Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Electronic Supplementary Information for:

Chemically-Engineered, Stable Oligomer Mimic of Amyloid β42 Containing Oxime Switch to Fibril Formation

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Experimental Procedures

General

Analytical HPLC was carried out using a SHIMADZU HPLC system equipped with a SPD-20A UV-VIS detector, LC-20AD pumps, a DGU-20A_{3R} degasser, a SIL-20AC auto sampler, a CTO-20AC column oven, and a CBM-20A system controller (Shimadzu, Co., Kyoto, Japan), or a HITACHI HPLC system equipped with an L-4200 UV–vis detector, an L-6210 pump or L-6200/L-6000 pumps, and an ERC-3510 or L-5090 degasser (Hitachi, Ltd., Tokyo, Japan). Preparative HPLC was conducted using a SHIMADZU HPLC system equipped with a SPD-20A UV-VIS detector, LC-6AD pumps, a CTO-20AC column oven, a FRC-10A fraction collector, and a CBM-20A system controller (Shimadzu, Co., Kyoto, Japan). LC/MS analysis was conducted using an Agilent Technologies LC/MS (ESI-Q) system equipped with a 1260 Infinity High Performance Degasser, 1260 Infinity Binary Pump, 1260 Infinity Standard Autosampler, a 1260 Infinity Thermostatted Column Compartment, a 1260 Infinity Variable Wavelength Detector, and an Agilent 6120 Single Quadrupole LC/MS (Agilent Technologies, Inc., Santa Clara, CA, U. S. A.). MALDI-TOF MS was measured on a Shimadzu Biotech Axima-ToF2 TM spectrometer (Shimadzu, Co., Kyoto, Japan) using α-cyano-4-hydroxy cinnamic acid (Sigma-Aldrich, Inc., St. Louis, MO, U. S. A.) as a matrix. ESI-Q-MS spectra were measured on a Waters ZQ4000 (Waters, Co., Milford, MA, U. S. A.) spectrometer (for LRMS). Water was purified in advance using a Millipore Milli-Q water purification system (Merck KGaA, Co., Darmstadt, Germany) to obtain filtered deionized water and distilled water.

Materials

All protected amino acids were purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan) and Peptide Institute, Inc. (Osaka, Japan). Fmoc-Ala-TrtA-PEG resin (100-200 mesh) was purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). 1-Hydroxybenzotriazole monohydrate (HOBt·H_2O), *N*,*N*-diisopropylethylamine (DIPEA), and *N*,*N*'-diisopropylcarbodiimide (DIC) were obtained from Watanabe Chemical Industries, Ltd. Triisopropylsilane (TIS) was purchased from Sigma-Aldrich, Inc. Piperidine was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Thioanisole and tris(2,2′-bipyridyl)dichlororuthenium(II) hexahydrate was purchased from Fluka Chemical Co., Ltd. *m*-Cresol and trifluoroacetic acid (TFA) were purchased from Tokyo Chemical Industry, Co., Ltd. (Tokyo, Japan). Ammonium persulfate was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). *N*,*N*-Dimethylformamide (DMF), tetrahydrofuran (THF), diethyl ether (Et_2O), dichloromethane (CH_2Cl_2), 1,2-dichloroethane, and acetonitrile (MeCN) were obtained from Kanto Chemical Co., Inc. and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and used without further manipulation unless otherwise stated. *O*-Acyl isopeptides of Aβ1–42 and Aβ42[Met^{35}(O)] were purchased from Peptide Institute, Inc. Other chemicals were purchased from the following commercial suppliers: Wako Pure Chemical Industries, Ltd., Nacalai Tesque, Inc., Kanto Chemical, Co., Inc., and Sigma-Aldrich, Inc. Flash column chromatography was performed using Kanto Chemical silica gel (spherical, 40–63 μm). Thin layer chromatography (TLC) was performed on Merck silica gel 60 F254 (0.25 mm) plates.

Analytical HPLC

Peptide compositions were evaluated by analytical HPLC using a C18 reverse phase column (4.6 × 150 mm; YMC-Triart C18; YMC Co., Ltd., Kyoto, Japan) with a linear gradient of 0–100% MeCN in 0.1%

aqueous TFA over 40 min at 40 °C at a flow rate of 1 mL min^{-1}, detected at 230 nm. LC/MS grade solvents were used.

Analytical LC/MS

LC/MS analysis was performed using a C18 reverse phase column (2.0 × 50 mm; YMC-Triart C18; YMC Co., Ltd.) with a linear gradient of 0–100% MeCN in 0.1% aqueous HCO_2H over 15 min at 37 °C at a flow rate of 0.2 mL min^{-1}, detected at 230 nm, with on-line ESI-Q MS. LC/MS grade solvents were used.

Preparative HPLC

Peptides were purified by preparative HPLC using a C18 reverse phase column (10 × 250 mm; YMC-Triart C18; YMC Co., Ltd.) with a linear gradient of 0–100% MeCN in 0.1% aqueous TFA over 100 min at 40 °C at a flow rate of 3.5 mL min^{-1}, detected at 230 nm. LC/MS grade solvents were used.

