Application of FITC-labeled Ternatin on Its Cellular Localization in 3T3-L1 Murine Preadipocytes

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The cellular localization of a potent fat-accumulation inhibitor (−)-ternatin was investigated. FITC-labeled ternatin, a chemical probe for current study, was synthesized from a highly bioactive ternatin analogue using Click Chemistry, which was found to be bioactive. The treatment of 3T3-L1 murine preadipocytes with synthetic probe revealed that (−)-ternatin is localized in a specific organelle of 3T3-L1 cells.

(−)-Ternatin (**1**, Figure 1) is a highly N-methylated cyclic heptapeptide that was isolated from the mushroom *Coriolus versicolor* in our continuing search for potential antiobesity drugs from natural sources.[1] The in vitro biological evaluation revealed that **1** potently inhibited fat-accumulation against 3T3-L1 murine adipocytes. In addition, we also demonstrated the inhibitory effect of fat accumulation in vivo, which led to suppression of body-weight gain in high-fat-diet induced obese mice by treatment with **1** (5 mg/kg/day) for 5 weeks.[2] Therefore, **1** could be considered as a plausible lead compound for antiobesity drugs. However, both the biological mechanism of **1** and its cellular target still remained unknown.

As an initial effort toward bioorganic studies on **1**, we recently reported its structure–activity relationships, which revealed key amino acid residues (Ile[1] and Leu[4]) responsible for potent bioactivity.[3] These SAR profiles enabled further chemical modification, i.e., installation of functional groups, in appropriate positions in the chemical structure of **1**. In a previous paper, we reported [*N*Me-D-ProGly[6]]-ternatin (**2**, Figure 1) as a highly bioactive analogue of **1**, which was applied to the synthesis of biotin-labeled ternatin as a chemical probe for identification of bio-molecules that bind to **1**.[4] As a part of our on-going bioorganic studies on **1**, we describe here the synthesis and biological evaluation of FITC-labeled ternatin **3**, and its cellular localization in 3T3-L1 murine preadipocytes.

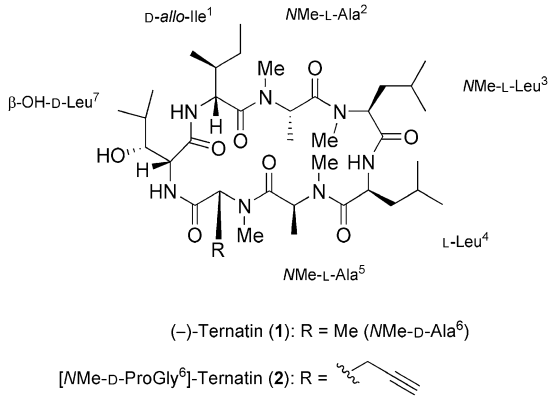
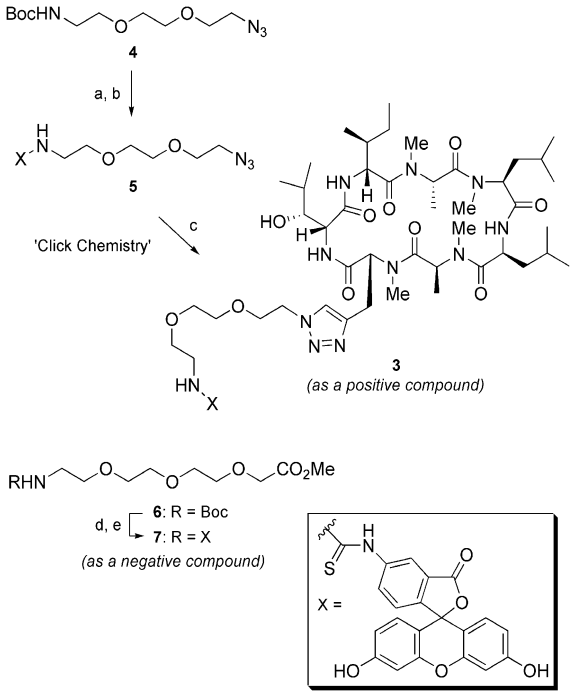


Figure 1. Structures of **1** and **2**.



Scheme 1. a) 50% TFA/CH_2Cl_2; b) FITC, NEt_3, DMF, 59% in 2 steps; c) **2** (1.0 equiv), $CuSO_4$, sodium ascorbate, *t*-BuOH, H_2O, 58%; d) 50% TFA/CH_2Cl_2; e) FITC, NEt_3, DMF, quant. in 2 steps.

On the basis of the synthetic route to biotin-labeled compounds[4] which feature the Cu-catalysed Huisgen reaction (Click Chemistry),[5] we thought that **3** could be synthesized from three components, **2**, FITC (fluorescent isothiocyanate), and linker **4**, which is shown in Scheme 1. First, compound **4**, prepared from triethylene glycol in five steps, was subjected to Boc deprotection under acidic conditions. The resulting amine (as a TFA salt) was coupled with FITC in the presence of NEt_3 to provide the FITC-linker conjugate **5**. Finally, the Cu-catalyzed Huisgen reaction of **5** with alkyne **2** using $CuSO_4$ and sodium ascorbate in *t*-BuOH/H_2O generated **3**. After HPLC purification of the reaction mixture, the desired **3** was obtained in 58% yield. On the other hand, another FITC-linker conjugate **7** which bears a methyl ester group instead of the peptide core in **3** was synthesized as a negative compound in our study. The Boc deprotection of **6** followed by coupling with FITC afforded **7**.

