation, was conducted using Click chemistry at the 6-position (*N*Me-D-ProGly moiety), which was a plausible location selected on the basis of our SAR studies. The compound displayed sufficient fat-accumulation inhibitory effect against 3T3-L1 adipocytes for further bio-organic studies.

Health problems caused by obesity have led to new crises in diseases such as cancer, cardiovascular disease, hypertension, hyperlipidemia and diabetes, and have shortened the life expectancy.[1] Considerable effort has been devoted to the discovery of anti-obesity drugs worldwide. At the cellular level, obesity is characterized by an increase in the number and size of adipocytes differentiated from fibloblastic preadipocytes in adipose tissues.[2] For basic studies on obesity, 3T3-L1 murine preadipocytes, which differentiate into mature adipocytes under appropriate conditions, have been used as a model for adipose cells. For instance, tea chatechins[2] and flavonoids[3] have been reported to suppress adipocyte differentiation (adipogenesis), which lead to the inhibition of fat accumulation in 3T3-L1 adipocytes. In addition, capsaicin induces apoptosis and suppresses adipogenesis in 3T3-L1 cells.[4] However, the effective concentrations of these compounds are rather high (>1 μM). In 2006, (−)-ternatin (**1**, Fig. 1), a highly *N*-methylated cyclic heptapeptide from the mushroom *Coriolus versicolor*, was isolated in the course of our continuing search for fat-accumulation-inhibitors from natural sources.[5] A biological evaluation revealed that **1** potently inhibited fat accumulation against 3T3-L1 adipocytes with an IC_{50} value of 27 nM. Recently, we also reported its novel in vivo bioactivity[6]: **1** suppresses body-weight gain and fat accumulation in diet-induced obese mice, which may be promising lead in the development of anti-obesity drugs. Due to its potent and unique bioactive profile, synthetic studies of **1** have been carried out, and these have enabled large-scale preparation and useful derivatization.[6] Continuing studies of the structure–activity relationship (SAR) provided additional insights regarding further chemical modifications.[7]

To clarify the detailed mechanism of the inhibitory effect of **1** on fat accumulation, we began bio-organic studies on **1**. In general, small molecules are thought to exhibit bioactivity by interacting with receptors in living cells, but this has not yet been clarified for **1**. Therefore, to identify the target bio-molecule that bind to **1**, we planned to use biotin–streptavidin affinity purification which is a classical but reliable solution.[8] For this purpose, biotinylated derivatives with considerable bioactivity must be synthesized.

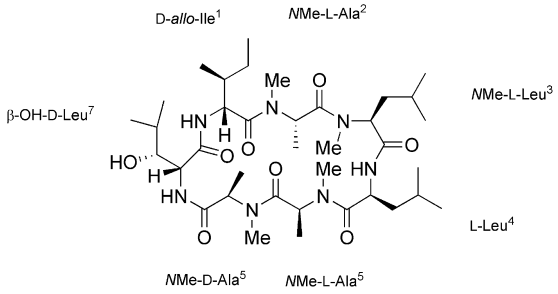

Figure 1. Structure of (−)-ternatin (**1**).