General protocol for solid phase peptide synthesis (SPPS)

Peptide synthesis was performed manually on a 0.1 mmol scale using Fmoc-Ala-TrtA-PEG resin (100-200 mesh). The peptide chains were assembled by the sequential coupling of activated *N*α-Fmoc-amino acid (5 eq.) in DMF in the presence of *N*,*N*'-diisopropylcarbodiimide (DIC, 5 eq.) and 1-hydroxybenzotriazole (HOBt, 5 eq.) with a reaction time of 1 h at room temperature. The resins were washed with DMF (× 5), and then *N*α-Fmoc deprotection was carried out by treatment with 20% piperidine/DMF (*v*/*v*) (1 min × 1 and 10 min × 1), followed by washing with DMF (× 10). The coupling and deprotection cycles were repeated. The peptide-resins were washed with MeOH (× 5) and dried for at least 2 h *in vacuo*. The peptides were cleaved from the resins with TFA in the presence of *m*-cresol, thioanisole, and distilled water (92.5:2.5:2.5:2.5) for 60 min at room temperature, concentrated *in vacuo*, and precipitated with Et_2O at 0 °C. The resulting precipitate was collected by filtration, dissolved with 0.1% aqueous TFA/MeCN (1:1), and lyophilized for at least 12 h to give the crude peptide. The crude peptide was purified by preparative reverse phase HPLC with 0.1% aqueous TFA/MeCN system as an eluent, immediately frozen using liq. N_2, and lyophilized for at least 24 h to afford the desired peptide.

Preparation of peptide stock solution

The powder of **3**, *O*-acyl isopeptide of **4**, or *O*-acyl isopeptide of **5** was dissolved in 0.1% aqueous TFA. These solutions were treated with an ultracentrifugation (100,000 rpm) at 4 °C for 3 h on an ultracentrifuge OptimaTM TLX (Beckman Coulter, Inc., Brea, CA) with a rotor TLA100 or TLA120.2 (Beckman Coulter, Inc.). The upper three-quarters fraction of the solution was collected, and an absorbance of the solution at 280 nm was measured by UV sepectrometer (UV-1800, SHIMADZU, Co., Ltd.) and the concentration was decided to obtain the following stock solutions: 200 μM of **3**, *O*-acyl isopeptide of **4**, and *O*-acyl isopeptide of **5**. Stock solution of *O*-acyl isopeptide of Aβ1–42 in 0.1% aqueous TFA was prepared as described in Ref S1. These solutions were stocked at –80 °C until use.

Atomic Force Microscopy (AFM)

Stock solution of **3**, *O*-acyl isopeptide of **4**, or *O*-acyl isopeptide of **5** was thawed and diluted with 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) buffer (0.1 M, pH 7.4) containing 0.1 M NaCl or 0.1% aqueous TFA to obtain 10 μM of the respective solution. The prepared samples were incubated

at 37 °C for the desired time periods (0 h, 3 h, 24 h). Aliquots (10 μL) of these samples were spotted on freshly cleaved mica (Nilaco, Co., Tokyo, Japan), incubated at room temperature for 3 min, rinsed with 20 μL of filtered water for three times and dried in air. Samples were imaged by an intermittent contact mode in air at room temperature on a NanoWizard III (JPK instruments, AG, Berlin, Germany).

SDS-PAGE

A stock solution of **3** or *O*-acyl isopeptide of **4** was thawed and diluted with an equal volume of phosphate buffer (0.2 M, pH 7.4), and additional phosphate buffer (10 mM, pH 7.4) was added to obtain 20 μM of the peptide solution. The prepared sample was incubated at 37 °C for the desired time periods (0 h, 3 h, 24 h, 72 h). To 5 μL of the solution, 0.8 μL of 0.25 mM Tris (2,2'-bipyridyl) dichlororuthenium (II) hexahydrate $[Ru(bpy)_3]^{2+}$ and 0.8 μL of 5 mM ammonium persulfate in 10 mM phosphate buffer were added.[12] The mixture solution was irradiated for 1 s with white visible light using a high-intensity illuminator (Valore, Kyoto, Japan), and the reaction was quenched by addition of 0.8 μL of 0.5 M DTT in water. Then, the solution was mixed with 2 μL of 5 × SDS sample buffer and the mixture solution was analyzed on Novex® 15% Tris-glycine gel (Nacalai tesque, Kyoto, Japan) with Tris-glycine SDS running buffer under non-reducing condition. Molecular weight was estimated with Precision Plus Protein Standards dual color (Bio-Rad, California, USA). The gel was silver-stained with Silver Xpress silver staining kit (Invitrogen, California, USA) according to a manufacture's protocol.

Circular dichroism (CD) spectroscopy

A stock solution of **3** or *O*-acyl isopeptide of **4** (10 μM) was prepared in the same way shown in the protocol of the ThT assay. The prepared samples were incubated at 37 °C for the desired time periods (0 h, 3 h, 6 h, 24 h). CD spectra were measured at room temperature on a stopped flow circular dichroism spectrometer Model 202SF (AVIV Biomedical, Inc., Lakewood, NJ) using a rectangular quartz cell (1 mm pathlength). Blank spectra were subtracted, respectively.

Thioflavin-T (ThT) fluorescence assay

Stock solution of **3**, *O*-acyl isopeptide of **4**, or *O*-acyl isopeptide of **5** was thawed and diluted with an equal volume of phosphate buffer (0.2 M, pH 7.4), and additional phosphate buffer (0.1 M, pH 7.4) was added to obtain a solution at a concentration of 10 μM. The prepared samples were incubated at 37 °C without agitation for the desired time periods (0 h, 1 h, 3 h, 6 h, 24 h). Aliquots (10 μL) of these samples were mixed with 3.7 μM ThT in 50 mM glycine-NaOH buffer (pH 8.5) and the mixture was subjected to ThT fluorescence assay. The fluorescence was measured at an emission wavelength of 480 ± 10 nm and an excitation wavelength of 440 ± 5 nm. The fluorescence intensity was measured on a spectrofluorophotometer RF-5300PC (Shimadzu, Co.) using a rectangular quartz cell (3 mm pathlength).

