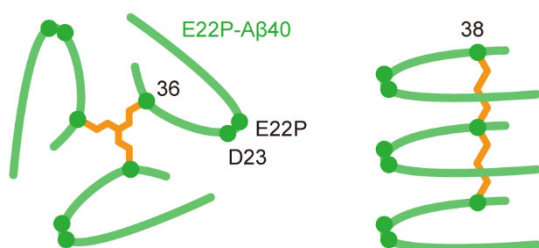


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Article Information

Synthesis and Characterization of Propeller and Parallel Type Full-length Amyloid β 40 Trimer Models

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ABSTRACT

Oligomers of the amyloid β (A β) protein play a critical role in the pathogenesis of Alzheimer's disease. However, their heterogeneity and lability deter the identification of their tertiary structures and mechanisms of action. A β trimers, as well as A β dimers, may represent the smallest aggregation unit with cytotoxicity. Although propeller-type trimer models of E22P-A β 40 tethered by an aromatic linker have recently been synthesized, they unexpectedly exhibited little cytotoxicity. To increase the flexibility of trimeric propeller-type models, we designed and synthesized trimer models with an alkyl linker, *tert*-butyltris-L-alanine (*t*ButA), at position 36 or 38. In addition, we synthesized two parallel-type trimer models tethered at position 38 using alkyl linkers of different lengths, α,α -di-L-norvalyl-L-glycine (di-nV-Gly) and α,α -di-L-homonorleucyl-L-glycine (di-hnL-Gly), based on the previously reported toxic dimer model. The propeller-type E22P,V36*t*ButA-A β 40 trimer (**4**), which was designed to mimic the C-terminal anti-parallel β -sheet structures proposed by the structural analysis of 150-kDa oligomers of A β 42, and the parallel-type E22P,G38di-nV-Gly-A β 40 trimer (**6**) showed significant cytotoxicity against SH-SY5Y cells as well as aggregative ability to form protofibrillar species. In contrast, the E22P,G38*t*ButA-A β 40 trimer (**5**) and E22P,G38di-hnL-Gly-A β 40 trimer (**7**) exhibited weak cytotoxicity though they formed quasi-stable oligomers observed by ion mobility-mass spectrometry and native polyacrylamide gel electrophoresis. These results suggest that **4** and **6** could have some phase of the structure of toxic A β oligomers with a C-terminal hydrophobic core, and that the conformation and/or aggregation process rather than the formation of stable oligomers contribute to the induction of cytotoxicity.

KEYWORDS

Alzheimer's disease, amyloid β , oligomer, SH-SY5Y cells, trimer, toxic conformation

INTRODUCTION

Alzheimer's disease (AD) is characterized pathologically by senile plaques consisting mainly of 40- and 42-mer amyloid β proteins (A β 40 and A β 42, Figure 1A).^{1,2} A β 42, with two more hydrophobic amino acid residues at the C-terminal region, is more aggregative and cytotoxic than A β 40.³ A β monomers aggregate to form oligomers, protofibrils,⁴ and finally, amyloid fibrils. Cellular membranes play a significant role in inducing the oligomerization of A β .^{5,6} Free lipids could affect the folding, stabilization, aggregation, and toxicity of A β .^{7,8} In addition, monosialoganglioside GM1 in membrane plays a critical role in A β aggregation.⁹ Membrane disruption by A β was suggested to be linked to the pathogenesis of AD.^{10,11} On the other hand, A β 40 and A β 42 can co-aggregate to form heterogenous fibrils,¹² and other amyloidogenic proteins such as tau and prion proteins,¹³ additionally, metals¹⁴ can also affect the aggregation process of A β .

It has been widely accepted that soluble oligomeric species including protofibrils of A β rather than mature A β fibrils induce cytotoxicity during AD progression, leading to the loss of neurons.^{5,15,16} A β oligomers can affect cells by binding to receptors¹⁷ or via oxidative stress through radicalization.¹⁸ Several A β oligomers, such as A β *56,¹⁹ A β -derived diffusible ligands (ADDLs),²⁰ amylospheroid,²¹ and protofibrils^{4,22} have been reported to be cytotoxic and/or synaptotoxic. Low molecular weight oligomers, even dimers, are reported to inhibit long-term potentiation (LTP),^{23,24} whereas high molecular weight oligomers may be more cytotoxic than low molecular weight oligomers.²² A β oligomers are aggregative and are in equilibrium among various species with different sizes and conformations, which hinders the determination of their tertiary structure and the mechanism of action of toxic A β oligomers. Furthermore, the aggregation process itself, rather than certain oligomeric species, could play an important role in the induction of cytotoxicity.^{25,26}

Structure determination of the transient A β oligomers is critical challenge to understand their mechanism of action. Recent investigations have revealed some phase of the structures of A β oligomers using nuclear magnetic resonance (NMR), mass spectrometry, and chemical and biological methods.²⁷⁻²⁹ Chemically pure and stable oligomeric models of A β would also be useful to investigate the characteristic features of toxic A β oligomers. Although A β dimers could represent a minimum unit of toxic oligomers,^{23,30} A β trimers could also contribute to the pathogenesis of AD. Trimerization is suggested to be an important step to form toxic oligomers as indicated by analysis of brain extracts of transgenic mice¹⁹ and chemically synthesized A β 40 in vitro.³¹ Furthermore, trimers inhibit LTP more potently compared with dimers and tetramers.³² Hitherto, several chemically pure trimer models of A β fragments have been synthesized.³³⁻³⁷ Models tethered with a disulfide bond³⁴ or with a cyclic peptide^{36,37} have shown certain

aggregative ability and cytotoxicity. However, such data for full-length A β trimer models are sparse.

Among the structural assembly of A β trimers, that with the intermolecular parallel β -sheet structure is most commonly observed, as determined by solid-state NMR^{38–41} and cryo-electron microscopy (cryo-EM).^{42–44} In contrast, the propeller-type trimer is also possible; Huang and coworkers found the existence of anti-parallel β -sheet structures at positions 32–40 in 150-kDa oligomers of A β 42 by using solid-state NMR.⁴⁵ Based on this result, they proposed a C₃-symmetric trimeric structure as a rational model. We have recently synthesized quasi-stable C₃-symmetrical propeller-type trimer models of full-length A β 40 according to the proposed structure.⁴⁶ Three E22P-A β 40 monomers were trimerized with a linker amino acid 1,3,5-phenyltris-L-alanine (PtA) at position 34, 36, or 38 (**1–3**, Figure 1B). Proline replacement at position 22 of A β 40 would accelerate the formation of the turn structure at positions 22/23, increasing its aggregative ability and cytotoxicity compared with wild-type A β 40 (WT-A β 40).^{47,48} Interestingly, four familial mutations were found at position 22 (E22Q: Dutch, E22K: Italian, E22G: Arctic, and E22 Δ : Osaka),^{49,50} and replacement of L-Glu with D-Glu at the position stabilized oligomers and increased their cytotoxicity.^{51,52} Among the three trimer models (**1–3**), **3** was the only model that exhibited weak cytotoxicity against SH-SY5Y cells.⁴⁶ This research suggests that these trimer models may not adopt the optimal conformation of toxic A β trimers possibly because of the poorly flexible structure of the aromatic ring in the linker.

In order to improve the flexibility of the linker, we replaced the benzene ring that binds three α -amino acid moieties in PtA with a flexible alkyl chain; that is, a C₃-symmetric *tert*-butyltris-L-alanine (*t*ButA, Figure 1C) was developed as reported previously.⁵³ In addition, a linear tris- α -amino acid, α,α -di-L-homonorleucyl-L-glycine (di-hnL-Gly), for a trimer model with a parallel β -sheet structure, was synthesized.⁵³ In this study, we newly synthesized a linear tris- α -amino acid, α,α -di-L-norvalyl-L-glycine (di-nV-Gly), which had a spacer shorter than di-hnL-Gly and corresponding to an L,L-diaminopimelic acid (DAP) linker previously used for the synthesis of a toxic dimer model.⁵⁴ This paper reports the synthesis and characterization of the trimer models with these alkyl linkers at position 36 or 38 of E22P-A β 40 (**4–7**).

