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'Click peptide' using production of monomer Aβ from the *O*-acyl isopeptide: Application to assay system of aggregation inhibitors and cellular cytotoxicity

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ABSTRACT

The *O*-acyl isopeptide of Aβ1–42 (**1**), possessing an ester bond at the Gly[25]-Ser[26] sequence, is a water-soluble and non-aggregative precursor molecule and is capable of production of monomer Aβ1–42. The SDS–PAGE result showed that the Aβ1–42, produced from **1**, adopted monomeric state at first and then self-assembled to oligomer. The oligomeric state was stabilized by nordihydroguaiaretic acid. The Thioflavin-T (ThT) fluorescence intensity derived from Aβ1–42 (generated from **1**) was suppressed by various aggregation inhibitors. Finally, **1** could generate Aβ1–42 via the *O*-to-*N* acyl migration under cellular medium conditions and the produced Aβ1–42 exhibited cytotoxicity against PC12 cells. These results suggest that the click peptide system, which enables us to predominantly produce monomer Aβ1–42 under physiological conditions, would be adoptable to various biochemical and biophysical experiments including cellular system to investigate the functions of Aβ1–42.

1. Introduction

Amyloid β peptides, which consist mainly of 40- and 42-residue peptides (designated Aβ1–40 and Aβ1–42, respectively), are associated with Alzheimer's disease (AD), a severe neurodegenerative disease present mostly in the elderly population.[1] Amyloid plaques (senile plaques), a hallmark for AD, are formed from the accumulation of aggregated Aβs. While Aβ1–40 is the predominant product, Aβ1–42 is far more aggregative. However, the toxicological mechanisms for Aβs to develop AD remain in controversies.

One major obstacle in elucidating the toxicological mechanisms is the intense and uncontrollable self-assembling nature of the Aβs, especially Aβ1–42, in experiments.[2–5] To study the physiological function, the Aβ1–42 samples at the beginning of the experiments should be in their monomer random coil states. Accordingly, because synthetic Aβ1–42 generally contain various oligomeric forms (the oligomeric degree might depend on the sources and/or lots), a variety of pre-treatments have been adopted to select only monomer Aβ1–42 for the experiments. However, the various pre-treatment methods lead to different outcomes among the Aβ studies. For example, residual disaggregating solvents such as DMSO and hexafluoroisopropanol (HFIP), which are often used during pre-treatments, would influence the experiment. Moreover, unintentionally, the obtained monomer Aβ1–42 start to self-assemble during storage and handling prior to their use. Thus, the intense and uncontrollable self-assembling nature of the Aβs lead to irreproducible or discrepant results.

As an experimental tool to overcome the problem, we have reported that monomer Aβ could be obtained in situ from its *O*-acyl isopeptide derivative (**1**, 'click peptide' concept, Fig. 1).[6–9] The presence of an *O*-acyl instead of *N*-acyl residue at a hydroxyamino acid residue in the peptide backbone generally changed the physicochemical property of the native peptide.[10–12] The *O*-acyl isopeptide of Aβ1–42 (**1**), possessing an ester bond (instead of an amide bond) at the Gly[25]-Ser[26] sequence (Fig. 1a), demonstrated 100-fold higher water-solubility than that of parent Aβ1–42 due to the protonated amino group at the isopeptide residues. In addition, the *O*-acyl isopeptide promptly converted to Aβ1–42 ($t_{1/2}$ ~10 s) at pH 7.4, via an *O*-to-*N* intramolecular acyl migration reaction.

Recently, our studies with size-exclusion chromatography (SEC), thioflavin-T (ThT) assay and circular dichroism spectrometry (CD) suggested that (1) *O*-acyl isopeptide **1** adopted a monomer random coil state (without forming amyloid fibril) under acidic conditions probably due to the ester structure of the *O*-acyl isopeptide and (2) the generated monomer Aβ1–42 under neutral pH conditions (as a result of the quick and irreversible *O*-to-*N* acyl migration) underwent oligomerization, random coil→β-sheet conformational transition and amyloid fibril formation with time.[13] Moreover, Bozso et al. reported studies on Aβ1–42 aggregation derived from **1** by CD, atomic force/transmission electron microscopy

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; Myr, myricetin; NDGA, nordihydroguaiaretic acid; Rif, rifampicin; TFA, trifluoroacetic acid; ThT, thioflavin-T; TA, tannic acid.