Cell viability assay with PC12 cells

Rat pheochromocytoma PC12 cells (Riken BioResouce Center, Ibaraki, Japan) were cultured on a collagen-I-coated 75 cm^2 vented flask (Becton, Dickinson and Co., Franklin, NJ) in 25 mM HEPES buffered Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Co., Carlsbad, CA) containing 5% (v/v) horse serum (HS) (Life Technologies, Co.) and 10% (v/v) fetal bovine serum (FBS) (Life Technologies, Co.) at 37 °C under 5% CO_2. For cell viability assays, the cells were seeded at a

density of 8000 cells/100 μL/well on a poly-D-lysine-coated 96-well plate (Becton, Dickinson and Co.) and incubated at 37 °C under 5% CO_2 for 3 days. After removal of the medium, the cells were washed with 150 μL of serum-free 25 mM HEPES buffered DMEM, and incubated in 100 μL of 25 mM HEPES buffered DMEM containing 0.1% (v/v) HS for 1 day. After removal of 50 μL of the conditioned medium from the wells, an aliquot (50 μL) of sample solutions (6 or 20 μM of **3**) was added to the wells (final volume: 100 μL, **3**: 3 or 10 μM), and incubated at 37 °C under 5% CO_2 for 3 d. Photography of cells was taken by the use of inverted microscope DMI6000 B (Leica Microsystems, GmbH, Wetzlar, Germany) equipped with a digital camera DFC360 FX (Leica Microsystems, GmbH). To each well, 10 μL of a cell count reagent SF including WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) (Nacalai Tesque, Inc.) was added, and incubated at 37 °C under 5% CO_2 for 2-3 h. The absorption values at 450 nm (reference: 655 nm) were measured on a microplate reader iMarkTM (Bio-Rad Laboratories, Inc., Hercules, CA).

Synthesis of 3

Fmoc-Ala-TrtA-PEG resin (0.1 mmol scale) was taken to the manual solid phase reactor and **3** was constructed by the general procedure of synthesis of *O*-acyl isopeptide using Fmoc-based SPPS.[S2] Fmoc-Gly($CH_2CH_2CH_2ONHBoc$)-OH, which was synthesized according to a literature[S3], was introduced at 28th position. After the coupling of Boc-Ser-OH at 26th position, the peptide-resin was washed with CH_2Cl_2 (× 5) and then subjected to the coupling with Fmoc-Gly-OH (15.0 eq.) in the presence of DIC (15.0 eq.) and *N*,*N*-dimethyl-4-aminopyridine (DMAP, 0.2 eq.) in CH_2Cl_2 for overnight (×2). Fmoc-Dap[Boc-Ser(*t*Bu)]-OH (Dap, diaminopropionic acid) was introduced at 23rd position. To this peptide-resin, removal of resin/protecting groups was performed and the crude peptide was purified using preparative HPLC to afford a white amorphous (2.24 mg, 0.89% isolation yield from the loading value in SPPS). To a solution of the resulting peptide in MeCN in 0.1% aqueous TFA (50% *v*/*v*), $NaIO_4$[S4] (0.31 mg, 1.46 μmol) was added, the mixture was left overnight at room temperature. After lyophilization of the reaction mixture, the obtained crude peptide was purified using preparative HPLC to obtain **3** (0.51 mg, 23%) as a white amorphous. MALDI-MS (TOF): found *m*/*z* 4541.9, calcd. 4542.1 $(M + H)^+$. Retention time: 20.8 min. Purity > 95% (based on HPLC analysis at 230 nm with a linear gradient of 0–100% MeCN in 0.1% aqueous TFA over 25 min, see Figure S2a).

Synthesis of O-acyl isopeptide of 5

The peptide was synthesized in a similar manner to that of **3**. Fmoc-Gly($CH_2CH_2CH_2ONHBoc$)-OH was introduced at 37th position and Fmoc-Dap[Boc-Ser(*t*Bu)]-OH was introduced at 15th position. After the SPPS, the aminoalcohol derivative was obtained with 1.2 % isolation yield (2.67 mg). After the oxidation using $NaIO_4$, purified *O*-acyl isopeptide of **5** was obtained with 19 % (0.52 mg) as a white amorphous. MALDI-MS (TOF): found *m*/*z* 4599.9, calcd. 4599.1 $(M + H)^+$. Retention time: 21.5 min. Purity > 95% (based on HPLC analysis at 230 nm with a linear gradient of 0–100% MeCN in 0.1% aqueous TFA over 25 min).

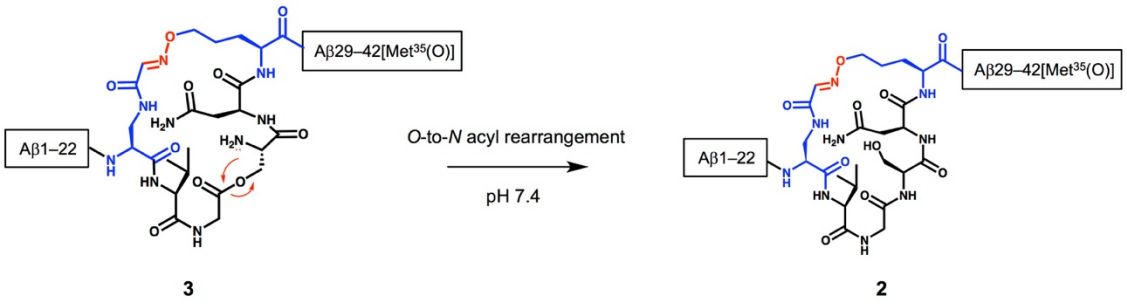
Oxime exchange reaction of 2 to convert to 6

3 in 0.1% aqueous TFA was diluted with 0.1 M phosphate buffer (final **3**: 0.1 mM) and the solution was incubated for 1 h at room temperature. To the solution, $MeONH_2$ hydrochloride in water was added to the final concentration of 0.3 mM, 1 mM, or 10 mM, and hydrogen chloride was added to adjust pH 4.