RESULTS AND DISCUSSION

Design and Synthesis of Trimer Models Tethered with Linker Amino Acids. Synthesis and purification of A β 42 is more difficult compared with those of A β 40 because of its continuous sequence of bulky hydrophobic residues, Val39, Val40, and Ile41, in the C-terminal region, and its highly aggregative nature.⁵⁵ Synthesis of trimer models is more challenging even in an A β 40

type, as exemplified in the model of 120 residues with a PtA linker.⁴⁶ Cross-linking at the C-terminal region of E22P-A β 40, a toxic conformer mimetic analog,^{47,48} could form a hydrophobic core to induce oligomerization to exhibit cytotoxicity. Based on this consideration, propeller-type trimer models were designed by tethering E22P-A β 40 with a linker amino acid *t*ButA, which is more flexible than PtA, at position 36 or 38 (**4** and **5**, Figure 1C). Position 36 is the central residue in the C₃-symmetric structure of the 150-kDa A β 42 aggregate proposed by Huang and coworkers,⁴⁵ and position 38 is that where the previous trimer model **3** with weak cytotoxicity is tethered.⁴⁶

The trimer models of intermolecular parallel β -sheet-types (**6** and **7**) were designed to mimic the trimers as substructures of aggregates with intermolecular parallel β -sheet structures proposed by solid-state NMR and cryo-EM.^{38–44} Our previous study on the corresponding dimer models suggested that a DAP linker of three carbons in the spacer could give a toxic dimer model by tethering E22P-A β 40 at position 38.⁵⁴ However, an L,L-diaminoazelaic acid (DAZ) linker of five carbon atoms in the spacer might be more suitable for the formation of intermolecular β -sheet structures as observed in the dimer model at position 30 with a DAZ linker, whose β -sheet content was higher than that of the corresponding dimer model with a DAP linker.⁵⁴ Based on these results, we designed two linear linker amino acids, di-nV-Gly and di-hnL-Gly, for the trimer models with intermolecular parallel β -sheets (Figure 1C). The number of methylene carbon atoms between adjacent α -carbon atoms of amino acid moieties was three for di-nV-Gly and five for di-hnL-Gly, respectively. The cross-linking position was set at position 38 (**6** and **7**, Figure 1C).

The linkers were synthesized as Fmoc-protected amino acids for Fmoc solid-phase peptide synthesis. Tri-Fmoc-*t*ButA and tri-Fmoc-di-hnL-Gly were synthesized as reported previously.⁵³ Tri-Fmoc-di-nV-Gly was synthesized according to the protocol of tri-Fmoc-di-hnL-Gly in 10 steps (Scheme 1). Two 3-butenyl groups were introduced at the α -position of isocyanide **8** to afford **9** (66%). Acidic hydrolysis of the isocyanide group and subsequent Cbz protection of a primary amino group gave a protected amino acid **10** (94% in 2 steps). Ozonolysis of the two terminal olefines produced a mixture containing dialdehyde and possibly hemiaminal, which was subjected to Horner-Wadsworth-Emmons reaction conditions without isolation to yield **11** (22% in 2 steps). Asymmetric hydrogenation of **11** with (*S,S*)-QuinoxP*-Rh catalyst⁵⁶ produced **12** (98%, 96%ee, 94%de; Figure S1, Supporting Information), which was converted to di-nV-Gly by acidic hydrolysis of a *tert*-butyl ester, alkaline hydrolysis of methyl esters, and subsequent removal of Cbz groups. Fmoc protection of primary amino groups of di-nV-Gly gave tri-Fmoc-di-nV-Gly in 62% yield from **12**.

With these linker amino acids in hand, we synthesized four trimer models of E22P-A β 40 (**4**–**7**) by solid-phase synthesis with a microwave peptide synthesizer.⁴⁶ One-third equivalent of each Fmoc-linker amino acid was employed, instead of Fmoc-L-Val at position 36 or Fmoc-L-Gly at

position 38, to reduce the formation of the mono-coupled and/or di-coupled peptides. Each peptide was extended in a stepwise fashion on preloaded Fmoc-L-Val-PEG-PS resin according to the synthesizer's protocol. A coupling reaction of the C₃-symmetric linker, Fmoc-*t*ButA, was carried out for 10 min at 75 °C with *O*-(7-aza-1*H*-benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU)⁵⁷ and *N,N*-diisopropylethylamine (DIPEA) in *N,N*-dimethylformamide (DMF).

In the synthesis of the intermolecular parallel β -sheet-type model **6** using Fmoc-Val Tentagel R PHB resin under the above linker coupling conditions, many di-coupled peptides were produced. An extension of the coupling reaction time to 20 min increased production of the desired tri-coupled peptide from 2.3% to 3.8% after HPLC purification using a C₄ column, and the improved conditions were used for the synthesis of **6** and **7**. Finally, the trimer models **4–7** were synthesized at yields of 1.5%, 1.2%, 0.53%, and 0.80%, respectively, after HPLC purification using C₄ and C₁₈ columns and lyophilization. The purity of each peptide was checked by HPLC analyses (>98%), and its molecular weight and formula were confirmed by HR-ESI-qTOF-MS measurements (Figure S2 and S3, Supporting Information).

Cytotoxicity of the Trimer Models against SH-SY5Y Neuroblastoma Cell Lines. The cytotoxicity of the trimer models **4–7** against human neuroblastoma SH-SY5Y cell lines was evaluated with MTT assay. The previous trimer models (**2** and **3**)⁴⁶ with a C₃-symmetric aromatic linker were also assayed for comparison. After a 24-h incubation with each trimer model or E22P-A β 40 as a positive control, cell viability was measured by quantifying the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduced by living cells according to the previously established protocol.^{46,54} Although MTT assay is commonly used to evaluate cellular metabolic activity, the results could be affected by various factors such as cell number of seeding, concentration of MTT reagent, incubation time in the presence of A β , serum starvation, and culture media composition.⁵⁸ Thus, caution should be used to interpret the data between different experiments.

Among the propeller-type trimers, the models linked at position 38 (**3** and **5**) showed weak cytotoxicity (Figure 2A). This means that substitution with the flexible alkyl linker did not enhance the cytotoxicity at all when the linker was bound at position 38. In contrast, the propeller-type trimer model with the alkyl linker at position 36 (**4**) exhibited significant cytotoxicity, while the corresponding trimer model with the aromatic linker (**2**) was almost inactive. Val36 is the central residue in the C₃-symmetric structure proposed by Huang and coworkers.⁴⁵ An increase in flexibility of the linker at position 36 might have allowed the formation of the proposed tertiary structure. This indicates that a C₃-symmetric trimeric structure proposed by the analysis of the 150-kDa A β 42 oligomers⁴⁵ could be one of the toxic conformations of the A β oligomers.

Unexpectedly, the intermolecular parallel β -sheet-type trimer model with a longer linker of 5 carbon atoms (**7**) showed little cytotoxicity (Figure 2B). It is quite noteworthy that the cytotoxicity of the trimer model with a shorter cross-linker of 3 carbon atoms (**6**) was almost equal to that of E22P-A β 40. This suggests that the shorter the length of the cross-linker, the higher the cytotoxicity.

Aggregative Ability of the Trimer Models. The aggregative abilities of the trimer models **4–7** were assessed using the fluorescent reagent thioflavin T (Th-T), which binds the β -sheet structure in A β aggregates.⁵⁹ Each A β peptide was incubated with Th-T, and the fluorescence was measured every 10 min over 24 h. WT-A β 42 and E22P-A β 40 exhibited high fluorescence after a 4- and a 8-h incubation, respectively, suggesting the formation of highly aggregated fibrils (Figure 3A). Among the propeller-type trimer models including the previous models (**2–5**), only the fluorescence of **4** with an alkyl linker at position 36 began to increase after a 7-h incubation, while the other propeller-type trimer models were negative after a 24-h incubation. Huang and coworkers suggested that the 32–40th residues formed anti-parallel β -sheet structures in the 150-kDa oligomers of A β 42.⁴⁵ Formation of C-terminal anti-parallel β -sheets in **4** could induce its aggregation to show significant cytotoxicity (Figure 2A). In addition, C₃-symmetric structural model of A β 40 proposed by Tycko and coworkers using solid-state NMR and electron microscopy could be another possible conformation of **4**.³⁸ Their model has intermolecular β -sheets formed by 11–22nd and 30–39th residues, and extended to form twisted fibrils with triangular cross-sections. Since position 36 is close to the center of a C-terminal triangular structure compared to position 38, **4** might suit for the formation of this C₃-symmetric structure including intermolecular β -sheets.