Table 1. Fat-accumulation inhibitory effects of compounds **1**–**3** and **7**, and cell viability of 3T3-L1 adipocytes[a]

Compound	Fat-acumulation inhibitory effect: IC_{50} /μM	Cell viability: IC_{50} /μM
(−)-Ternatin (**1**)	0.027 ± 0.003	0.28 ± 0.03
2	0.019 ± 0.001	>5.2[b]
3	23 ± 1.5	>75[b]
7	>164[b]	>164[b]

[a]Values are means of quadruplicate determinations. [b]Not tested at higher concentrations.

The in vitro fat-accumulation inhibitory effects for synthetic compounds **3** and **7** were then assessed (Table 1). 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, control cells were differentiated into mature adipocytes. Both the rates of fat accumulation and cell viability were calculated to identify undesired fat-accumulation inhibition caused by cytotoxicity.

On the basis of the results, **3** showed moderate inhibitory effect on fat accumulation with an IC_{50} value 23 μM and no significant cytotoxicity at tested concentrations (IC_{50} > 75 μM), which suggested that **3** was potent enough to be applied for further bio-organic investigation. Meanwhile, **7** did not show any inhibitory effect or cytotoxicity (IC_{50} > 164 μM).

Visualization of the intracellular localization of synthesized compounds was conducted using fluorescence microscopy (Eclipse TE200; Nikon, Tokyo, Japan), which is shown in Figure 2.[6] For this examination, we employed 3T3-L1 murine preadipocytes. Our recent research demonstrated that **1** shows its inhibitory effects against fat-accumulation by suppressing the differentiation of 3T3-L1 cells in the early stage. Therefore, **1** was thought to show its biological effects in 3T3-L1 murine preadipocytes.

By the fluorescence microscopic analysis, a certain rate of 3T3-L1 cells treated with **3** showed strong fluorescence under UV (Figure 2b). The pattern of the fluorescence was partial and spot-like inside the cells. On the other hand, no fluorescence was observed when cells were treated with **7** (Figure 2d). These results suggested that **3** was incorporated into cells and localized in a specific organelle of 3T3-L1 cells. It was confirmed that the organelle where **3** accumulated was not identical to nucleus by **3** and DAPI co-staining analysis (data not shown). The part of localization is now under investigation. Thus, FITC-labeled (−)-ternatin was demonstrated to localize in a specific organelle of 3T3-L1 cells, and then, (−)-ternatin is likely to function there to show its biological activity.

In summary, we synthesized FITC-conjugated ternatin **3** using Click Chemistry, and examined its inhibitory effect against the fat accumulation in 3T3-L1 cells. Next, we surveyed its intracellular localization in 3T3-L1 cells using fluorescence microscopic analysis. **3** was observed to localize in a specific organelle of 3T3-L1 cells, and thus, **3** will be a useful tool for the analysis of the mechanism of action and the identification of the target molecules of **1**.

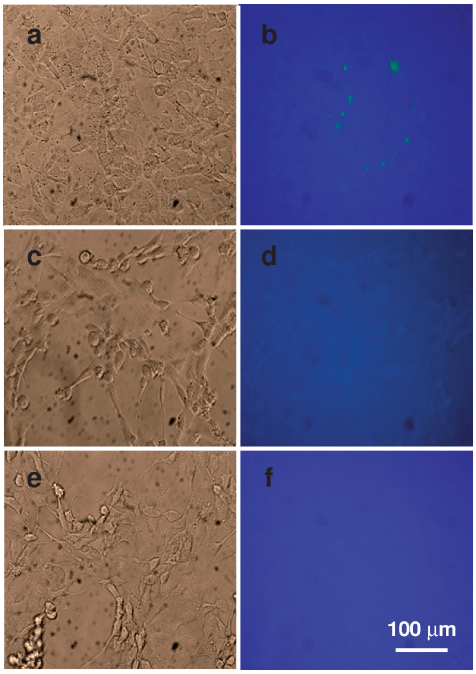


Figure 2. Cellular localization of FITC-labeled ternatin **3** in 3T3-L1 murine preadipocytes analyzed by fluorescence. (a) Cells treated with **3** for 18 h; (b) fluorescence image of a; (c) cells treated with **7** for 18 h; (d) fluorescence image of c; (e) control cells (no additive); (f) fluorescence image of e.

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6 3T3-L1 preadipocytes were inoculated at 5 × 10^4 cells/well in 24-well plates, and incubated with 25 μM of **3** or **7** for 18 h. After washing, cells were analyzed by phase contrast (left panels) and fluorescence (right panels) microscopy. The cells were photographed at a magnification of 200×.

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