The solution was incubated at room temperature for 24 h. As a control experiment, $MeNH_2$ hydrochloride was used instead of $MeONH_2$. For AFM analysis, HEPES buffer containing 0.1 M NaCl was used instead of phosphate buffer. MALDI-MS (TOF): see Figure S8.

Removal of methoxyamine

After preparation of the solution in which **6** was dominant according to the procedure above ("**Oxime exchange reaction of 2 to convert to 6.**"), the reaction mixture was subjected to ultrafiltration (2 h) using centrifuge 5424R (eppendorf Japan, Tokyo, Japan). To the resulting protein solution, TFA was added (1% *v*/*v*), and the solution was incubated at room temperature for 16 h. MALDI-MS (TOF): see Figure S11.



3 2

Fig. S1. Structure of *O*-acyl isopeptide of **2** (**3**) and the conversion to **2** under a neutral aqueous solution via an *O*-to-*N* acyl rearrangement.

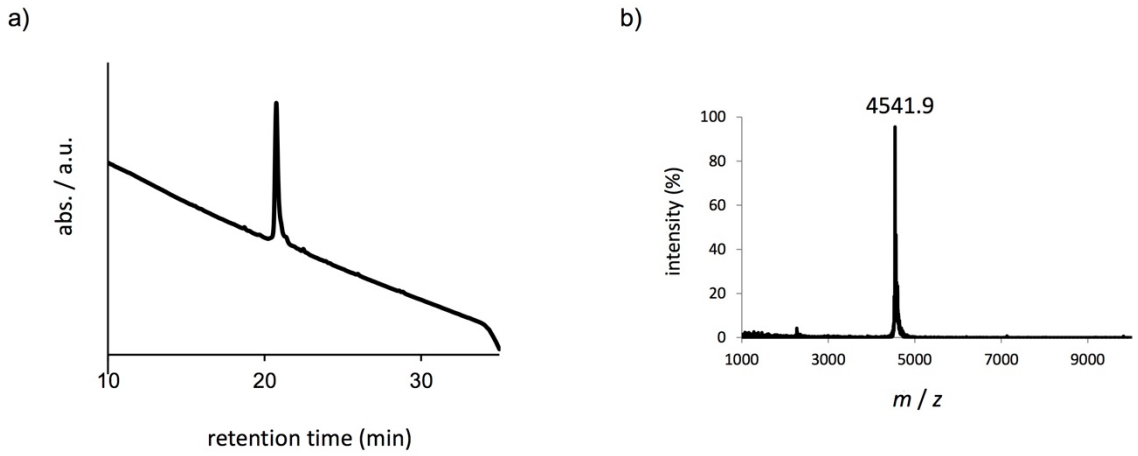


Fig. S2. (a) The HPLC chart of purified **3**. HPLC analysis was performed with a linear gradient of 0–100% MeCN in 0.1% aqueous TFA over 40 min a 40 ˚C at a flow rate of 1 mL min^{-1}, detected at 230 nm; (b) MALDI-TOF MS spectrum of purified **3**.

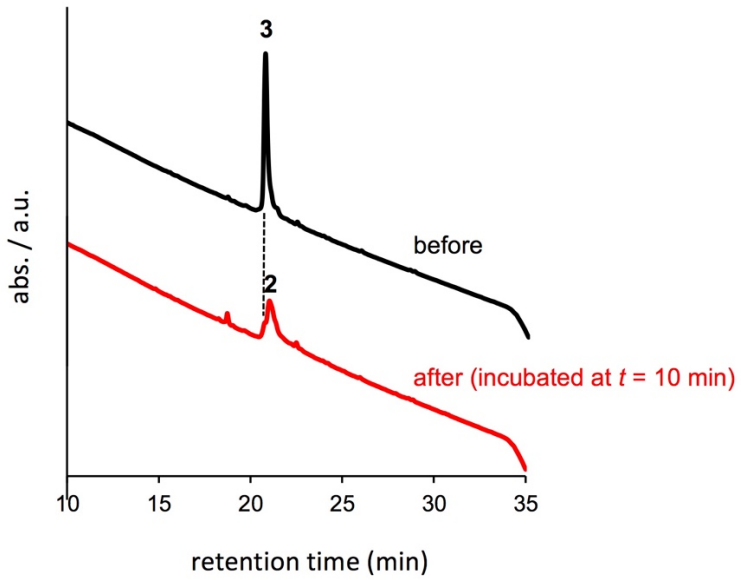


Fig. S3. HPLC profiles of the conversion of **3** to **2** in phosphate buffer (pH 7.4, 25˚C). Chromatographic separations were performed as described in Figure S2a legend.

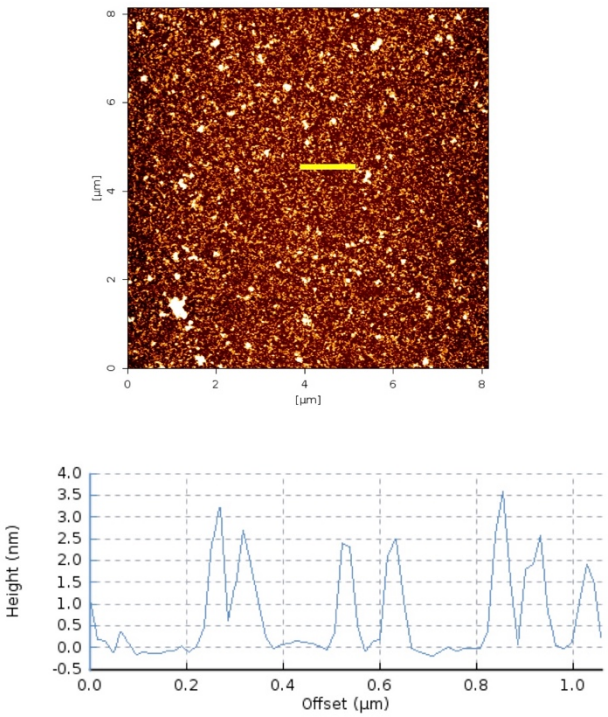


Fig. S4. Atomic force microscopy (AFM) image of **2** (10 μM). The sample was incubated in 0.1 M HEPES buffer containing 0.1 M NaCl (pH 7.4) at 37 ˚C for 10 min. The image is shown in height mode. Lower graph indicates z-heights obtained at the yellow bar of upper image.

