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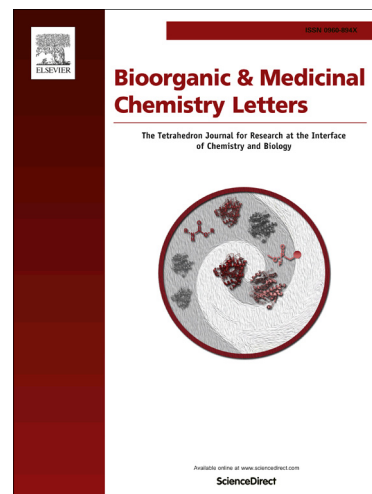
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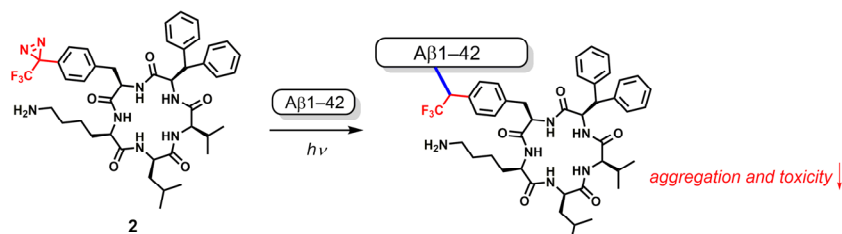
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Covalent modifier-type aggregation inhibitor of amyloid- β based on a *cyclo*-KLVFF motif

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ABSTRACT

Inhibition of amyloid- β (A β) aggregation could be a drug development target for treating Alzheimer disease. Insufficient activity to inhibit aggregation, however, remains a key issue. Here, we report a covalent modifier-type aggregation inhibitor of A β , diazirine-equipped *cyclo*-KLVF(β -Ph)F (**2**). Due to the affinity of the *cyclo*-KLVFF motif for A β , **2** selectively reacted with A β 1–42 under UV-light irradiation to form an irreversible covalent bond. The Tyr-10 residue of A β 1–42 was identified as the covalent modification site with **2**. The extent of cross- β -sheet structure, characteristics of amyloid aggregation, and toxicity of A β 1–42 were strongly attenuated by this chemical modification.

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A pathologic hallmark of Alzheimer disease (AD), an age-related neurodegenerative disorder characterized by progressive memory loss and cognitive impairment, is the depositions of senile plaque in the brain. Senile plaque results from the aggregation of amyloid- β (A β), produced by proteolytic processing of amyloid precursor protein.¹ A β comprises mainly 40- and 42-residue peptides, designated A β 1–40 and A β 1–42, respectively, and A β 1–42 is far more aggregative and toxic than A β 1–40.² Thus, A β 1–42 aggregation is considered to be associated with the onset and development of AD, and based on this hypothesis, a number of A β -targeted therapeutic approaches have been investigated.³ Some compounds that inhibit A β -producing enzymes are currently under clinical investigation.⁴ Continued A β -targeted drug development is crucial to improving the treatment of this debilitating disease.

A compound that inhibits A β aggregation is a potential drug candidate for treating AD. In this context, Nordstedt *et al.* reported that Lys–Leu–Val–Phe–Phe (KLVFF), corresponding to the A β 16–20 fragment, inhibits the aggregation of full-length A β .⁵ The fragment peptide seems to bind with the corresponding self-recognition region of A β via hydrogen bonding and hydrophobic interactions to interfere with A β aggregation. In an effort to improve the inhibitory activity, a number of derivatizations have been reported.⁶ We recently reported that

stronger inhibitory activity could be realized via cyclization of KLVFF in a head-to-tail fashion.⁷ A more potent inhibitor (**1**, Figure 1), in which an additional phenyl group is introduced to the side chain of Phe-4 residue, has been also identified.⁸ Nevertheless, pursuing a stronger inhibitor of A β aggregation remains a key issue in drug development for the treatment of AD.

One strategy for increasing the anti-aggregation activity includes derivatization to a covalent modifier.⁹ Strong inhibitory activity can be realized by forming irreversible covalent bonds between the inhibitor and target protein. For example, a papain-affinity molecule equipped with a Michael acceptor (β -substituted methyl acrylate) unit exhibits a potent papain inhibitory activity via its reaction with a Cys thiol group of the protein.¹⁰ As the reactivity of the Michael acceptor is low enough to avoid reactions with thiols of other proteins without any affinity to the molecule, high selectivity for the target protein compared to off-target molecules is achieved. In addition, in combination with X-ray crystallography, Solca *et al.* designed a sophisticated covalent modifier derived from an anti-cancer drug afatinib, in which the Michael acceptor unit is placed proximate to a thiol group of the target protein.¹¹ We considered that the covalent modifier approach would be effective for potent inhibition of protein-protein interactions, in which effective inhibition by small molecules is often difficult to achieve.^{12, 13, 14}

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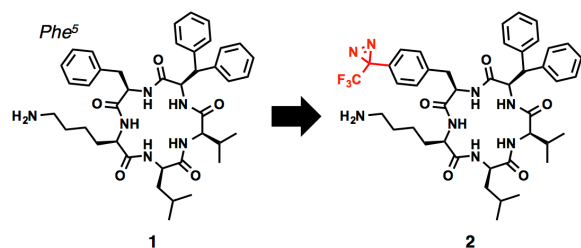


Figure 1. Structures of **1** and **2**.