On the other hand, the intermolecular parallel β -sheet-type trimer model with a shorter linker (**6**) showed an increase in fluorescence, albeit weakly, soon after the initiation of incubation, while **7** with a longer linker did not (Figure 3B). This suggests that a trimer model with a shorter linker could easily form a C-terminal hydrophobic core to develop larger aggregates. In accordance with these results, the corresponding dimer model (E22P,G38DAP-A β 40 dimer) formed protofibrils after a 48-h incubation.^{46,54} Stabilizing intermolecular β -sheet structures and growing up to larger oligomers would be important, as shown in the study reported that tetramers and octamers forming β -sheets showed cytotoxicity by membrane disruption.⁶⁰

Transmission electron microscopy (TEM) analysis confirmed the formation of aggregates (Figure 4). After a 48-h incubation of each peptide in the absence of Th-T, WT-A β 42 and E22P-A β 40 formed typical amyloid fibrils. In contrast, protofibrillar aggregates were observed for the cytotoxic trimer models (**4** and **6**), while only amorphous aggregates were detected for the less toxic models (**5** and **7**). This morphology is in good agreement with the Th-T intensity in Figure

3. In our previous data on several dimer and trimer models, only the cytotoxic models tended to form protofibrillar aggregates.^{46,54} However, the morphology of aggregates of **4** was not like typical fibrils regardless of its positive response in Th-T assay. The aggregation process and/or the tertiary structure might be different from that of WT-A β 42 and E22P-A β 40.

Oligomerization of the Trimer Models. We attempted to evaluate the oligomer size of the trimer models **4–7** by ion mobility-mass spectrometry (IM-MS) (Figure 5). Recently, IM-MS combined with native ionization techniques and ion mobility spectrometry (IMS) made it possible to separate each multivalent ion derived from various A β oligomers.^{61,62} The previously reported trimer models with an aromatic linker at position 36 or 38 (**2** and **3**) were also measured for comparison. The two-dimensional heat maps with m/z and drift time after a 4-h incubation are shown in Figure 5. As reported previously,^{46,54} a 4-h incubation was required for the definitive formation of higher molecular weight oligomers. Cross peaks on the maps were assigned to the series of multivalent ions after deconvolution based on the measured and calculated masses ($n = 1, 2, 3, \dots$ of each trimer model denotes trimer, hexamer, nonamer, ..., respectively). The propeller-type trimer models with an aromatic linker (**2** and **3**) formed 3–9-mer ($n = 1–3$) and 3–18-mer ($n = 1–6$), respectively, which is consistent with previous results.⁴⁶ Among the alkyl-linked propeller-type trimer models cross-linked at position 36 or 38 (**4** and **5**), 3–6-mer ($n = 1–2$) were detected for **4**, while higher molecular weight oligomers (9–18-mer, $n = 3–6$) were observed for **5**. In contrast, both the intermolecular parallel β -sheet-type trimer models (**6** and **7**) formed relatively high molecular weight oligomers up to 15-mer ($n = 5$) and 18-mer ($n = 6$), respectively. These results indicate that the trimer models tethered at position 38 tended to form larger oligomers.

IM-MS could detect up to 20–30-mer oligomers, but peaks greater than 30-mer could not be identified because of their weak intensity as reported previously.^{46,54,63} We then performed polyacrylamide gel electrophoresis (PAGE) under nondenaturing conditions to assess oligomerization of the trimer models beyond the range detectable by IM-MS (Figure 6). After a 24-h incubation, little bands ascribable to oligomers were detected in WT-A β 42 and E22P-A β 40 that formed typical fibrils and exhibited significant cytotoxicity.⁴⁷ The propeller-type trimer model **4** showed a band of over 1000 kDa along with few bands corresponding to oligomers after a 24-h incubation, suggesting that **4** could form over 30-mer aggregates that could not be identified by IM-MS. A similar pattern was observed for the intermolecular parallel β -sheet-type trimer model **6**. It is quite noteworthy that **4** and **6** exhibited significant cytotoxicity against SH-SY5Y cells (Figure 2).

In contrast, strong smears ranging from ca. 50–700 kDa were detected for the trimer models **5** and **7**. As shown in Figure 2, cytotoxicity of **5** and **7** were extremely weak. This indicates that

the ability to form quasi-stable oligomers less than 30-mer could not correlate to the cytotoxicity. The ability to form larger aggregates over 700 kDa (ca. 150-mer) would be more important to induce cytotoxicity.

CONCLUSIONS

In summary, we synthesized two types of trimer models of E22P-A β 40, the propeller-type trimer models (**4** and **5**) and the intermolecular parallel β -sheet-type models (**6** and **7**), tethered by flexible alkyl linkers at position 36 or 38 based on the recent structural studies on A β aggregates (Figure 1). Their biological and physicochemical properties were quite different from each other. The propeller-type trimer model that mimicked the anti-parallel β -sheet at the C-terminal region of 150-kDa oligomers of A β 42 (**4**)⁴⁵ formed protofibrillar aggregates to exhibit significant cytotoxicity (Figure 2), suggesting that **4** could become one of the toxic trimer models. These results also suggest that the flexible linker can make the model adopt a toxic conformation.

On the other hand, only the trimer model of the intermolecular parallel β -sheet-type with a shorter linker (**6**) formed protofibrillar aggregates to exhibit significant cytotoxicity, while the model with a longer linker (**7**) remained as globular aggregates of less than 50-mer, and was not as cytotoxic as **6**. The linker length between each E22P-A β 40 of **6** is the same as that of a dimer model at position 38 with a DAP linker, which exhibited significant cytotoxicity comparable to that of the E22P-A β 40 monomer.⁵⁴ These results suggest that the length of the linker is quite important to make the model adopt a toxic conformation and to afford toxic oligomers. Further study is needed to optimize the length of the linker to develop an ideal trimer model with intermolecular parallel β -sheets. Shorter linkers have an advantage in the formation of hydrophobic core to form larger aggregates. In both propeller- and parallel-type trimer models, the formation of an aggregation core at the C-terminal region would play an important role in aggregation to induce cytotoxicity. Since A β 42, unlike A β 40, is hypothesized to form the C-terminal hydrophobic core by radicalization,⁶⁴ cross-linking at the appropriate C-terminal position of A β 40 could make its aggregative ability similar to A β 42.

Although the trimer models **5** and **7** formed quasi-stable oligomers detected by IM-MS and PAGE under nondenaturing conditions, they were not as cytotoxic as **4** and **6**. This indicates that the formation of stable oligomers is not always necessary for cytotoxicity, and that the conformation of A β in toxic oligomers could be one of the critical factors. The importance of the conformation rather than the oligomer size in cytotoxicity has also been suggested by recent studies using sonicated A β 42 fibrils,⁶⁵ the chiral mutant library of A β 42,^{66,67} and fixed-conformation analogs of A β 42 with an intramolecular disulfide bond.⁶³ Moreover, the aggregative process could be another possible contributor to the pathogenesis of AD. Nucleated

polymerization is reported to induce cytotoxicity.^{25,26} The trimer models **4** and **6** formed protofibrillar species, which could be the seeds for further aggregation.

This study showed that **4** and **6** could be useful as toxic trimer models. We are planning to produce antibodies against these trimer models, which might contribute to AD diagnosis and/or therapeutics in its early stage. However, caution should be used since they function in heterogeneous conditions with metals, membranes, and proteins. Interactions between the models and other constituents of the biological environment should be considered in further investigations.