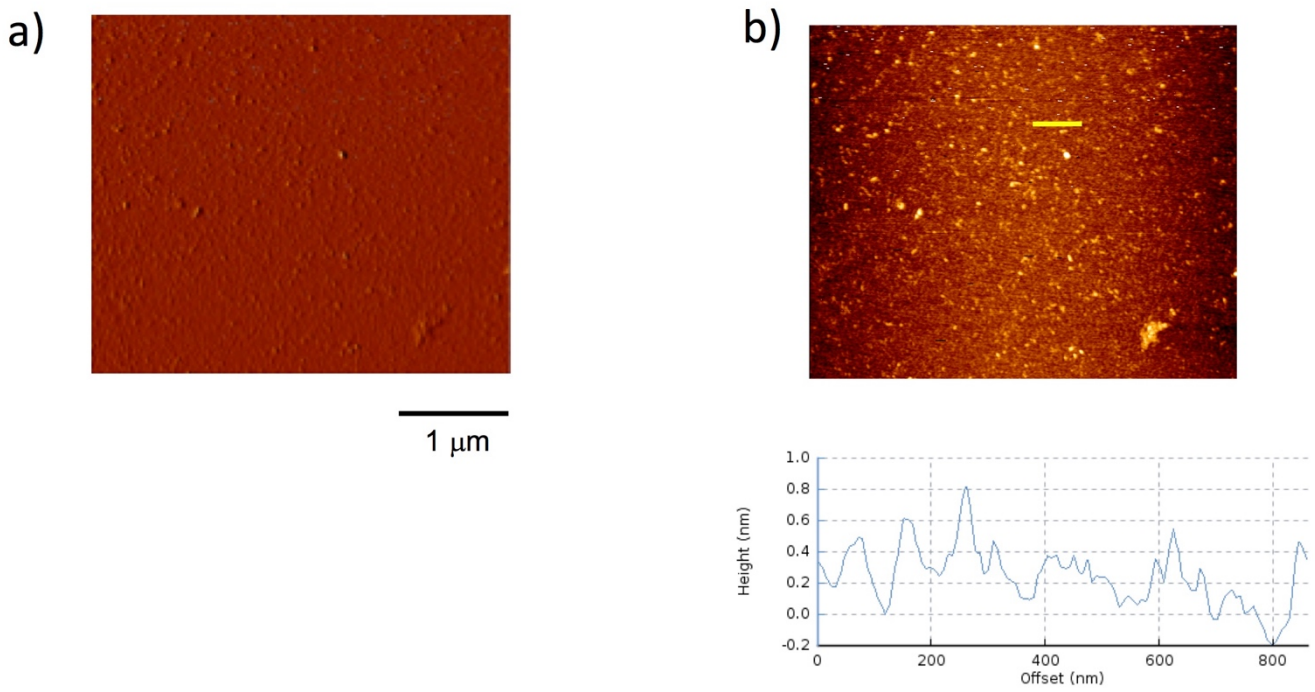


Fig. S5. AFM images of **3** (10 μM) as a 0.1% aqueous TFA. (a) The image is shown in amplitude mode. Scale bar measures 1 μm; (b) The image is shown in height mode. Lower graph indicates z-heights obtained at the yellow bar of upper image.