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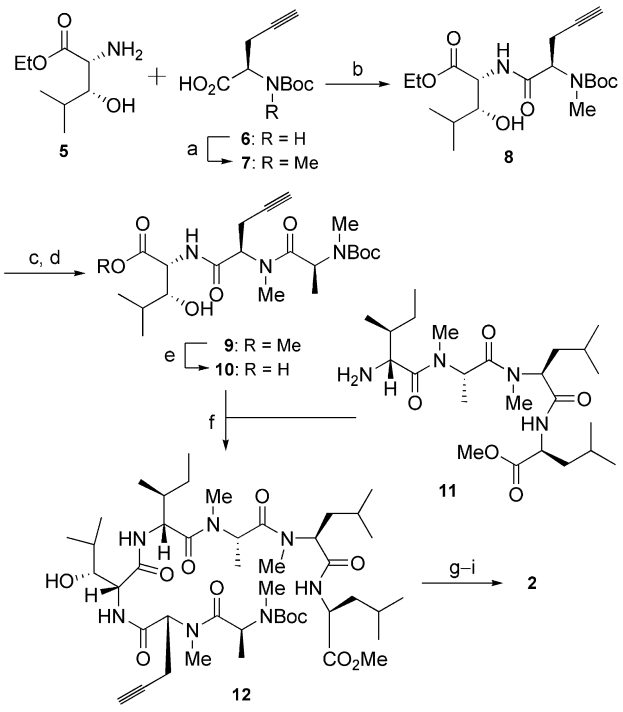
We describe here the design, synthesis, and fat-accumulation inhibitory effects of [*N*Me-D-ProGly (*N*Me-propargylglycine)[6]]-ternatin (**2**) and biotin-labeled ternatin **3** which would be useful in further bio-organic studies.

Initially, we sought to install new functional groups at the 7-position, since the hydroxy group was pre-formatted in natural compound **1**. As described in our 1st-generation SAR study,[7a] the unusual β-OH-D-Leu[7] amino acid moiety was a plausible candidate that did not show a significant loss of bioactivity by replacement with normal amino acids such as Ala, Leu, and Ser. However, esterification between **1** and a tag (biotin-linker conjugate) bearing the carboxyl group was not successful due to the lower reactivity of the hydroxyl group. Similarly, neither of the sterically less-hindered synthetic analogues [D-Ser[7]]-ternatin (IC_{50} 12 μM) and [D-Thr[7]]-ternatin (IC_{50} 3.1 μM) gave adequate results. Recently, however, we clarified the essential and inessential amino acid moieties in **1** for a potent fat-accumulation-inhibitory effect against 3T3-L1 adipocytes, in the 2nd-generation SAR study.[7b] Based on the SAR profile, the 6-position was the best location, since potent bioactivity is believed to retain after chemical modification.

Thus, we planned a concise synthesis of biotin-labeled probe **3**, which is outlined in Figure 2. We envisaged that **3** would be assembled from three key components; cyclic peptide **2**, triethylene glycol-based linker **4**, and (+)-D-biotin. Use of the newly designed analogue [*N*Me-D-ProGly[6]]-ternatin (**2**) enables the rapid installation of a biotin-linker conjugate via Click chemistry, that is, [3 + 2] azide–alkyne cycloaddition, at the final stage.

The synthesis of **2** began with the *N*-methylation of Boc-D-Pro-Gly-OH (**6**) (Scheme 1). The use of an appropriate amount of NaH (2.2 equiv) gave the desired Boc-*N*Me-D-ProGly-OH (**7**).[9] Next, **7** was coupled with β-OH-D-Leu-OEt (**5**)[6] in the presence of HATU to provide dipeptide **8**. Removal of the Boc group of **8** with 50% TFA/CH_2Cl_2 followed by a second HATU-mediated condensation with Boc-*N*Me-L-Ala-OH gave tripeptide **9**. After alkaline hydrolysis of the ester group, the left fragment **10** was obtained. Next, the formation of an amide bond between the two fragments, the carboxylic acid **10** and the amine **11**[6] (a key common fragment), generated heptapeptide **12** in medium yield. Methyl ester hydrolysis followed by Boc deprotection of **12** gave the cyclic precursor. Finally, the key macrolactamization between the amino group in the *N*Me-L-Ala[5] moiety and the carboxyl group in the L-Leu[4] moiety was performed in the presence of HATU (2.0 equiv) and HOAt (2.0 equiv) at low concentration (1.5 mM). After HPLC purification of the reaction mixture, [*N*Me-D-ProGly[6]]-ternatin (**2**)[10] was obtained in 28% yield (from **12**).