MATERIALS AND METHODS

General remarks. Analytical thin-layer chromatography was performed with TLC Silica gel 60 F₂₅₄ (Merck, Darmstadt, Germany). Silica gel column chromatography was carried out with Wakogel C-200 (Wako Pure Chemical Industries, Osaka, Japan) or Chromatorex BW-300 (Fuji Silica Chemical, Aichi, Japan). Preparative HPLC was performed with a model 600E with a model 2487 UV detector (Waters, Milford, MA, USA). Gel permeation chromatography was performed with a LaboACE LC-5060 with a JAIGEL-1HR+2HR column, a UV-Vis 4ch 800LA UV/Visible detector, and an RI-700LA differential refractometer (Japan Analytical Industry, Tokyo, Japan). Proton nuclear magnetic resonance (¹H NMR) spectra were recorded on an AVANCE III 500 (Bruker, Germany) at 500 MHz. Chemical shifts were reported relative to Me₄Si (δ 0.0) in CDCl₃, and residual solvent of CD₃OD (δ 3.31) and D₂O (δ 4.79). Multiplicity is indicated by one or more of the following: s (singlet); d (doublet); t (triplet); m (multiplet); br (broad). Carbon nuclear magnetic resonance (¹³C NMR) spectra were recorded on an AVANCE III 500 (Bruker, Germany) at 126 MHz. Chemical shifts were reported relative to CDCl₃ (δ 77.0) and CD₃OD (δ 49.0). Infrared spectra were recorded on a FT/IR-470 Plus Fourier-transform infrared spectrometer (Jasco, Tokyo, Japan). Specific rotations were recorded on a P-2200 digital polarimeter (Jasco, Tokyo, Japan). Low- and high-resolution mass spectra were recorded on a JMS700 mass spectrometer (JEOL, Tokyo, Japan) for FAB-MS (matrix, *m*-nitrobenzyl alcohol). The trimer models were synthesized using an Initiator+ Alstra peptide synthesizer (Biotage, Uppsala, Sweden). Analytical HPLC was performed with a Waters model 1525 binary HPLC pump with a model 2489 UV/Visible detector and EmpowerTM3 software (Waters, Milford, MA, USA). HR-ESI-qTOF-MS spectra were recorded on a Waters Xevo G2-S qTOF (Waters, Milford, MA, USA). Absorbance and Fluorescence on microplates were recorded on microplate readers, Multiskan FC and Fluoroskan Ascent (Thermo Fisher Scientific, Waltham, MA, USA). Transmission electron microscopic observation was carried out with a JEM-1400 transmission electron microscope (JEOL, Tokyo, Japan). HPLC was carried out on YMC PROTEIN-RP column (20 mm I.D. x 150 mm, 5 μ m, YMC, Kyoto, Japan) and XBridge Peptide BEH C18 OBDTM Prep Column (10 mm I.D. x 250 mm, 300 Å, 5 μ m, Waters, Milford, MA, USA).

Materials for synthesis of tri-Fmoc-di-nV-Gly were obtained from Tokyo Chemical Industry (Tokyo,

Japan), Sigma-Aldrich (St. Louis, MO, USA), FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), and other commercial suppliers and used without further purification. HATU,⁵⁷ CH₂Cl₂, and NH₄OH (25%) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). DIPEA and Fmoc-L-Val-PEG-PS resin were purchased from Applied Biosystems (Foster City, CA, USA). DMF, trifluoroacetic acid (TFA), *m*-cresol, 1,2-ethanedithiol, thioanisole, and diethyl ether (peroxide free) were purchased from Nacalai Tesque (Kyoto, Japan). Piperidine was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fmoc amino acids were purchased from Applied Biosystems (Foster City, CA, USA), Watanabe Chemical Industries (Hiroshima, Japan), Merck (Kenilworth, NJ, USA), or PerSeptive Biosystems (Framingham, MA, USA).

Synthesis of Tri-Fmoc-di-nV-Gly. Compound 9. To a suspension of NaH (60%) (1.38 g, 34.5 mmol) in dry Et₂O/dry DMSO (100 mL/5 mL) was added **8** (1.40 g, 9.94 mmol) dropwise under an argon atmosphere at room temperature. The mixture was stirred for 30 min. 4-Bromo-1-butene (3.35 g, 24.8 mmol) was added and the reaction mixture was stirred at room temperature overnight. Ten mL of H₂O was added dropwise to the reaction mixture at room temperature. The two layers were separated, and the aqueous layer was extracted with Et₂O 3 times. The organic layers were washed with brine, dried over Na₂SO₄, filtered, and concentrated. The crude product was purified by silica gel column chromatography (hexane/Et₂O = 1/0 to 9/1) to afford **9** (1.65 g, 66%) as a pale yellow oil. ¹H NMR: (500 MHz, CDCl₃, 0.079 M): δ 5.78 (ddt, *J* = 17.0, 10.3, 6.5 Hz, 2H), 5.10–5.06 (m, 2H), 5.03–5.01 (m, 2H), 2.38–2.30 (m, 2H), 2.09–1.96 (m, 4H), 1.84 (ddd, *J* = 13.3, 12.6, 4.6 Hz, 2H), 1.51 (s, 9H) ppm; ¹³C NMR: (126 MHz, CDCl₃, 0.079 M): δ 167.3, 159.0, 136.2 (2C), 116.0 (2C), 83.8, 68.1, 38.4 (2C), 28.3 (2C), 27.8 (3C) ppm; IR (neat on KBr plate): ν_{max} 3081, 2979, 2933, 2856, 2136, 1748, 1644, 1370, 1148, 916, 843 cm⁻¹; HRMS (FAB): *m/z* calcd for C₁₅H₂₄NO₂: 250.1807 [M+H]⁺; found: 250.1810.

Compound 10. Isocyanide **9** (1.53 g, 6.13 mmol) was dissolved in EtOH (60 mL) containing 12 M aqueous HCl solution (0.5 mL) and the resulting mixture was stirred for 1 h at room temperature. The solvent was removed under reduced pressure and the remaining oil was taken up in toluene (30 mL). The mixture was cooled to 0 °C, and CbzCl (3.12 g, 18.29 mmol) and 2 M aqueous Na₂CO₃ solution (15 mL) were added. The reaction mixture was stirred at room temperature for 3 h. The two layers were separated, and the aqueous layer was extracted with EtOAc 3 times. The organic layers were washed with 0.1 M aqueous HCl solution and saturated aqueous NaHCO₃ solution, dried over Na₂SO₄, filtered, and concentrated. The crude product was purified by silica gel column chromatography (hexane/Et₂O = 1/0 to 9/1) to afford **10** (2.14 g, 94% for 2 steps) as a colorless oil. ¹H NMR: (500 MHz, CDCl₃, 0.079 M): δ 7.38–7.28 (m, 5H), 5.90 (s, 1H), 5.75 (ddt, *J* = 16.7, 10.0, 6.7 Hz, 2H), 5.07 (s, 2H), 4.98–4.95 (m, 2H), 4.93–4.90 (m, 2H), 2.48–2.42 (m, 2H), 2.08–1.99 (m, 2H), 1.81–1.73 (m, 4H), 1.48 (s, 9H) ppm; ¹³C NMR: (126 MHz, CDCl₃, 0.079 M): δ 172.5, 153.8, 137.7 (2C), 136.8, 128.5 (2C), 128.0, 127.8 (2C), 114.9 (2C), 82.5, 66.1, 63.6, 34.9 (2C), 28.5 (2C), 27.9 (3C) ppm; IR (neat on KBr plate): ν_{max} 3418, 3077, 2977, 2940, 1718, 1642, 1496, 1158, 912, 697 cm⁻¹; HRMS (FAB): *m/z* calcd for C₂₂H₃₂NO₄: 374.2331 [M+H]⁺; found:

374.2335.