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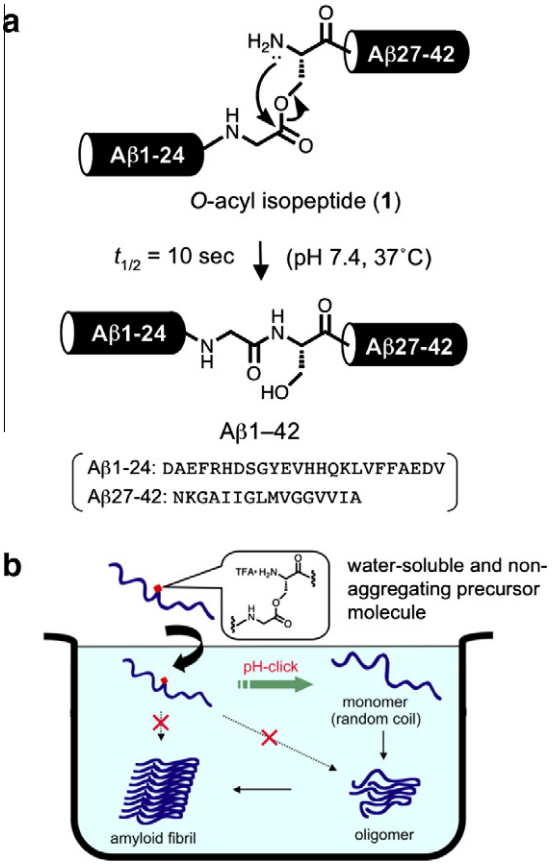

Figure 1. (a) Chemical structure of *O*-acyl isopeptide (**1**) and its conversion to Aβ1–42 via an *O*-to-*N* intramolecular acyl migration reaction (pH 7.4, 37 °C). (b) 'Click peptide' concept: In situ production of monomeric random coil Aβ1–42 from the *O*-acyl isopeptide and its subsequent self-assembly (to form the oligomer and amyloid fibril) of the produced Aβ1–42.

and dynamic light scattering.[14] This research group also proved that Aβ1–42 (derived from **1**) impaired long-term potentiation in hippocampal slices and caused memory deficit in rats.

These data supported the advantages of **1** as a precursor molecule of Aβ1–42 in experiments. Indeed, **1** is becoming popular as a tool in AD research.[15–17] The click peptide concept on islet amyloid polypeptide (IAPP), associating with diabetes mellitus type 2, has been also reported.[18]

Herein, the results from western blot analyses with SDS–PAGE supported that monomer Aβ1–42 generated from **1** was predominant at the starting point of the experiment and assembled to form oligomers/fibrils over time. The oligomeric state was significantly stabilized by nordihydroguaiaretic acid (NDGA). ThT studies suggest that the aggregation of Aβ1–42 (generated from **1**) were suppressed in varying degrees by aggregation inhibitors. Moreover, **1** could generate Aβ1–42 in cell culture medium and the produced Aβ1–42 showed cytotoxicity against PC12 cells.

2. Results and discussion

2.1. Oligomerization of Aβ1–42 derived from 1 verified by SDS–PAGE

O-Acyl isopeptide **1** in 0.1% aqueous TFA solution (as a stock solution) was neutralized by dilution with 0.1 M phosphate buffer (pH 7.4) and the buffer was incubated at 37 °C (final Aβ concentration: 10 μM). The sample solution after incubating for the desired periods of time (2 min, 3 h, 18 h, 2 days and 4 days) were applied to Nu-PAGE® Bis–Tris 4–12% gel, detected using 6E10 monoclonal antibody (Fig. 2, left side). At an incubation time of 2 min, in which the *O*-to-*N* acyl migration mostly completed, a band at 4.5 kDa (corresponding to the monomer molecular weight) was predominantly observed. A weak band detected around 10 kDa (corresponding to a dimer to trimer) might be an SDS-induced artifact in light of a reported observation on SDS–PAGE.[19] The result thus suggested that the Aβ1–42 generated from **1** adopted monomeric state at the start of the experiment. This is in agreement with a previous result with SEC in which a monomer-derived peak was sorely detected in the sample solution after 2 min incubation.[13]