Scheme 1. Synthesis of [*N*Me-D-ProGly[6]]-ternatin (**2**). Reagents and conditions: (a) MeI (2.2 equiv), NaH (2.2 equiv), THF, rt, quant.; (b) HATU, DIPEA, CH_2Cl_2, rt, 65%; (c) 50% TFA/CH_2Cl_2, 0 °C; (d) Boc-*N*Me-L-Ala-OH, HATU, DIPEA, CH_2Cl_2, rt, 62%; (e) 1 M NaOH aq, 1,4-dioxane, 0 °C, 48%; (f) right fragment **11** (1.0 equiv), HATU, DIPEA, CH_2Cl_2, 76%; (g) LiOH, H_2O, *t*-BuOH, THF; (h) 50% TFA/CH_2Cl_2, 0 °C; (i) HATU, HOAt, DIPEA, DMF, CH_2Cl_2 (1.5 mM), 2 days, 28% in 3 steps.

Synthesis of the probe **3** is shown in Scheme 2. First, triethylene glycol derivative **4** was prepared as a linker. We anticipated that **4** would be helpful for increasing the solubility of the target probes in aqueous solution. The azide alcohol **14**, readily prepared from commercially available triethylene glycol (**13**),[11] was subjected to hydrogenation followed by Boc protection of the resulting amino group to afford alcohol **15**. Next, **15** was converted into azide **16** by sequential mesylation and azidation. The Boc group of **16** was cleaved under acidic conditions to provide linker **4** as a TFA salt.

Next, the functional unit, that is, biotin, was incorporated. Biotin-linker conjugate **17** was synthesized by HATU-mediated coupling of linker **4** and (+)-D-biotin in quantitative yield after HPLC

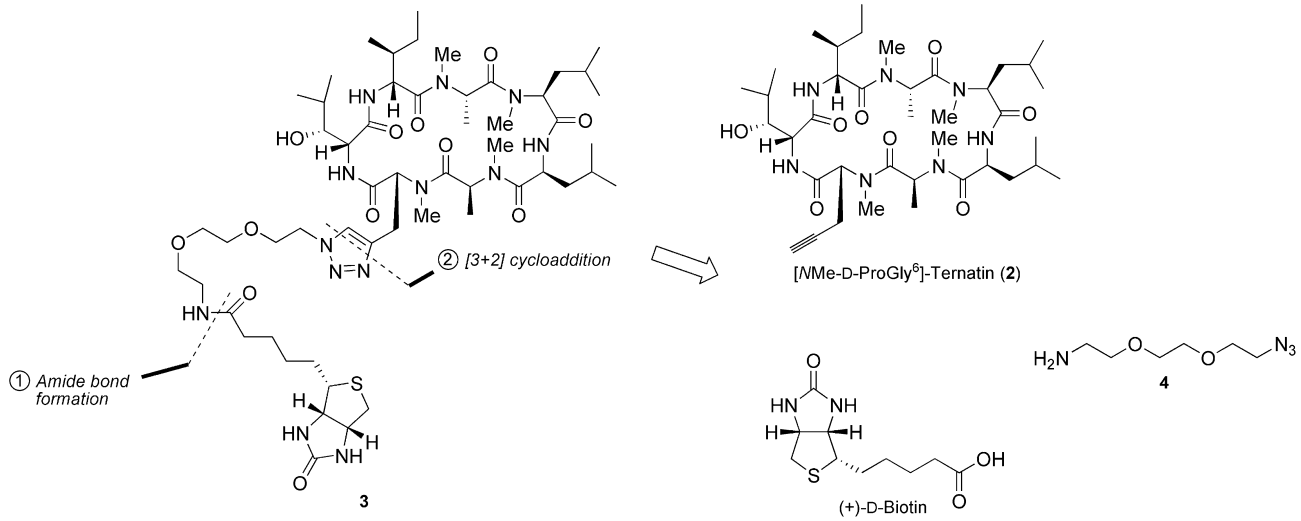
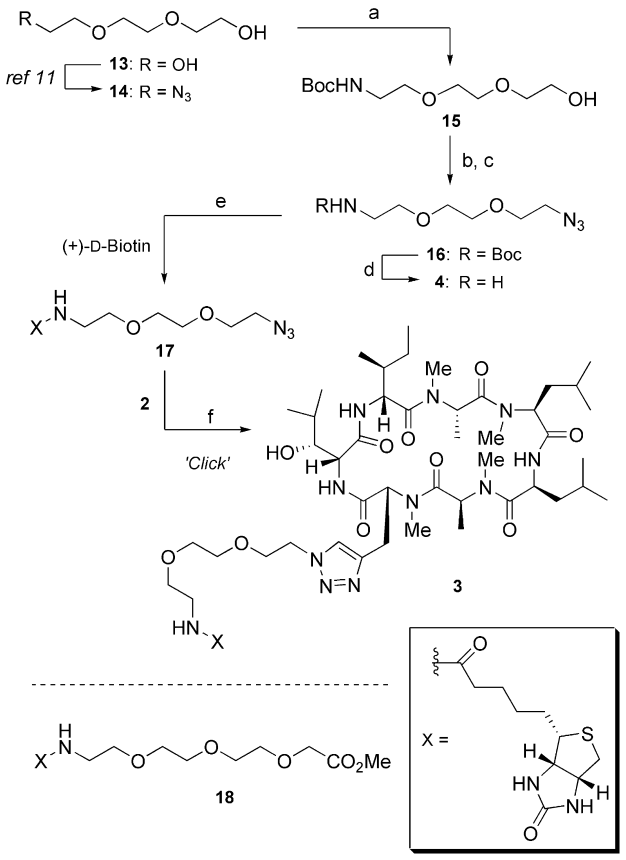