Compound 11. A solution of **10** (1.05 g, 2.80 mmol) in CH₂Cl₂ (30 mL) was cooled to -78 °C. The solution was bubbled with O₃ until the color of it turned blue (12 min). Excess ozone was removed from the reaction mixture by purging with O₂ for 5 min. To the mixture was added Ph₃P (2.21 g, 8.43 mmol) at -78 °C. The reaction mixture was stirred at -78 °C for 10 min and warmed to 0 °C with stirring for an additional 2 h. The reaction mixture was washed with water, and the aqueous layer was extracted with CHCl₃ 3 times. The combined organic layers were washed with brine, dried over Na₂SO₄, filtered, and concentrated. The crude product was purified by silica gel column chromatography (hexane/EtOAc = 4/1 to 1/1) to afford a mixture containing dialdehyde as a colorless oil. To a solution of (Z)- α -phosphonoglycine trimethyl ester (1.63 g, 4.91 mmol) in CH₂Cl₂ (8 mL) was added DBN (624 mg, 5.02 mmol). The mixture was stirred at 0 °C for 10 min, to which a solution of the above mixture containing dialdehyde in CH₂Cl₂ (12 mL) was then added slowly at 0 °C. The reaction mixture was gradually warmed to room temperature with stirring overnight and was washed with 1 M aqueous HCl solution. The aqueous layer was further extracted with EtOAc 3 times. The organic layers were washed with brine, dried over Na₂SO₄, filtered, and concentrated. The crude product was purified by silica gel column chromatography (hexane/EtOAc = 13/7 to 3/2). The fractions containing **11** were concentrated and further purified by gel permeation chromatography (CHCl₃) to afford **11** (488 mg, 22% for 2 steps) as a colorless oil. ¹H NMR: (500 MHz, CDCl₃, 0.035 M): δ 7.36–7.27 (m, 15H), 6.55 (t, *J* = 7.4 Hz, 2H), 6.29 (br s, 2H), 5.92 (s, 1H), 5.09 (s, 4H), 5.00 (s, 2H), 3.71 (s, 6H), 2.46–2.41 (m, 2H), 2.21–2.12 (m, 2H), 2.07–2.00 (m, 2H), 1.91–1.85 (m, 2H), 1.42 (s, 9H) ppm; ¹³C NMR: (126 MHz, CDCl₃, 0.035 M): δ 171.8, 164.9 (2C), 154.3, 154.1 (2C), 136.4, 136.2 (2C), 135.9 (2C), 128.54 (4C), 128.50 (2C), 128.3 (2C), 128.2 (4C), 128.0, 127.8 (2C), 125.9 (2C), 83.2, 67.4 (2C), 66.5, 63.4, 52.4 (2C), 34.1 (2C), 27.8 (3C), 23.4 (2C) ppm; IR (neat on KBr plate): ν_{max} 3329, 3033, 2962, 1714, 1660, 1504, 1260, 1063, 799, 698 cm⁻¹; HRMS (FAB): *m/z* calcd for C₄₂H₅₀N₃O₁₂: 788.3394 [M+H]⁺; found: 788.3391.

Compound 12. A solution of [Rh(cod)₂]BF₄ (5.2 mg, 12.8 μ mol) and (*S,S*)-QuinoxP* (5.6 mg, 16.7 μ mol) in degassed MeOH (1 mL) was stirred vigorously at room temperature under nitrogen atmosphere. After 35 min, additional degassed MeOH (4 mL) was added to the mixture. Compound **11** (407 mg, 516 μ mol) was charged in a hydrogenation bottle. After the bottle was evacuated and filled with hydrogen several times, the catalyst solution was added. The hydrogen pressure was adjusted to 4 atm, and the mixture was stirred vigorously overnight at room temperature. The reaction mixture was concentrated, and the crude product was purified by silica gel column chromatography (hexane/EtOAc = 7/3 to 3/2) to afford **12** (399 mg, 98%) as a colorless oil; ee and de were determined by HPLC (Daicel CHIRAL CEL OD-RH, λ = 254 nm, CH₃CN/H₂O = 11/9, 0.5 mL/min, 96% ee, 94% de). [α]_D²⁵ +12.8 (*c* = 1.05, CHCl₃); ¹H NMR: (500 MHz, CDCl₃, 0.020 M): δ 7.37–7.27 (m, 15H), 5.86 (s, 1H), 5.24 (d, *J* = 8.2 Hz, 1H), 5.20 (d, *J* = 7.9 Hz, 1H), 5.11–5.08 (m, 4H), 5.04–5.03 (m, 2H), 4.35–4.28 (m, 2H), 3.693 (s, 3H), 3.689 (s, 3H), 2.34–2.25 (m, 2H), 1.82–1.74 (m, 2H), 1.72–1.57 (m, 4H), 1.41 (s, 9H), 1.37–1.23 (m, 2H), 1.10–0.97 (m, 2H) ppm; ¹³C

NMR: (126 MHz, CDCl₃, 0.020 M): δ 172.8, 172.7, 172.1, 155.8 (2C), 153.9, 136.6, 136.2 (2C), 128.51 (4C), 128.49 (2C), 128.17, 128.16, 128.1 (4C), 128.0, 127.8 (2C), 82.7, 67.0 (2C), 66.2, 63.7, 53.7, 53.6, 52.3 (2C), 35.2, 35.0, 32.4, 32.3, 27.7 (3C), 19.8, 19.6 ppm; IR (neat on KBr plate): $\nu_{\max}$ 3345, 3033, 2953, 2868, 1714, 1503, 1215, 1065, 698 cm⁻¹; HRMS (FAB): m/z calcd for C₄₂H₅₄N₃O₁₂: 792.3707 [M+H]⁺; found: 792.3711.

Tri-Fmoc-di-nV-Gly. To a solution of **12** (364 mg, 460 μ mol) in CH₂Cl₂ (4.5 mL) was added TFA (4.5 mL) at room temperature. The mixture was stirred for 2 h. The volatile component in the reaction mixture was removed under reduced pressure. The residue was reconstituted in Et₂O and washed with saturated aqueous NH₄Cl solution. The aqueous layer was further extracted twice with Et₂O. The combined organic layers were dried over Na₂SO₄, filtered, and concentrated. Residual solvent was removed by azeotropic drying with pentane to afford monocarboxylic acid as a colorless oil. To a solution of the monocarboxylic acid in MeOH (15 mL) was added 2 M aqueous NaOH solution (2.30 mL). The reaction mixture was stirred at room temperature for 2 h. The volatile component in the reaction mixture was removed under reduced pressure. The residual water layer was adjusted to a pH of 1 using 1 M aqueous HCl solution and extracted with EtOAc 3 times. The organic layers were washed with brine and dried over Na₂SO₄. Filtration and concentration afforded a crude tricarboxylic acid as a white solid. To a solution of the tricarboxylic acid in MeOH (15 mL) was added Pd/C (10 wt%, 88.5 mg). The reaction mixture was stirred overnight under a hydrogen atmosphere at room temperature. The reaction mixture was filtered through celite and washed with H₂O to afford di-nV-Gly as a white solid. ¹H NMR: (500 MHz, D₂O, 0.017 M): δ 3.86 (t, J = 5.9 Hz, 1H), 3.81 (t, J = 6.4 Hz, 1H), 1.97–1.81 (m, 8H), 1.62–1.53 (m, 1H), 1.50–1.42 (m, 1H), 1.41–1.29 (m, 2H) ppm; ¹³C NMR: (126 MHz, D₂O, 0.017 M): δ 174.9, 173.73, 173.68, 64.4, 54.0, 53.7, 35.7, 35.3, 30.3, 30.0, 19.3, 18.7 ppm.