A broad smear band around 60 kDa was generated after 3 h incubation and a 40–160 kDa band was increased in intensity with incubation time (3 h, 18 h and 2 days) at the expense of the monomer band (Fig. 2, left side). A band at top of the gel, which is increased over incubation time, would be due to the formation of a larger oligomer. In addition, in the 4 days-incubated sample, there is no significant detectable band, probably due to the aggregation of Aβ1–42 into fibrils during incubation. These results agree with those of SEC analyses in which a peak derived from oligomer (>8 mer) was amplified with incubation time,[13] although the correlation difference between the oligomer sizes determined by the Nu-PAGE® and SEC experiments is not clear.

When nordihydroguaiaretic acid (NDGA), possessing potent inhibitory activity for Aβ aggregation,[20] was co-incubated with Aβ1–42 derived from **1** (10 μM each), the SDS–PAGE analysis showed a considerable increase in intensity for the oligomer band throughout the incubation period (3 h, 18 h, 2 days and 4 days) (Fig. 2, right side). The result from SDS–PAGE is in agreement with a report that the level of Aβ oligomer was significantly increased in NDGA-treated mice.[21] The increase in intensity for the oligomer band would suggest that NDGA significantly slowed oligomer→fibril transformation, although the binding mode with Aβ has not been solved. The aggregation inhibitory mechanism for NGDA would be associated with the stabilization of the oligomeric states of Aβ.

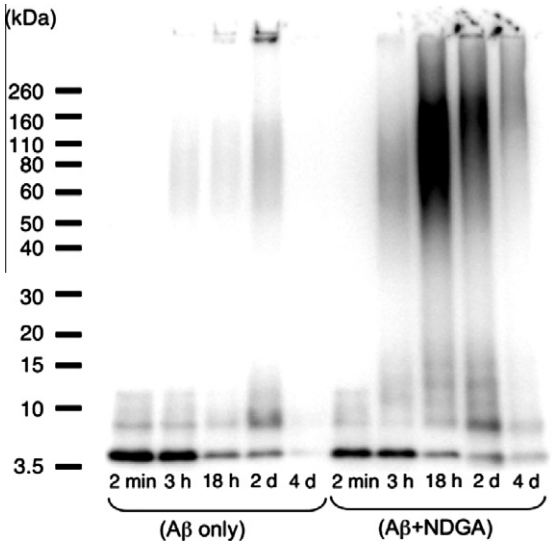

Figure 2. Western blot analyses with SDS–PAGE: The *O*-acyl isopeptide **1** was incubated in pH 7.4 buffer at 37 °C (10 μM) and the sample solution was analyzed on Nu-PAGE® Bis–Tris 4–12% gel, detected using 6E10 monoclonal antibody. Incubation time: 2 min, 3 h, 18 h, 2 days and 4 days, left side: Aβ1–42 alone (from **1**), right side: Aβ1–42 (from **1**) + NDGA (10 μM).

2.2. Inhibition of amyloid fibril formation of Aβ1–42 derived from 1

ThT fluorescence intensity generally corresponds to the extent of amyloid fibril formation and most likely also large oligomers. When **1** in a pH 7.4 buffer solution was incubated and applied to the ThT assay, the Aβ1–42, which was produced from **1** via the *O*-to-*N* acyl shift, induced increases in fluorescence intensity over time. This signifies the fibrils formation of Aβ1–42 derived from **1**.[13]