In the present study, to improve the inhibitory activity of **1**, we introduced a covalent modifier unit, diazirine¹⁵, to the side chain of the Phe-5 residue (**2**, Figure 1). The diazirine moiety usually does not react with any functional groups of natural proteins, but under UV-light irradiation, it generates a carbene intermediate, that irreversibly reacts with proximate bonds to form a covalent bond. Due to the short lifetime of carbene, it reacts with only proximate amino acid residues, and otherwise a reaction occurs with environmental water to quench the reactivity. Based on these features, the diazirine moiety attached to a ligand can be utilized to probe target proteins.¹⁶ Due to unknown three-dimensional structures of A β aggregates and the lability of the A β conformation during aggregation, fine-tuning to place conventional and less reactive covalent modifier units, such as a β -lactam unit targeting Ser side chain hydroxy group,¹⁷ on a

specific residue of A β is difficult. Thus, we considered that the diazirine moiety might be a suitable covalent modifier unit to inhibit A β aggregation, due to the high reactivity of the generated carbene with a variety of bonds.

To synthesize diazirine-equipped **2** (Scheme S1), the corresponding linear peptide was prepared by 9-fluorenylmethyloxycarbonyl (Fmoc)-based solid phase peptide synthesis, and cyclization of the resulting peptide was achieved using a condensation reagent under high-dilution conditions. Removal of the Fmoc group at the Lys side chain, and final purification using reverse-phase high-performance liquid chromatography gave the desired **2**.

A β 1–42 (10 μ M) and **2** (200 μ M) were dissolved in a phosphate buffer (0.1 M, pH 7.4), and incubated at 37 $^{\circ}$ C for various periods of time (0 min, 20 min, 60 min, and 120 min). The solutions were irradiated with UV light (365 nm) for 10 min, and then the reaction mixtures were analyzed using MALDI-TOF MS after enzymatic digestion with endoproteinase Lys-C (C-terminal side of Lys residue is cleaved). A peak derived from the **2**-adduct of the A β 1–16 fragment was observed, and the relative intensity of the peak to unreacted A β 1–16 tended to increase with an increase in the incubation time (10% at 0 h; 20% at 20 min; 36% at 60 min; 24% at 120 min; Figure 2). On the other hand, the peaks from **2**-adducts of A β 17–28 and A β 29–42 were below the detection limit (Figure 2a). These findings suggest that **2** reacted with the A β 1–16 fragment of A β 1–42, and the reaction efficiency increased as the degree of aggregation of A β 1–42 increased (up to 60 min) possibly due to the higher affinity of **2** with the aggregates than monomers. As controls, when *cyclo*-A β 1–5 (Asp-Ala-Glu-Phe-Arg) containing diazirine at Phe-4 and Boc-protected Phe with diazirine (for the structures: see Figure S2) were subjected to the identical reaction conditions, no adduct