Figure 2. Synthetic design of biotin-labeled ternatin **3**.



Scheme 2. Synthesis of biotin-labeled ternatin **3**. Reagents and conditions: (a) H_2, 5% Pd/C, Boc_2O, MeOH, quant.; (b) MsCl, NEt_3, THF; (c) NaN_3, DMF, 50 °C, 64% in 2 steps; (d) 50% TFA/CH_2Cl_2; (e) (+)-D-biotin, HATU, DIPEA, DMF, quant. in 2 steps; (f) $CuSO_4$, sodium ascorbate, *t*-BuOH, H_2O, 57%.

purification. Finally, the alkyne–azide cycloaddition reaction[12] of **2** and **17** was performed by using $CuSO_4$ and sodium ascorbate in water, which provided desired **3**[13] in moderate yield. In addition, we prepared biotin-linker conjugate **18**[14] to examine the influence of its chemical structure on bioactivity (as a negative control).

The three synthetic derivatives, [*N*Me-D-ProGly^6]-ternatin (**2**), biotin-labeled ternatin **3** and biotin-linker conjugate **18**, were assessed with regard to their fat-accumulation-inhibitory effect against 3T3-L1 adipocytes (Table 1). The bioassay consisted of the treatment of confluent 3T3-L1 preadipocytes with each sample and further incubation for 7 days. After this period, control cells were completely differentiated into mature adipocytes. Both the rate of fat accumulation and the cell viability of adipocytes treated with each sample were calculated. Among the samples tested, no cytotoxicity was observed at the concentration that gave 50% fat-accumulation inhibition (IC_{50}).

Table 1
Fat-accumulation inhibitory effect of synthetic compounds **2**, **3**, and **18** and cell viability of 3T3-L1 adipocytes.[a]

Compound	Fat-accumulation inhibitory effect: IC_{50} (μM)	Cell viability: IC_{50} (μM)
(−)-Ternatin (**1**)	0.027 ± 0.003	0.28 ± 0.03
[*N*Me-D-ProGly^6]-ternatin (**2**)	0.019 ± 0.001	> 5.2[b]
3	46 ± 2.6	>86
18	>160	>160

[a] Values are means of quadruplicate determinations.
[b] 54% inhibition at 5.2 μM. Not tested at higher concentrations.