Here, in the assay system with *O*-acyl isopeptide of Aβ1–42 (**1**), the inhibitory activities of known aggregation inhibitors (tannic acid: TA,[22,23] nordihydroguaiaretic acid: NDGA,[20,23] myricetin: Myr,[24,25] rifampicin: Rif,[23,26] Aβ fragments L-Aβ16–21[27] and D-Aβ16–21[27]) were evaluated (Fig. 3a). When each inhibitor (10 μM) was co-incubated with Aβ1–42 (10 μM) (generated from **1**) in pH 7.4 buffer at 37 °C for 48 h, the fluorescence intensities of Aβ1–42 were suppressed in varying degrees by a series of compounds. The intensity was reduced by more than 90% (as compared to the control) in the presence of TA and NDGA. Myr and Rif reduced the intensity by 60–70%. L-Aβ16–21 inhibited a relative intensity of approximately 20%, while the D-form did ~35%. In addition, TA inhibited Aβ aggregation in a dose-dependent manner (Fig. 3b). TA also maintained its suppressing activities for 48 h (Fig. 3c). D-Aβ16–21 slowed the increase of the intensity and reduced the maximum ThT fluorescence intensity.

An assay system utilizing the click peptide, in which monomer Aβ1–42 could be predominantly produced from water-soluble *O*-acyl isopeptide precursor, would be useful to identify aggregation inhibitors in a highly reproducible manner. Based on this concept, Lashuel and co-workers reported a ThT assay system using the *O*-acyl isopeptides of Aβ14–24 derivatives.[28] We herein further demonstrated a screening system with the *O*-acyl isopeptide of whole Aβ1–42 sequence to evaluate a wider range of aggregation inhibitors, which interacts not only with the middle sequence region (i.e., Aβ14–24) of Aβ but also the *N*- and *C*-terminal regions.

2.3. Cytotoxicity of Aβ1–42 derived from 1 for PC12 cells

At first, we confirmed that **1** was converted to Aβ1–42 ($[M+H]_{calcd}$: 4515.0, $[M+H]_{found}$: 4514.8; the retention time on the HPLC analysis was identical to the authentic sample), with a half-life of ~1 min (more than 95% conversion after 5 min), in HEPES buffered DMEM containing 1% horse serum (pH 7.4, 37 °C) (Fig. 4a). Furthermore, Aβ1–42 was quantitatively recovered from the PC12 cells-containing medium in the poly-D-lysine-coated well. The near quantitative production of Aβ1–42 demonstrated that the *O*-to-*N* intramolecular acyl migration reaction of **1** took place in a similar manner as in a simple phosphate buffer[13] without any interruption from the cells and components of cell culture medium such as serum.

To examine the cytotoxicity of Aβ1–42 generated from **1**, a solution with **1** was applied to rat pheochromocytoma PC12 cells in HEPES buffered Dulbecco's modified Eagle's medium (DMEM) containing 1% horse serum (final pH 7.4, concentration of **1**: 1–20 μM). The cellular culture medium was then incubated at 37 °C under 10% CO_2 and the viability was examined after 48 h incubation, estimated by the absorbance of soluble formazan dye formed from WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt) catalyzed by mitochondrial reductase. As shown in Figure 4b, the viability of PC12 cells was reduced in a dose-dependent manner for Aβ1–42 (generated from **1** in situ) with an EC_{50} value of ~7 μM. Compared to the control, apparent morphological changes of the cells, with a characteristic of apoptotic-like cell death, were observed in the presence of 10 μM Aβ1–42 (Fig. 4c).