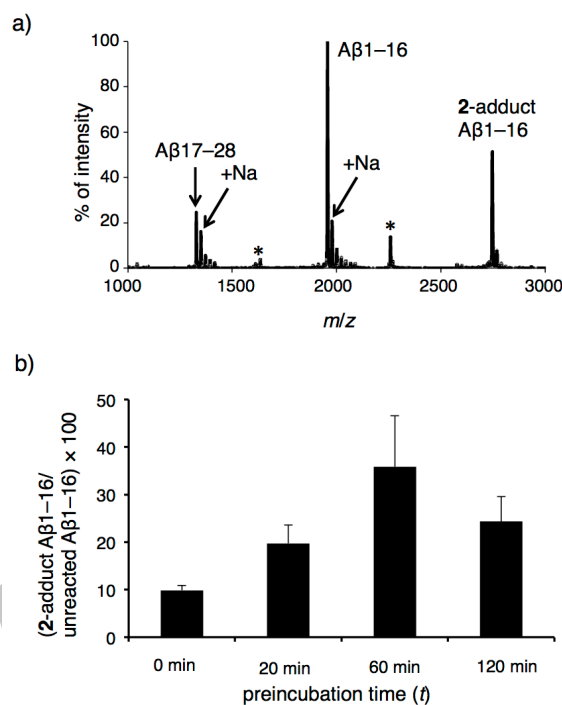


Figure 2. (a) MALDI-TOF MS spectra of the reaction mixture after enzymatic digestion with Lys-C (A β 1–42: 10 μ M, **2**: 200 μ M, pH 7.4, 37 $^{\circ}$ C, preincubation time: 120 min, UV-light irradiation: 10 min). *unidentified peaks. As A β 29–42 was not detected in MALDI-TOF MS due to its poorly ionizable character, the fragment was analyzed using ESI MS, and intact A β 29–42 was detected, but not the **2**-adduct derivative (Figure S1); (b) relative MS peak intensity of **2**-adduct A β 1–16 versus unreacted A β 1–16 of the reaction mixtures with various preincubation times ($n = 3$, mean \pm s.d.).

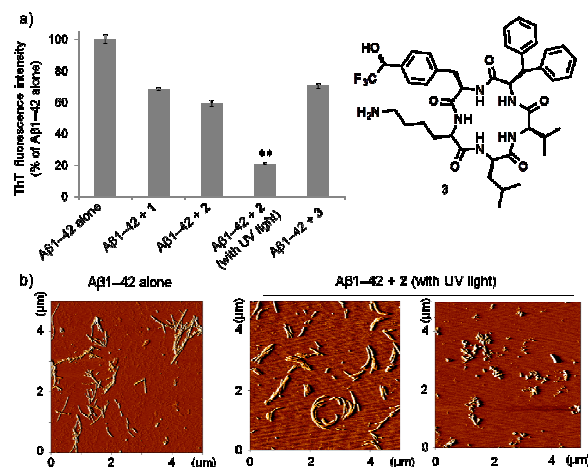


Figure 3. (a) Relative ThT fluorescence intensity to that of A β 1–42 alone (10 μ M). A β 1–42 (10 μ M) was incubated with **1**, **2** or **3** (50 μ M) in 0.1 M phosphate buffer (pH 7.4, 2% v/v DMSO) at 37 $^{\circ}$ C for 60 min. In “A β 1–42 + **2** (with UV light)”, UV light was irradiated for 10 min, from $t = 20$ min to 30 min after starting the incubation. We checked that the value of ThT fluorescence intensity derived from A β was not changed with or without UV light irradiation. $n = 3$, mean \pm s.d., *** $p < 0.01$ (vs. “A β 1–42 + **1**”, “A β 1–42 + **2**”, and “A β 1–42 + **3**”, Dunnett’s test); (b) Atomic force microscopy images of A β 1–42 alone (10 μ M, left panel) and A β 1–42 + **2** (with UV light, 10 μ M + 50 μ M, center and right panels), incubated in 0.1 M HEPES-0.1 M NaCl buffer (pH 7.4, 1% v/v DMSO) at 37 $^{\circ}$ C for 12 h. Two representative images are shown for A β 1–42 + **2** (with UV light).

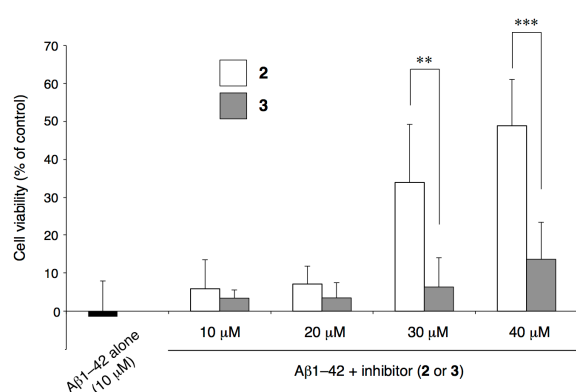


Figure 4. Concentration-dependent effects of **2** and **3** on the toxicity of Aβ1-42. The phosphate buffer saline solution (1% v/v DMSO) with Aβ1-42 alone (10 μM) or Aβ1-42 (10 μM) + **3** (10–40 μM) was incubated at 37 °C for 30 min. Aβ1-42 (10 μM) + **2** (10–40 μM) was incubated at 37 °C for 20 min, and UV light was successively irradiated for 10 min. Then, the solutions were added to the culture medium with the PC12 cells, incubated at 37 °C for 48 h, and cell viability was measured ($n = 5$, mean \pm s.d.; ** $p < 0.01$ and *** $p < 0.001$ by Student's t -test).

of the reactants into Aβ1-42 was detectable. These findings suggest that the *cyclo*-KLVF(β-Ph)F motif of **2** serves as an affinity ligand to Aβ1-42. Additionally, when Aβ1-16 was used instead of Aβ1-42, no **2**-adduct was produced, suggesting that the full-length component of Aβ to aggregate is necessary for the reaction with **2**. Finally, LC-MS/MS analysis was performed to determine the covalently modified site of Aβ1-42 with **2**. Because we could not determine the modified site with the Aβ1-16 fragment due to insufficient intensity, we used Glu-C to digest the Aβ1-42 derivative, and LC-MS/MS analysis of the resulting **2**-adduct of Aβ4-11 fragment revealed that the insertion of **2** occurred at the Tyr-10 residue (Figure S3).