To a suspension of di-nV-Gly in H₂O/acetone (5 mL/5 mL) were added Fmoc-OSu (498 mg, 1.48 mmol) and Na₂CO₃ (273 mg, 2.58 mmol) at 0 °C. The reaction mixture was gradually warmed to room temperature with stirring overnight. The reaction mixture was adjusted to a pH of 2 using 1 M aqueous HCl solution and extracted 3 times with EtOAc. The organic layers were washed with brine, dried over Na₂SO₄, filtered, and concentrated. The crude product was purified by silica gel column chromatography (hexane/EtOAc = 2/1 to 1/2, 1% acetic acid). The central amino group of the product could not be protected. The compound was suspended in H₂O/acetone (5 mL/5 mL), and Fmoc-OSu (221 mg, 654 μ mol) and Na₂CO₃ (282 mg, 2.64 mmol) were added at 0 °C. The reaction mixture was gradually warmed to room temperature with stirring overnight. The reaction mixture was adjusted to a pH of 1 using 1 M aqueous HCl solution and extracted 3 times with EtOAc. The organic layers were washed with brine, dried over Na₂SO₄, filtered, and concentrated. The crude product was purified by silica gel column chromatography (hexane/EtOAc = 7/3 to 3/7, 1% acetic acid) and HPLC (YMC Pack ODS-A No.2015000608, λ = 254 nm, CH₃CN/H₂O = 7/3, 0.1% TFA, 8.0 mL/min, retention time 19.4 min) to afford tri-Fmoc-di-nV-Gly (278 mg, 62% for 4 steps) as a white solid. $[\alpha]_D^{26}$ +23.4 (c = 1.15, CHCl₃); ¹H NMR: (500 MHz, CD₃OD, 0.013 M): δ 7.80–7.56 (m,

12H), 7.41–7.22 (m, 12H), 4.34–3.92 (m, 11H), 2.22–0.82 (m, 12H) ppm; ^{13}C NMR: (126 MHz, CD_3OD , 0.013 M): δ 176.5, 175.9 (2C), 158.7 (2C), 156.3, 145.2 (6C), 142.5 (6C), 128.7 (6C), 128.1 (6C), 126.3 (6C), 120.9 (6C), 68.1, 68.0, 67.4, 64.0, 55.2, 55.0, 48.3 (3C), 35.7, 35.5, 32.63, 32.57, 21.7, 21.4 ppm; IR (neat on KBr plate): ν_{max} 3399, 3326, 3067, 3019, 2952, 2591, 1715, 1516, 1450, 1337, 1218, 1802, 741 cm^{-1} ; HRMS (FAB): m/z calcd for $\text{C}_{57}\text{H}_{54}\text{N}_3\text{O}_{12}$: 972.3707 $[\text{M}+\text{H}]^+$; found: 972.3716.

Synthesis and Preparation of A β Peptides. The E22P-A β 40 trimer models were synthesized in a stepwise fashion on 0.1 mmol of preloaded Fmoc-L-Val-PEG-PS (loading: 0.18 mmol/g) resin by Initiator+Alstra (Biotage) using the Fmoc method as reported previously.⁴⁶ Each coupling reaction was carried out using each Fmoc amino acid (0.4 mmol, 0.5 M in DMF), HATU (0.4 mmol, 0.5 M in DMF), and DIPEA (0.8 mmol, 1.0 M in DMF) for 5 min at 75 °C with microwave irradiation. The coupling reaction of histidine was carried out for 10 min at 50 °C to avoid racemization. The coupling reaction of tri-Fmoc-*t*ButA was carried out for 10 min at 75 °C. The coupling reactions of tri-Fmoc-di-nV-Gly and tri-Fmoc-di-hnL-Gly were carried out for 20 min at 75 °C. After each coupling reaction, the *N*-terminal Fmoc group was deblocked with 20% piperidine in DMF. The one-third molar equivalent of each trimer linker (0.033 mmol) was employed instead of Fmoc-L-Val at position 36 or Fmoc-L-Gly at position 38 in order to avoid formation of the mono-coupled and/or di-coupled peptides.

After the completion of chain elongation followed by Fmoc deblocking, the resultant peptide resin, washed with DMF and CH_2Cl_2 , was shaken for 2 h at room temperature in a mixture containing TFA (16 mL), *m*-cresol (0.4 mL), 1,2-ethanedithiol (1.2 mL), and thioanisole (2.4 mL) for final deprotection and cleavage from the resin. Each crude peptide was precipitated by diethyl ether at 4 °C, followed by purification using HPLC on a YMC PROTEIN-RP column with elution at 8.0 mL/min by an 80-min linear gradient (curve 6) of 20–60% CH_3CN containing 0.1% TFA. Subsequent purification was carried out using XBridge Peptide BEH C18 OBDTM Prep Column with elution at 3.0 mL/min by a 40-min linear gradient (curve 6) of 20–40% CH_3CN containing 0.1% TFA for **4** and **5**, at 5.5 mL/min by a 60-min exponential gradient (curve 8) of 30–60% CH_3CN containing 0.1% TFA for **6**, or at 5.5 mL/min by a 60-min exponential gradient (curve 7) of 30–50% CH_3CN containing 0.1% TFA for **7**. Lyophilization produced each peptide, the purity of which was checked by HPLC (>98%, Figure S2). Molecular weights and formulae were confirmed by HR-ESI-qTOF-MS measurements (Figure S3).

MTT Assay. MTT assay was carried out using the method reported previously.^{46,54} A human neuroblastoma SH-SY5Y cell line (ATCC, Manassas, VA, USA) was maintained in a 1:1 mixture of Eagle's minimum essential medium and Ham's F12 medium (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) containing 10% fetal bovine serum (Biological Industries, Beit Heamek, Israel) and 1% MEM non-essential amino acid (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Each A β was dissolved in 0.15% NH_4OH to give a 110 μM stock solution, which was diluted with 0.15% NH_4OH to appropriate concentrations (11 and 36 μM), and each 10 μL aliquot was subsequently added to 100 μL of the culture medium containing near-confluent cells (2×10^4 cells/well) to give final concentrations, 1, 3.3, and 10 μM .

After being treated at 37 °C for 24 h, 15 µL/well of Dye solution in CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) was added to the cells, followed by a 4-h incubation at 37 °C. The solubilization/stop solution (100 µL/well) was subsequently added to the cells. Next, the resultant cell lysate was incubated overnight in the dark at room temperature before performing measurements at 570 nm using a microplate reader (Multiskan FC, Thermo Fisher Scientific). Absorbance obtained by the addition of vehicle (0.15% NH₄OH) was taken as 100%.

Thioflavin-T (Th-T) Assay. The aggregative ability of each A β was assessed by Th-T fluorescence assay.⁵⁹ Th-T fluorescence was measured automatically based on the previously described procedure^{63,68,69} with slight modifications. For instance, 390 µL of 50 mM sodium phosphate and 100 mM NaCl was aliquoted into a 1.5 mL tube, and 10 µL of 1 mM Th-T solution in distilled water was added. Each A β was dissolved with 0.15% NH₄OH and adjusted to the desired concentration. One hundred µL of each A β solution was added to the tube so as to become the final desired concentration (WT-A β 42: 10 µM, E22P-A β 40: 20 µM, trimer models 2–7: 6.7 µM). Each solution was aliquoted into a 96-well black plate and fluorescence was measured automatically every 10 min at 430 nm excitation and 485 nm emission using a microplate reader (Fluoroskan Ascent, Thermo Fisher Scientific). For each assay, a vehicle control without A β solution was subtracted as blank to calculate substantial fluorescence.

Transmission Electron Microscopy (TEM) Analysis. The aggregates of each A β solution (WT-A β 42, E22P-A β 40: 25 µM, trimer models 4–7: 8.3 µM) in 50 mM sodium phosphate and 100 mM NaCl (pH 7.4) after a 48-h incubation were examined under a transmission electron microscope (JEM-1400, JEOL) based on the previously described procedure.^{46,54} After each A β aggregate was centrifuged (4 °C, 15,000 rpm, 10 min), the supernatant was removed from the pellet. The resultant pellet was centrifuged (4 °C, 15,000 rpm, 10 min) again, and resuspended in water (WT-A β 42, E22P-A β 40: 100 µL, trimer models 4–7: 50 µL) using a vortex for 1 min. Following this, the sample was spun down (room temperature, 6,000 rpm, 0.5 min). The sample suspension (15 µL) was applied to a 400-mesh carbon-coated cooper grid (thickness: 15–20 nm; Veco, Eerbeek, Netherlands), allowed to incubate for 5 min, and negatively stained twice with 2% uranyl acetate (Merck, Kenilworth, NJ, USA). Stained samples were subsequently subjected to TEM.