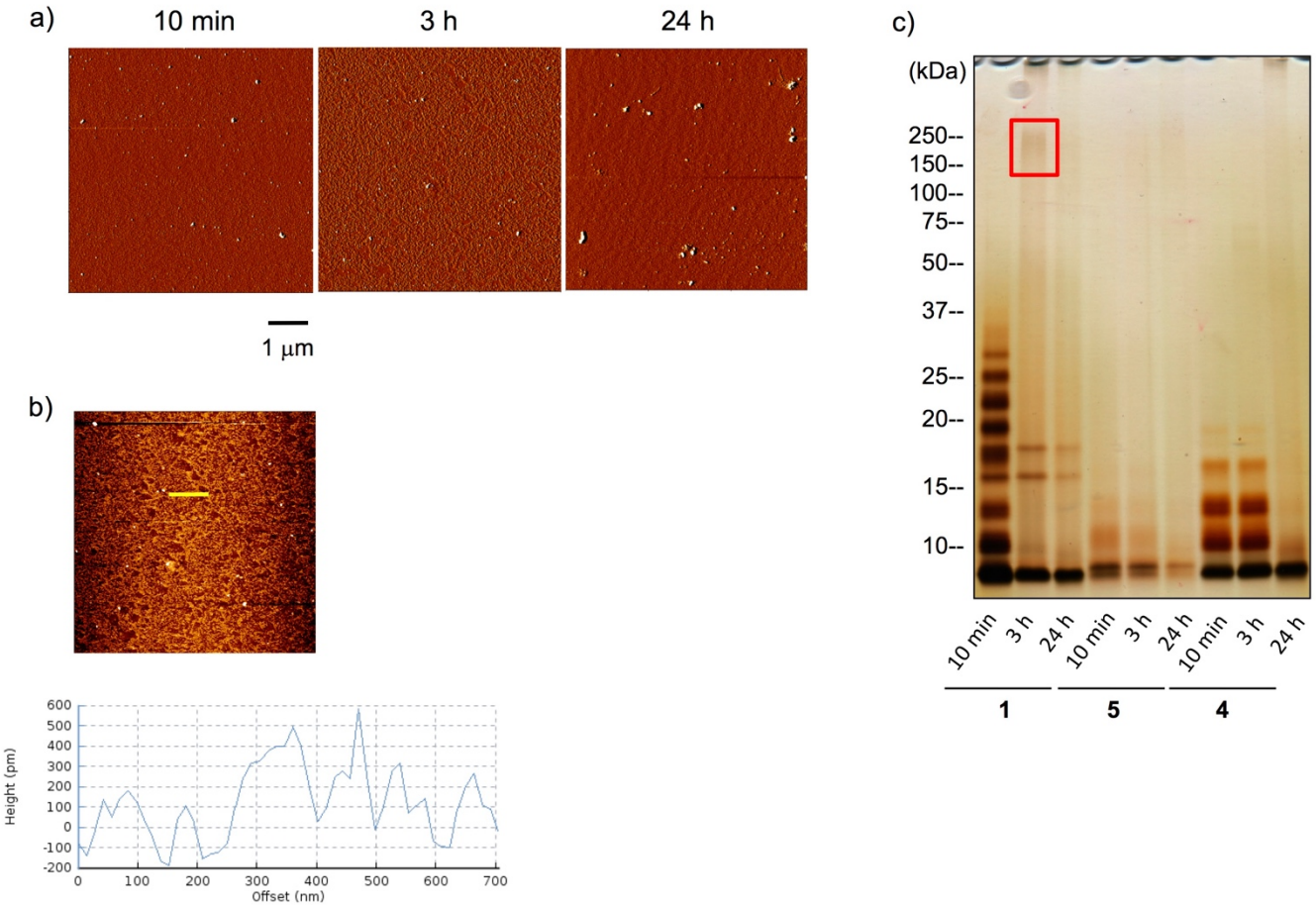


Fig. S6. (a, b) AFM images of **5** (10 μM). (a) The sample was incubated in 0.1 M HEPES buffer containing 0.1 M NaCl, pH 7.4, at 37 ˚C for the indicated time periods and then analyzed. The images are shown in amplitude mode. Scale bar measures 1 μm; (b) The image is shown in height mode. Incubate time = 3 h. Lower graph indicates z-heights obtained at the yellow bar of upper image; (c) SDS–PAGE analysis of **1** (20 μM), **5** (20 μM), and **4** (20 μM) after PICUP. Samples were incubated in phosphate buffer (10 mM, pH 7.4) at 37 ˚C for the indicated time periods and then analyzed. The aggregates were separated using a 15% Tris-glycine gel and detected with silver staining.

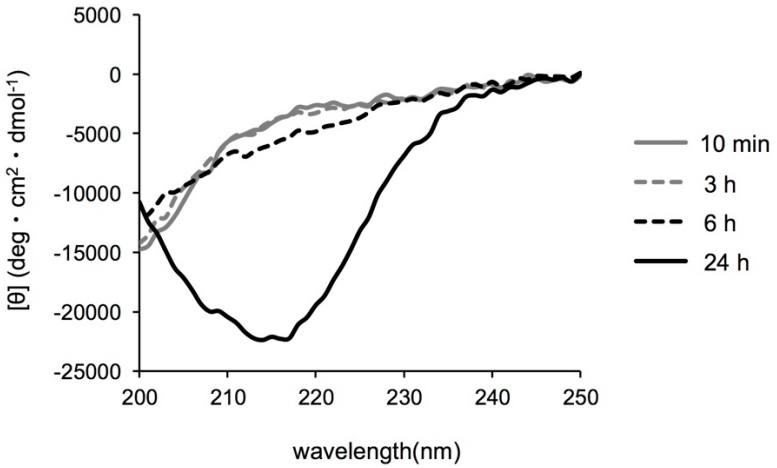


Fig. S7. CD spectroscopy of **4**. A phosphate buffer (pH 7.4) that contained **4** (10 μM) was incubated at 37 ˚C for the indicated time periods and then analyzed.

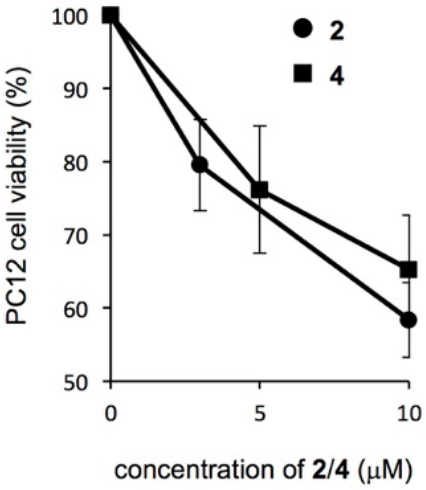
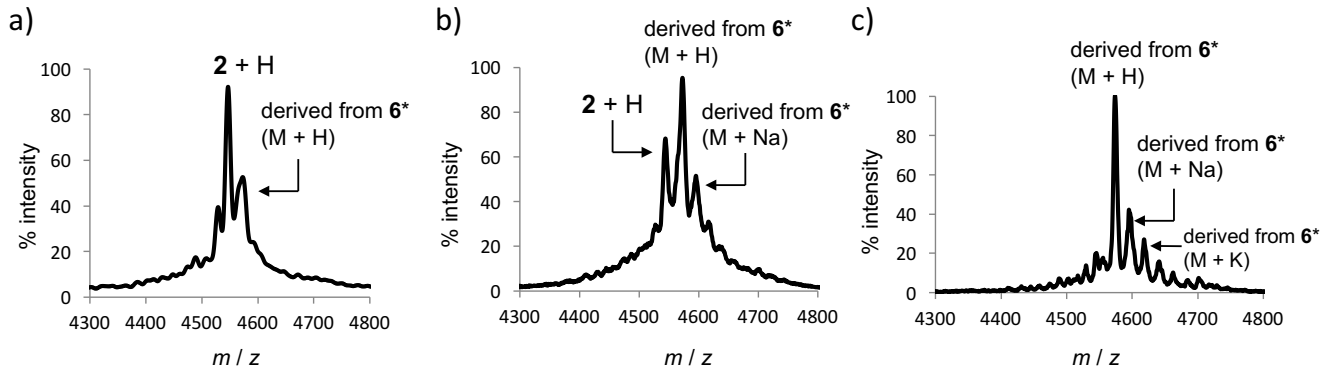