We next examined the Aβ-selectivity of **2** using angiotensin-IV, neuropeptide S, and human insulin as off-targets (Figures S4). When a buffer solution (pH 7.4) containing each peptide substrate (10 μM) and **2** (200 μM) was irradiated with UV light at 37 °C, no **2**-adduct was produced in any case. Thus, **2** reacted with Aβ1-42 in a highly selective manner.

The effect of the covalent modifier on the aggregation of Aβ1-42 was evaluated. The fluorescence intensity (FI) of thioflavin T (ThT) dye corresponds to the extent of cross-β-sheet structure characteristics of amyloid aggregation.¹⁸ When **2** (50 μM) was co-incubated with Aβ1-42 (10 μM) for 60 min (0.1 M phosphate buffer, pH 7.4, 37 °C), the FI was 59% compared to the control without inhibitor (100%, designated "Aβ1-42 + **2**", Figure 3a). The FI of **2** was similar to that of **1** (68%). The decreased FI suggests potent inhibitory activity of **2** on Aβ aggregation. At $t = 20$ min after starting the incubation, the solution was irradiated with UV light for 10 min, and the FI at $t = 60$ min was evaluated [designated as "Aβ1-42 + **2** (with UV light)", Figures 3a and S5]. The FI value was 21% (Figure 3a), and the decreased FI value was maintained for up to $t = 4$ h (Figure S5). Additionally, we checked the inhibitory activity of an alcohol derivative (**3**), produced by the reaction of the carbene intermediate of **2** with water, prepared via UV-light irradiation (10 min) of **2**-containing buffer solution in the absence of Aβ. When Aβ1-42 was added to the **3**-containing solution, the time-course profile of the FI was indistinguishable from that of **2** without UV-light irradiation (Figures 3a and S5). Thus, the FI value of **2** with UV light (21%) at 60 min was significantly lower

than that of **2** without UV light or **3**, suggesting that stronger inhibitory activity to the cross-β-sheet formation was induced by the formation of covalent bonds between Aβ and **2**. Atomic force microscopy analysis also indicated that the formation of typical amyloid fibrils in Aβ1-42 alone was not observed by the presence of **2** with UV-light irradiation (Figure 3b). The modification at Tyr-10 by **2** would associate with the attenuation of the Aβ aggregation as it was suggested that Tyr-10 participated in the pathogenicity of Aβ.^{19,20}

We further examined effects of the covalent modification on the toxicity of Aβ1-42, using rat PC12 cells (Figure 4). Phosphate buffer saline solution with Aβ alone or Aβ + **3** was incubated at 37 °C for 30 min. Aβ + **2** was incubated at 37 °C for 20 min, and UV light was successively irradiated for 10 min. The solutions were then added to the culture medium with the cells, incubated at 37 °C for 48 h, and the cell viabilities were measured. Whereas most of the cells died when treated with Aβ1-42 alone, cell viability was increased with the addition of **2** in a dose-dependent manner. As cell viability also increased with the addition of **3** in a dose-dependent manner, in a sample of Aβ + **2**, inhibition of Aβ aggregation by **3** (which should be present after UV-light irradiation of sample Aβ + **2**) would partly contribute to the increased cell viability. Nevertheless, based on the finding that cell viability with Aβ in the presence of **2** at 30 μM and 40 μM was significantly higher than that in Aβ with **3**, the toxicity of Aβ1-42 was attenuated by the covalent modification with **2**.

In conclusion, here we synthesized a covalent modifier-type aggregation inhibitor of Aβ, diazirine-equipped *cyclo*-KLVF(β-Ph)F (**2**). Due to the Aβ-affinity of the *cyclo*-KLVFF motif, under the UV light irradiation, **2** could selectively react with Aβ1-42. The Tyr-10 residue of Aβ1-42 was identified as the covalent modification site with **2**. The extent of cross-β-sheet structure, characteristics of amyloid aggregation, and toxicity of Aβ1-42 were all significantly attenuated by the covalently linked modification. We report the first successful demonstration of the utility of the covalent modifier approach to strengthen the inhibitory activity against Aβ aggregation, one of the most challenging targets of protein-protein interaction.

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Supplementary Material

Supplementary data associated with this article can be found, in the online version, at doi:XXX/j.bmcl.20XX.

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