Based on the results, compound **2** showed 1.4-fold more potent bioactivity than natural **1**, which entirely agreed with our previous SAR profile. On the other hand, it was found that **2** is considerably less toxic compared to **1**. The bioactivity of probe **3** (IC_{50} for fat accumulation: 46 μM) was 1700-fold weaker than that of **1**, however, it is still potent enough to be used in further applications as a chemical probe. Consequently, the modification at the 6-position, which was defined in our whole SAR study, provided generally potent bioactivity. Meanwhile, **18** did not exhibit any bioactivity with regard to fat-accumulation-inhibition or cytotoxicity (both IC_{50}: > 160 μM). This evidence shows the chemical structure responsible for bioactivity is the peptide core.

In summary, we designed highly bioactive [*N*Me-D-ProGly^6]-ternatin (**2**) bearing an alkyne group for chemical modification. The use of 'Click Chemistry' enabled the rapid and reliable incorporation of a biotin unit. Bioactivity of bioin-labeled ternatin **3** was potent enough for its application in bio-organic studies. Further studies on this topic are now in progress.

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9. The use of excess NaH resulted in complex mixture of *N*-methylated and *C*,*N*-dimethylated products (*C*-methylation was occurred at the terminal alkyne group). For example, the use of 3.3 equiv NaH afforded the mixture in 1:1 ratio, which cannot be separated by silica-gel column chromatography.
10. Spectroscopic data for **2**: $[\alpha]^{28}_{D}$ −53.1° (*c*, 0.58, $CHCl_3$); IR ($CHCl_3$) 3346, 3308, 3008, 2963, 2870, 1635, 1507, 1411, 1078 cm^{-1}; ^1H NMR (600 MHz, C_6D_6) δ 7.92 (d, *J* = 9.5 Hz, 1H), 7.54 (d, *J* = 8.8 Hz, 1H), 6.21 (d, *J* = 7.0 Hz, 1H), 5.88 (d, *J* = 3.7 Hz, 1H), 5.77 (dd, *J* = 11.3, 4.7 Hz, 1H), 5.67 (q, *J* = 6.6 Hz, 1H), 5.25–5.17 (m, 1H), 5.08 (t, *J* = 9.5 Hz, 1H), 4.48 (dd, *J* = 7.3, 3.0 Hz, 1H), 4.36 (q, *J* = 7.3 Hz, 1H), 4.29 (dd, *J* = 11.3, 4.0 Hz, 1H), 3.95–3.90 (m, 1H), 3.29 (s, 3H), 3.05 (ddd, *J* = 17.6, 4.7, 2.9 Hz, 1H), 2.79 (s, 3H), 2.694 (s, 3H), 2.689 (s, 3H), 2.48 (ddd, *J* = 17.6, 11.3, 2.9 Hz, 1H), 2.38–2.32 (m, 1H), 2.14 (quint., *J* = 6.6 Hz, 1H), 2.10–2.04 (m, 1H), 1.83–1.73 (m, 2H), 1.67–1.60 (m, 1H), 1.60 (t, *J* = 2.9 Hz, 1H), 1.50–0.60 (m, 4H) 1.48 (d, *J* = 7.0 Hz, 3H), 1.43 (d, *J* = 6.6 Hz, 3H), 1.25 (d, *J* = 6.6 Hz, 3H), 1.16 (d, *J* = 6.6 Hz, 3H), 0.97 (d, *J* = 6.6 Hz, 3H), 0.93 (d, *J* = 6.6 Hz, 3H), 0.90 (d, *J* = 7.0 Hz, 3H), 0.88 (d, *J* = 7.3 Hz, 3H), 0.60 (d, *J* = 7.0 Hz, 3H), 0.58 (t, *J* = 7.3 Hz, 3H); ^{13}C NMR (150 MHz, C_6D_6) 175.3, 174.4 (2C), 174.3, 173.7, 168.8, 168.2, 76.1, 71.8, 69.9, 59.2, 56.2, 55.18, 55.11, 51.4, 49.84, 49.79, 40.5, 38.9, 37.9, 33.7, 30.7, 30.3, 29.7, 29.3, 26.7, 26.0, 25.3, 23.8, 23.3, 22.6, 21.34, 21.30, 18.5, 15.8, 14.8, 13.5, 13.2, 11.6; HRMS (FAB) calcd for $C_{39}H_{67}N_7O_8Na$ $(M+Na)^+$ 784.4949, found 784.4954.
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13. Spectroscopic data for **3**: ^1H NMR (600 MHz, CD_3OD) δ 7.94 (d, *J* = 8.5 Hz, 1H), 7.77 (s, 1H), 7.72 (d, *J* = 9.2 Hz, 1H), 5.54 (dd, *J* = 11.7, 4.4 Hz, 1H), 5.52 (q, *J* = 7.0 Hz, 1H), 5.94–5.86 (m, 3H), 4.62 (d, *J* = 3.7 Hz, 1H), 4.55 (s, 1H), 4.50 (q,