Ion Mobility-Mass Spectrometry (IM-MS). IM-MS was measured using the method reported previously.^{46,54} Each trimer model (2–7) was dissolved in 0.2% NH₄OH at 80 µM, followed by a 10-fold dilution with 25 mM ammonium acetate before incubation for 4 h at 37 °C. The incubated sample (8 µM) was centrifuged for 5 min at 18,000 g before infusion into the MS apparatus using a glass capillary (Nanoflow Probe Tip, Waters, Milford, MA, USA). Mass spectra and ion mobility experiments were conducted on SYNAPT XS HDMS (Waters, Milford, MA, USA) using a nano-electrospray as an ionization source. The instrument was operated in negative ion mode with a capillary voltage of 0.7–1.0 kV, a sample cone voltage of 150 V, and a source temperature of 50 °C. For the ion mobility measurement, nitrogen gas was used in the ion mobility cell, and the cell pressure was maintained at approximately 2.83 mbar with a wave velocity of 650 m/s and a wave height of 40 V. Data acquisition and processing were performed with

MassLynx (V4.2) and DriftScope (V3.0) software supplied with the instrument. The CsI cluster ions were used for m/z scale as a calibrator.

Nondenaturing PAGE. A β aggregates under nondenaturing conditions were observed using nondenaturing PAGE based on a previously described procedure⁷⁰ with slight modifications. A β peptides prepared at 400 μ M in DMSO were diluted to make final concentrations (WT-A β 42, E22P-A β 40: 2 μ M, trimer models **4–7**: 0.67 μ M) in phosphate-buffered saline (PBS: 137 mM NaCl, 8.1 mM Na₂HPO₄, 2.68 mM KCl, 1.47 mM KH₂PO₄, pH 7.4) in protein LoBind tubes. The samples were incubated at 37 °C for 24 h. Then 7.5 μ L of each sample mixed with 2.5 μ L of NativePAGE 4 $\times$ sample buffer (Thermo Fisher, Waltham, MA, USA) and 3.75 μ L of NativeMark unstained protein standard (Thermo Fisher, Waltham, MA, USA) mixed with 1.25 μ L of NativePAGE 4 $\times$ sample buffer were loaded onto a NativePAGE 4–16%, Bis-Tris gel (Thermo Fisher, Waltham, MA, USA), and electrophoresed for 30 min at 150 V using the dark blue running buffer of the manufacturer. At that time, proteins had migrated through a third of the gel length, and buffer was then exchanged for light blue running buffer. The gel was further electrophoresed at 250 V for 60 min. After electrophoresis, proteins were transferred to PVDF membrane (Immobilon-P, Merck Millipore, Burlington, MA, USA). Membranes were blocked in 5% skim milk dissolved in Tris-buffered saline with 0.1% Tween 20 (TBST: 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature. Membranes were incubated with 82E1 antibody⁷¹ at 0.3 μ g/mL in blocking buffer for 16 h at 4 °C. On the following day, membranes were washed for 10 min $\times$ 3 with TBST and incubated for 1 h in HRP-labeled anti-mouse immunoglobulin G (IgG) (Thermo Fisher, Waltham, MA, USA) at 1:5000 in TBST. Membranes were washed for 10 min $\times$ 3 with TBST and exposed to Chemi-Lumi One chemiluminescent substrate (Nacalai Tesque, Kyoto, Japan). The chemiluminescent signal was recorded with ImageQuant LAS 500 (GE Healthcare, Chicago, IL, USA).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: xxxxxxxx.

¹H and ¹³C NMR spectra of new compounds; HPLC profiles for ee and de determination of compound **12**; HPLC profiles for purity check and ESI-qTOF-MS data of A β 40 trimers (**4–7**); projection of 2D spectra of **2–7** in Figure 5 in the main text on the mass spectra axis; the calculated and observed masses of **2–7** in Figure 5 in the main text (PDF).

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Author Contributions

K.I. designed the project. A.U. and K.I. analyzed and interpreted the experiments. A.U. synthesized the trimer linkers under the guidance of C.T. A.U. synthesized the trimer models under the guidance of K.I. and Y.I. A.U. performed the Th-T and TEM analysis. Y.I. performed the MTT assay and nondenaturing PAGE analysis. T.K. and K.H. performed the IM-MS measurements. Y.K. and I.T. supervised the nondenaturing PAGE. R.C.Y. performed the molecular modeling of analogs **3** and **4**. A.U. and K.I. drafted the manuscript, and K.I., C.T. and I.T. reviewed the manuscript.

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Notes

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ABBREVIATIONS

AD, Alzheimer's disease; A β , amyloid β ; di-hnL-Gly, α,α -di-L-homonorleucyl-L-glycine; di-nV-Gly, α,α -di-L-norvalyl-L-glycine; DAP, L,L-2,6-diaminopimelic acid; DAZ, L,L-2,8-diaminoazelaic acid; IM-MS, ion mobility-mass spectrometry; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PtA, 1,3,5-phenyltris-L-alanine; *t*ButA, *tert*-butyltris-L-alanine; TEM, transmission electron microscopy; Th-T, thioflavin-T.

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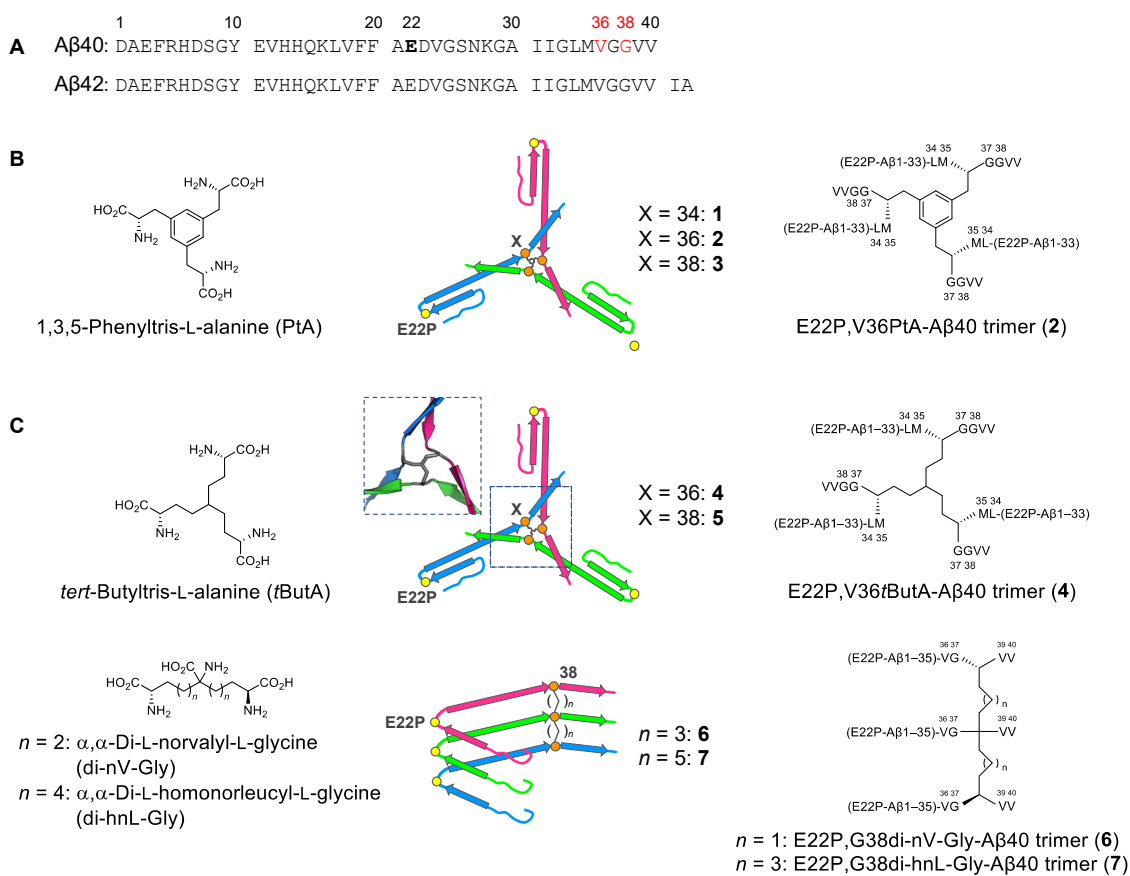


Figure 1. (A) Sequence of WT-A β 40 and 42. (B) Structures of 1,3,5-phenyltris-L-alanine (PtA) and propeller-type A β 40 trimer models (**1–3**) synthesized in the previous study.⁴⁶ (C) Structures of *tert*-butyltris-L-alanine (*t*ButA), α, α -di-L-norvalyl-L-glycine (di-nV-Gly), α, α -di-L-homonorleucyl-L-glycine (di-hnL-Gly), and propeller-type and intermolecular parallel β -sheet-type A β 40 trimer models (**4–7**) synthesized in this study.