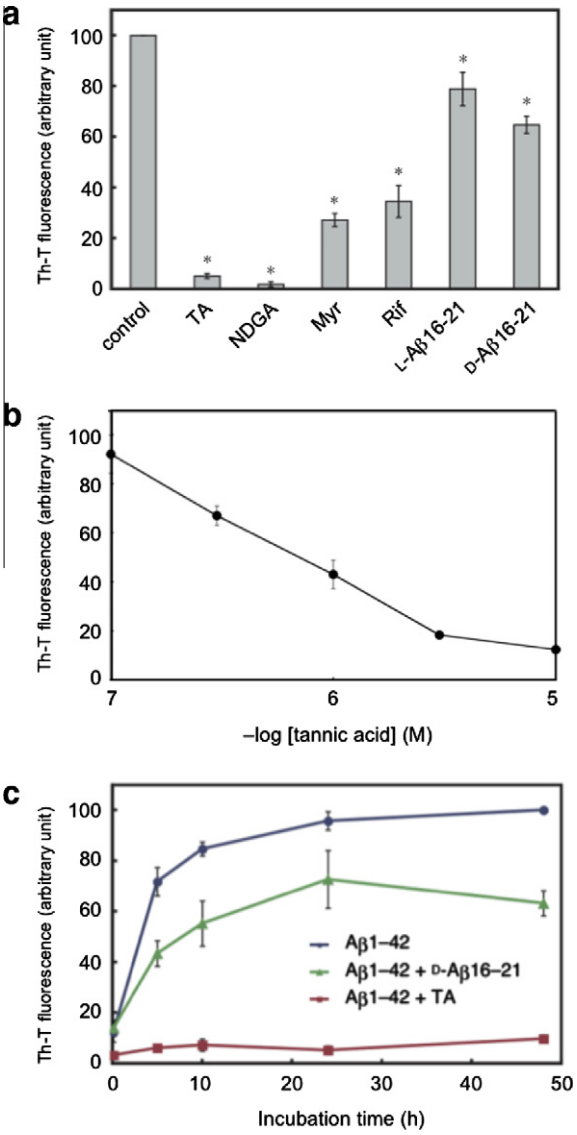

Figure 3. Thioflavin-T (ThT) assay with **1** (10 μM): (a) inhibitory activities of TA (tannic acid), NDGA (nordihydroguaiaretic acid), Myr (myricetin), Rif (rifampicin), L-Aβ16–21 and D-Aβ16–21 (10 μM), control: Aβ1–42 (from **1**) alone, $^{*}p$ <0.01 versus control (one-way analysis of variance followed by Dunnett's test was used for statistical analyses, $n = 3$); (b) dose-dependency of TA; (c) profiles of ThT fluorescence intensities in the absence or presence of TA and D-Aβ16–21.

3. Conclusion

We herein reported studies on the *O*-acyl isopeptide of Aβ1–42 (**1**), possessing an ester bond at the Gly^{25}-Ser^{26} sequence, with SDS–PAGE, ThT assay and cellular assay. SDS–PAGE study showed that the Aβ1–42, produced from **1** via the *O*-to-*N* intramolecular acyl migration reaction, adopted monomeric state at first and then self-assembled to form oligomers with incubation time. This result further supports the advantage of the click peptide concept, that is, monomer Aβ could be predominantly obtained in situ from the *O*-acyl isopeptide, to overcome the intense and uncontrollable self-assembling nature of Aβs. The oligomeric state was remarkably stabilized by the presence of NDGA, suggesting that NDGA significantly slowed oligomer→fibril transformation. The ThT fluorescence intensity derived from Aβ1–42 (generated from **1**) was suppressed by TA, NDGA, Myr, Rif, L-Aβ16–21 and D-Aβ16–21. Moreover, **1** could generate Aβ1–42 via *O*-to-*N* acyl migration in cell culture medium (as well as a simple buffer system) and the