J = 5.1 Hz, 2H), 4.47 (dd, J = 7.7, 4.0 Hz, 1H), 4.29 (dd, J = 7.7, 4.4 Hz, 1H), 4.10 (dd, J = 11.0, 4.0 Hz, 1 H), 3.85 (t, J = 5.1 Hz, 2H), 3.61–3.55 (m, 6H), 3.50 (t, J = 5.5 Hz, 2H), 3.38–3.32 (m, 4H), 3.22–3.16 (m, 1H), 3.09 (s, 3H), 3.07 (s, 3H), 2.98 (s, 3H), 2.94 (s, 3H), 2.91 (dd, J = 12.8, 4.7 Hz, 1H), 2.69 (d, J = 12.8 Hz, 1H), 2.19 (t, J = 7.7 Hz, 2H), 2.09 (ddd, J = 15.8, 11.0, 4.7 Hz, 1H), 1.94–1.86 (m, 1H), 1.85–1.81 (m, 1H), 1.76–1.39 (m, 12H), 1.22–1.17 (m, 1H), 1.20 (d, J = 6.0 Hz, 3H), 1.15 (d, J = 7.3 Hz, 3H), 1.03 (d, J = 7.7 Hz, 3H), 1.02 (d, J = 7.0 Hz, 3H), 0.99 (d, J = 7.3 Hz, 3H), 0.98 (t, J = 7.3 Hz, 3H), 0.97 (d, J = 6.6 Hz, 3H), 0.93 (d, J = 6.6 Hz, 3H), 0.91 (d, J = 5.8 Hz, 3H), 0.90 (d, J = 6.6 Hz, 3H), two amide protons in biotin moiety and a hydroxy proton were not observed; ^{13}C NMR (150 MHz, CD_3OD) 183.5, 177.6, 175.0, 174.2, 174.1, 171.3, 171.2, 170.3, 170.2, 151.9, 145.0, 124.6, 77.6, 76.7, 71.5, 71.3, 70.7, 70.5, 63.4, 61.6, 60.3, 57.9, 57.0, 56.8, 56.1, 53.0, 51.3, 51.2, 50.7, 41.0, 40.9, 40.4, 39.2, 36.8, 35.0, 31.9, 31.5, 30.4, 30.2, 29.8, 29.7, 29.5, 27.6, 26.8, 26.6, 26.2, 24.0, 23.7, 23.2, 21.22, 21.19, 15.5, 15.0, 14.3, 12.1; HRMS (FAB) calcd for $C_{55}H_{96}N_{13}O_{12}S$ $(M+H)^+$ 1162.7022, found 1162.7008.

14. Compound **18** was synthesized as shown below.