Fig. S8. Cell viability assay. PC12 cells were treated with **2** or **4** at 37 ˚C under 5% CO_2 for 3 d. Mean ± SEM, n = 3.

A



B

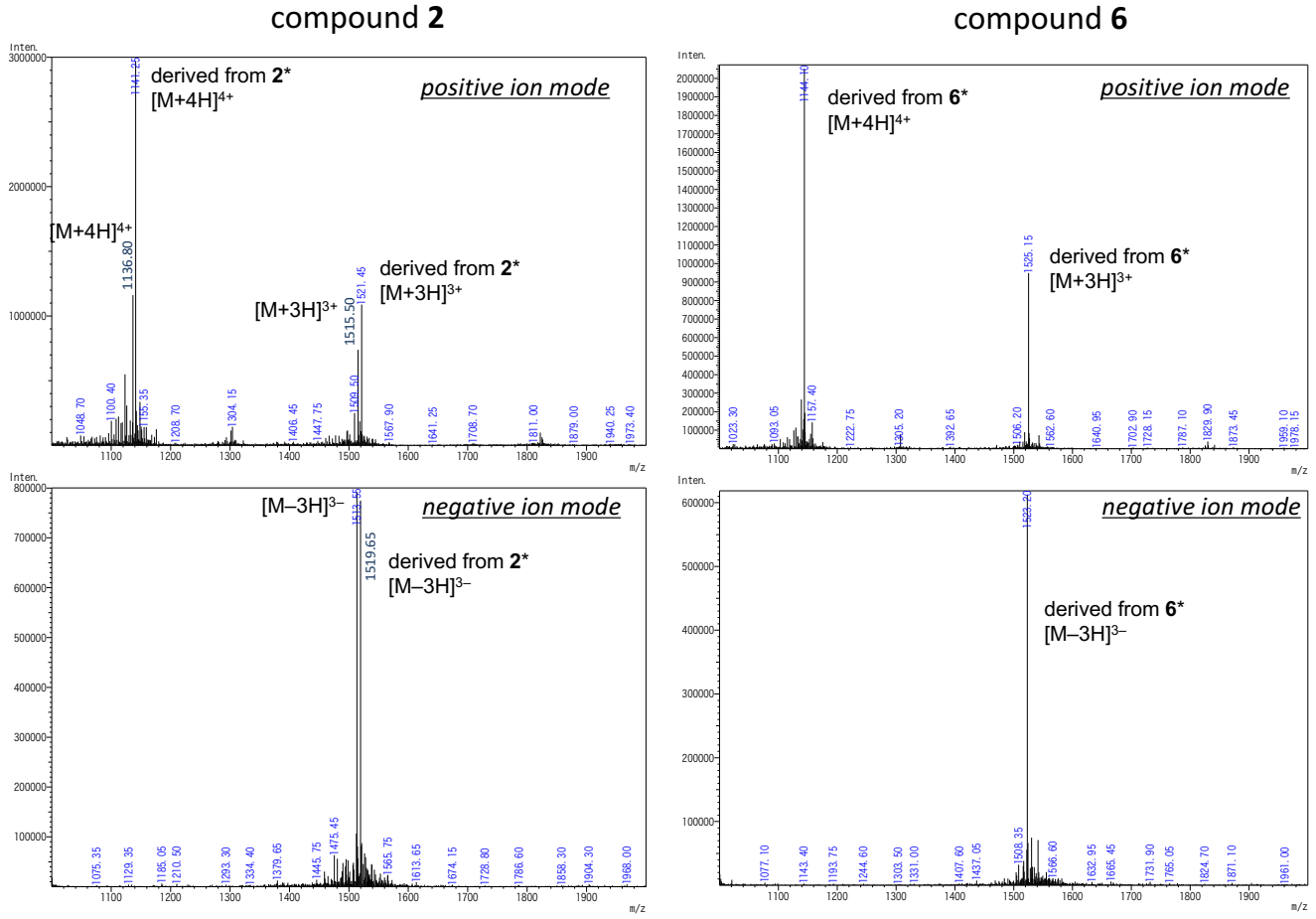


Fig. S9. **A.** MALDI-TOF MS spectra of the reaction mixture between **2** (0.1 mM) and varied concentrations of methoxyamine•HCl (a: 0.3 mM; b: 1 mM; c: 10 mM) in phosphate buffer at pH 4. derived from **6***: found M = 4571 (detected as dehydrated form of **6**).
B. ESI-MS spectra of **2** and **6** (**2***: detected as hydrated form of **2**). "Compound **2**": a sample before addition of methoxyamine•HCl was analyzed. "Compound **6**": identical sample to c) in **A** was analyzed.