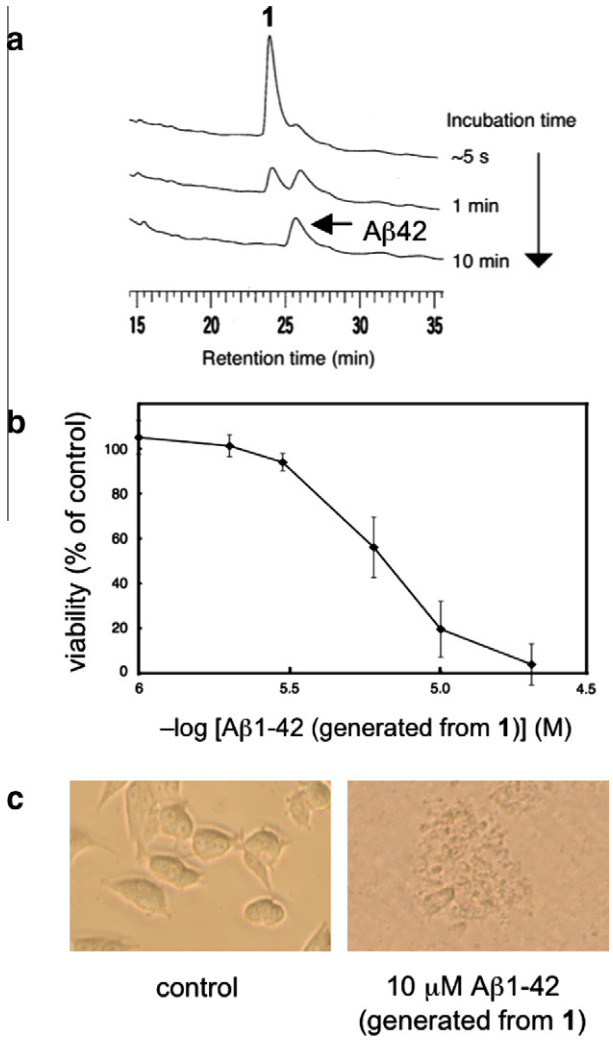

Figure 4. (a) HPLC of *O*-to-*N* acyl migration of **1** to give Aβ1–42 in 25 mM HEPES buffered DMEM containing 1% (v/v) horse serum (pH 7.4), incubated at 37 °C; (b) dose dependency of **1** (1, 2, 3, 6, 10 or 20 μM) for viability of PC12 cells. Cell viability was examined using WST-8; (c) microscopic photographs of the cells in the absence (left panel) or presence (right panel) of **1**.

produced Aβ1–42 exhibited cytotoxicity against PC12 cells in a dose-dependent manner. This demonstrates that the *O*-acyl isopeptide could be also applied to cellular assay systems. The intense and uncontrollable self-assembling nature of Aβ1–42 leads to irreproducible and discrepant experimental results. This is a major issue in elucidating the mechanisms of Aβ in Alzheimer's disease research. The click peptide system, which enables us to predominantly produce monomer Aβ1–42 under physiological conditions, would be adoptable to various biochemical and biophysical experiments to investigate the functions of Aβ1–42.

4. Experimental

4.1. General

HEPES buffered Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and horse serum were purchased from Invitrogen (Carlsbad, CA), MP Biomedicals, Inc. (Irvine, CA) and SAFC Biosciences (Lenexa, KS), respectively. Other chemicals were purchased from commercial suppliers, Wako Pure Chemical Ind., Ltd (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), Dojin Laboratories (Kumamoto, Japan) and Sigma–Aldrich, Inc., and were used without further purification. Rat pheochromocytoma PC12 cells were obtained from Riken BioResource Center (Ibaraki, Japan). The colorimetric determination was made on a Microplate Reader SH-1000 Lab (Corona Electric Co., Ltd, Ibaraki, Japan).

4.2. Sample preparation

The stock solution of **1** as a 0.1% aqueous TFA was prepared in a same manner that described in Ref. 13. 0.1% aqueous TFA solution with **1** was ultra-centrifuged (435,000*g*) at 4 °C for 3 h on a TL-100 (Beckman Instruments, Inc., Palo Alto, CA, USA) with a TLA-100.1 rotor (Beckman Instruments, Inc.) for SDS–PAGE experiments. Instead, a milder centrifugation condition (20,000*g*, 4 °C, 1 h) on a MC-150 (Tomy Seiko Co., Ltd, Tokyo, Japan) with a TMA-2 rotor (Tomy Seiko Co., Ltd) was used for ThT and cell assays. We confirmed that the samples prepared by the two centrifugal conditions give identical experimental results, as verified by CD, SDS–PAGE, ThT assay and cell assay (data not shown). The upper three-quarters fraction was collected. The peptide concentration of each solution was estimated from a UV absorption at 280 nm on a BioSpec-1600 (Shimadzu Co., Kyoto, Japan), using a Tyr extinction coefficient of 1490 $M^{-1}cm^{-1}$. The solution of **1** was further diluted with aqueous TFA (0.1%, 220 μM **1**). Each solution was stored at −80 °C until use. Just before the experiments, the stock solution of **1** was thawed and diluted with an equal volume of phosphate buffer (0.2 M, pH 7.4). Then additional phosphate buffer (0.1 M, pH 7.4) was immediately added to obtain the peptide solution (10 μM, pH 7.4).