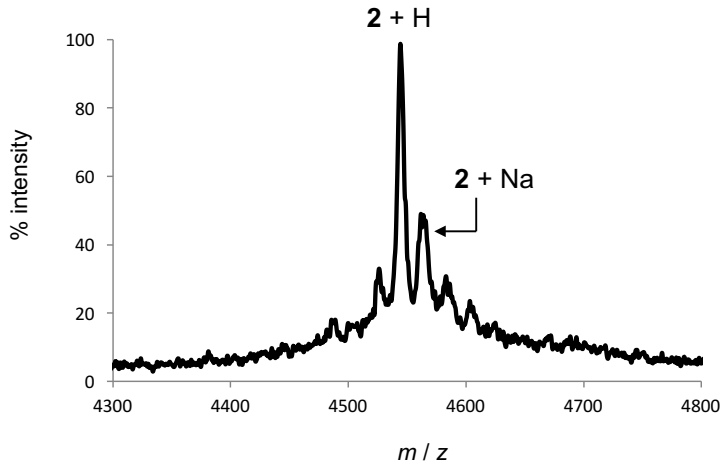


Fig. S10. MALDI-TOF MS spectrum of the reaction mixture between **2** (0.1 mM) and methylamine•HCl (10 mM) in phosphate buffer at pH 4.

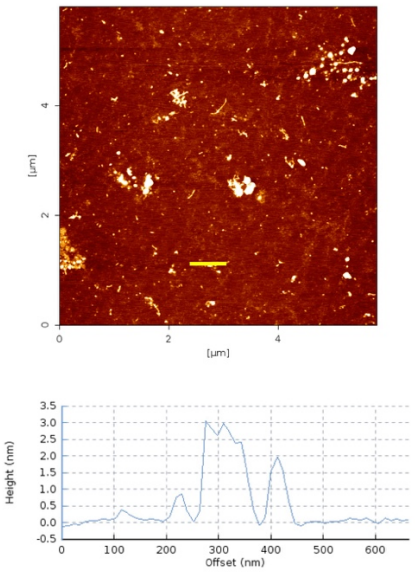


Fig. S11. AFM image of **2** (0.1 mM) in the presence of 10 mM $MeNH_2$. The sample was incubated in 0.1 M HEPES buffer containing 0.1 M NaCl (pH 7.4) at 37 ˚C. The image is shown in height mode. Lower graph indicates z-heights obtained at the yellow bar of upper image.

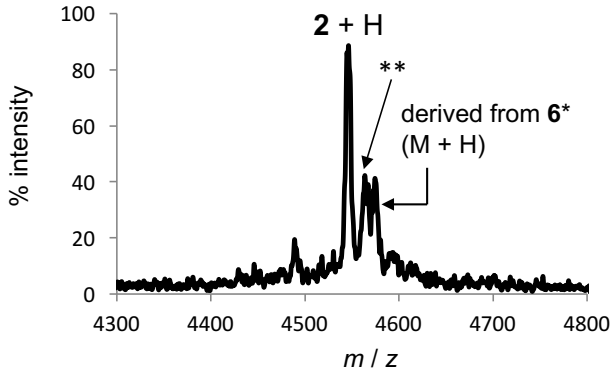


Fig. S12. MALDI-TOF MS spectra after removal of methoxyamine. After predominant conversion of **2** to **6** was confirmed (phosphate buffer, pH 4, **2**: 0.1 mM, methoxyamine: 10 mM, guanidinium chloride: 6 M to suppress possible aggregation during the following ultrafiltration) (see Figure S8c), methoxyamine was removed using ultrafiltration for 2 h. The resulting solution was acidified to pH 1, and the solution was incubated at room temperature for 16 h. The peak designated as "derived from **6***": found M = 4571 (detected as dehydrated form of **6**). The peak designated as "**": found (M + H) = 4560 (corresponding to a mass of hydrolyzed aldehyde product at the oxime bonds of **2**/**6**).

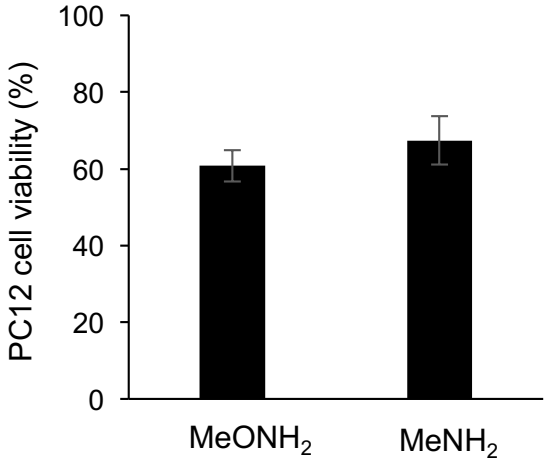


Fig. S13. Comparative toxicity of the oligomer formed from compound **2** (x-axis: $MeNH_2$) and fibril formed from compound **6** (x-axis: $MeONH_2$), corresponding to closing and opening forms of the oxime linker, respectively. Compound **2** (in 0.1% TFA) was added to PBS containing $MeONH_2$ or $MeNH_2$ (**2**, 40 μM; $MeONH_2$/$MeNH_2$, 2 mM; pH 7.4; we confirmed that **6**/**2** was dominant in the condition with $MeONH_2$/$MeNH_2$, respectively), incubated at 37 ˚C for 1 d, and the solutions were added to PC12 cell plates (final **2**, 10 μM; $MeONH_2$/$MeNH_2$, 500 μM). Then, the cells were incubated at 37 ˚C under 5% CO_2 for 2 d. Mean ± SEM, n = 3. Cell toxicity was not observed in the presence of $MeONH_2$/$MeNH_2$ at the concentration of 500 μM. Cell viability in the presence of $MeONH_2$ or $MeNH_2$ only (500 μM) is designated as “100” at y-axis.

References

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[S4] For a review of aldehyde functionalities for peptides, see: O. Melnyk, J. A. Fehrentz, J. Matinez and H. Gras-Masse, *Biopolymers (Peptide Science)*, 2000, **55**, 165–186.