4.3. Western blot with Nu-PAGE®

The isopeptide **1** in pH 7.4 buffer was incubated at 37 °C (*O*-to-*N* acyl migration mostly completed after ~2 min incubation to afford Aβ1–42), and the incubated sample solution was mixed with lithium dodecyl sulfate and the mixture was heated at 70 °C for 10 min. Then the sample was analyzed on Nu-PAGE® Bis–Tris 4–12% gel using MES SDS running Buffer (Invitrogen) under non-reducing conditions. The sample was transferred to 0.45 μm polyvinylidene difluoride membrane (BIO-RAD). The membrane was blocked in 5% skim milk in Tris-buffered saline containing 0.1% Tween 20. The membrane was incubated with the primary antibody 6E10 (Covance), a mouse monoclonal Aβ antibody to residues 1–16, and then incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody. Finally, we developed the membrane using enhanced chemiluminescence (ECL; GE Healthcare). Molecular weight values were estimated using Novex® sharp pre-stained protein standard (Invitrogen).

4.4. ThT assay

The fluorescence intensity was obtained in a similar manner as that described in Ref. 13.

In the assays with aggregation inhibitors, 0.12 μL of DMSO solution containing TA, NDGA, Myr, Rif, L-Aβ16–21 or D-Aβ16–21 (0.1–10 mM each) was added to 120 μL of pH 7.4 buffer which was pre-incubated for 2 min after adding 10 μM **1** (final inhibitor concentration: 0.1–10 μM; final DMSO: 0.1% each). The solution was further incubated at 37 °C. At the desired time points (~2 min and 1 h, 5 h, 10 h, 24 h, and 48 h), 15 μL of the aliquot was applied to the ThT system as described in Ref. 13.

4.5. Cell culture

Rat pheochromocytoma PC12 cells were cultured on a collagen-I-coated 75 cm^2 vented flask (Beton Dickinson Labware, Bedford, MA) in 25 mM HEPES buffered DMEM containing 5% (v/v) horse

serum and 10% (v/v) FBS at 37 °C under 10% CO_2. The cells were seeded at a density of 5000 cells/100 μL/well on a poly-D-lysine-coated 96-well plate (Beton Dickinson Labware) and incubated for 3 days at 37 °C under 10% CO_2. After the medium was removed, the cells were washed with 150 μL of serum-free 25 mM HEPES buffered DMEM.

4.6. Cytotoxicity assay with PC12 cells

The cells were pre-incubated in 100 μL of 25 mM HEPES buffered DMEM (pH 7.4) containing 1% (v/v) horse serum at 37 °C under 10% CO_2 for 1 day. Ten μL of 0.1% aqueous TFA solution of **1** (11–220 μM) was added to the well containing 100 μL cell culture medium (final **1** concentration: 1–20 μM; pH 7.4) and incubated for 48 h at 37 °C under 10% CO_2. Morphology of cells was observed with an Eclipse TE300 Microscope (Nikon Co., Tokyo, Japan). Ten μL of a cell count reagent SF including WST-8 (Nacalai Tesque) was added to each well and the plate was incubated for 2–4 h at 37 °C under 10% CO_2. The absorption values at 450 nm (reference: 655 nm) were measured. Values are expressed as mean ± SE (n = 3).

4.7. *O*-to-*N* Acyl migration in culture medium

Ten μL of the stock solution of **1** (as a 0.1% aqueous TFA solution) was added to 100 μL of 25 mM HEPES buffered DMEM containing 1% (v/v) HS (pH 7.4) and the mixture was incubated at 37 °C. At the desired time points (~5 s and 1 min, 2 min, 5 min, 10 min, and 30 min), the solution (80 μL) was diluted with HFIP (80 μL) and injected to analytical RP-HPLC.

The stock solution of **1** was also added to the poly-D-lysine-coated well containing the culture medium with PC12 cells (pH 7.4) and incubated at 37 °C under 10% CO_2 for 10 min. The mixture was taken, diluted with HFIP (2×) and injected to analytical RP-HPLC. The obtained peak area was compared to that of a control experiment in which the cellular medium was replaced with a phosphate buffer.

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