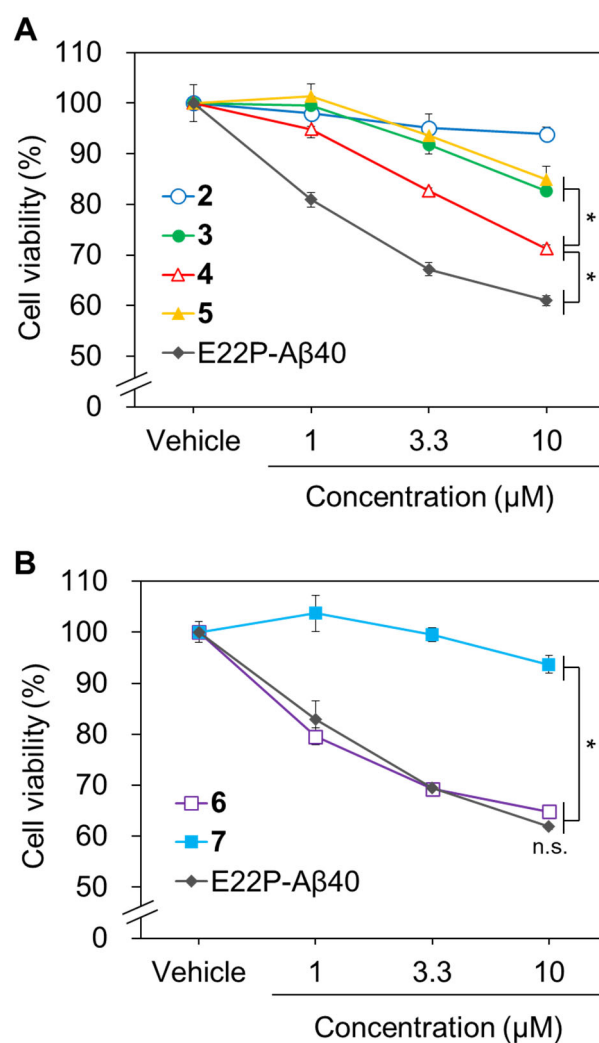


Figure 2. Cytotoxicity against SH-SY5Y cells of (A) the trimer models 2–5 and (B) the trimer models 6 and 7, along with that of E22P-Aβ40 monomer after a 24-h incubation at 37 °C. Absorbance obtained after adding vehicle (0.15% NH₄OH) was taken as 100%. * $p < 0.001$; n.s., not significant. Data are expressed as the mean $\pm$ SD ($n = 3$).

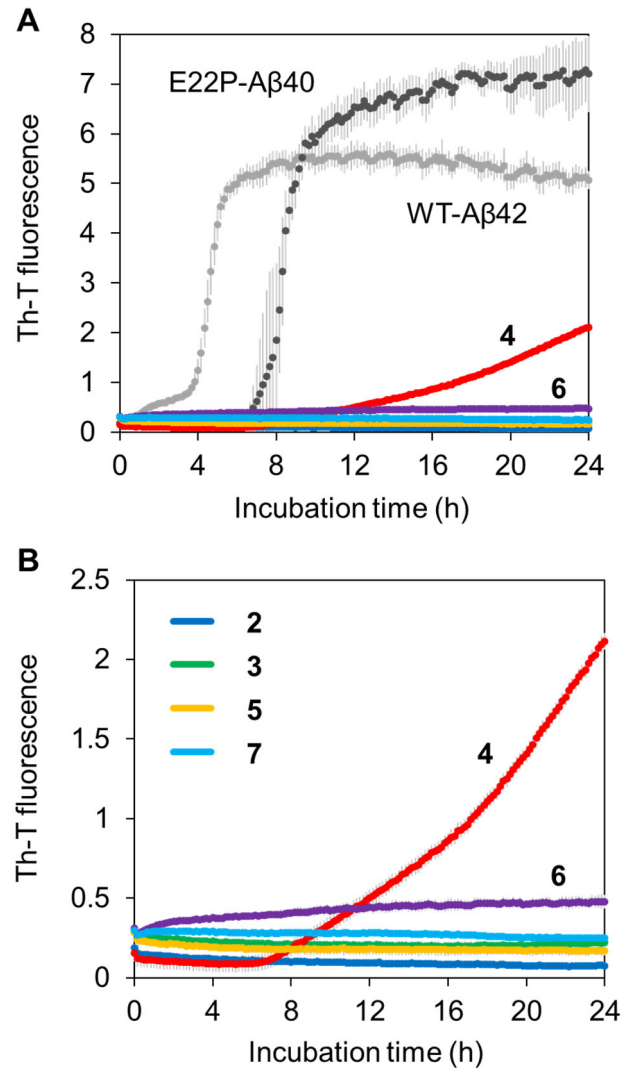


Figure 3. (A) Th-T fluorescence of the trimer models 3–7 (6.7 μ M) along with that of WT-A β 42 (10 μ M) and E22P-A β 40 (20 μ M) in the presence of Th-T (20 μ M) measured automatically every 10 min. Data are expressed as the mean $\pm$ SD ($n = 4$). (B) Enlarged figure of the fluorescence range 0 to 2.5 in the figure 3A, except for the data of WT-A β 42 and E22P-A β 40.

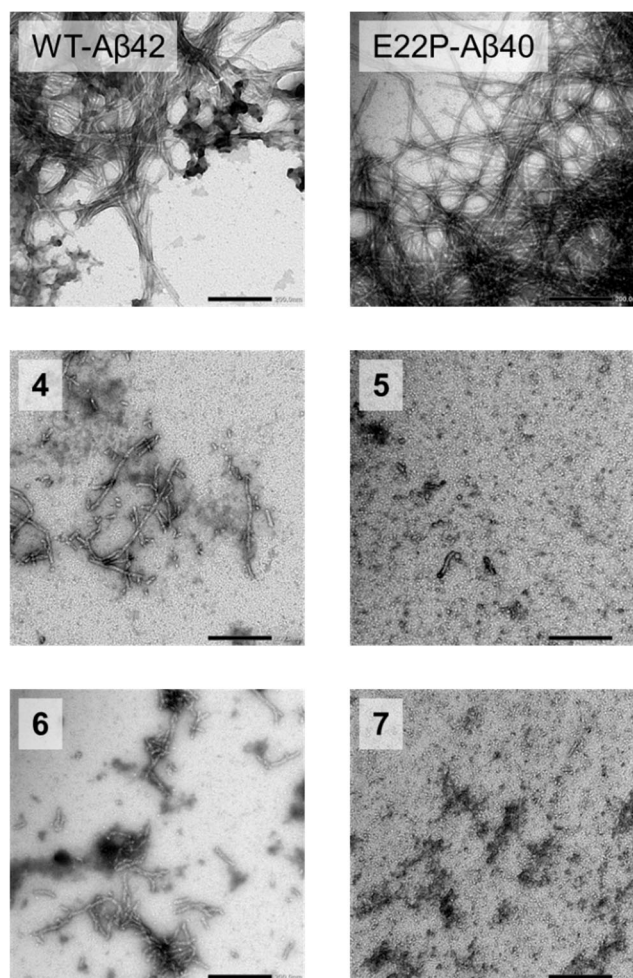


Figure 4. TEM analysis of the aggregates of the trimer models 4–7 (8.3 μ M) along with that of WT-A β 42 and E22P-A β 40 (25 μ M) after a 48-h incubation at 37 °C. Scale bar = 200 nm (magnification: 30 k). WT-A β 42 and E22P-A β 40 formed mature fibrils. The trimer models 4 and 6 formed protofibrillar species, and 5 and 7 remained as amorphous aggregates.

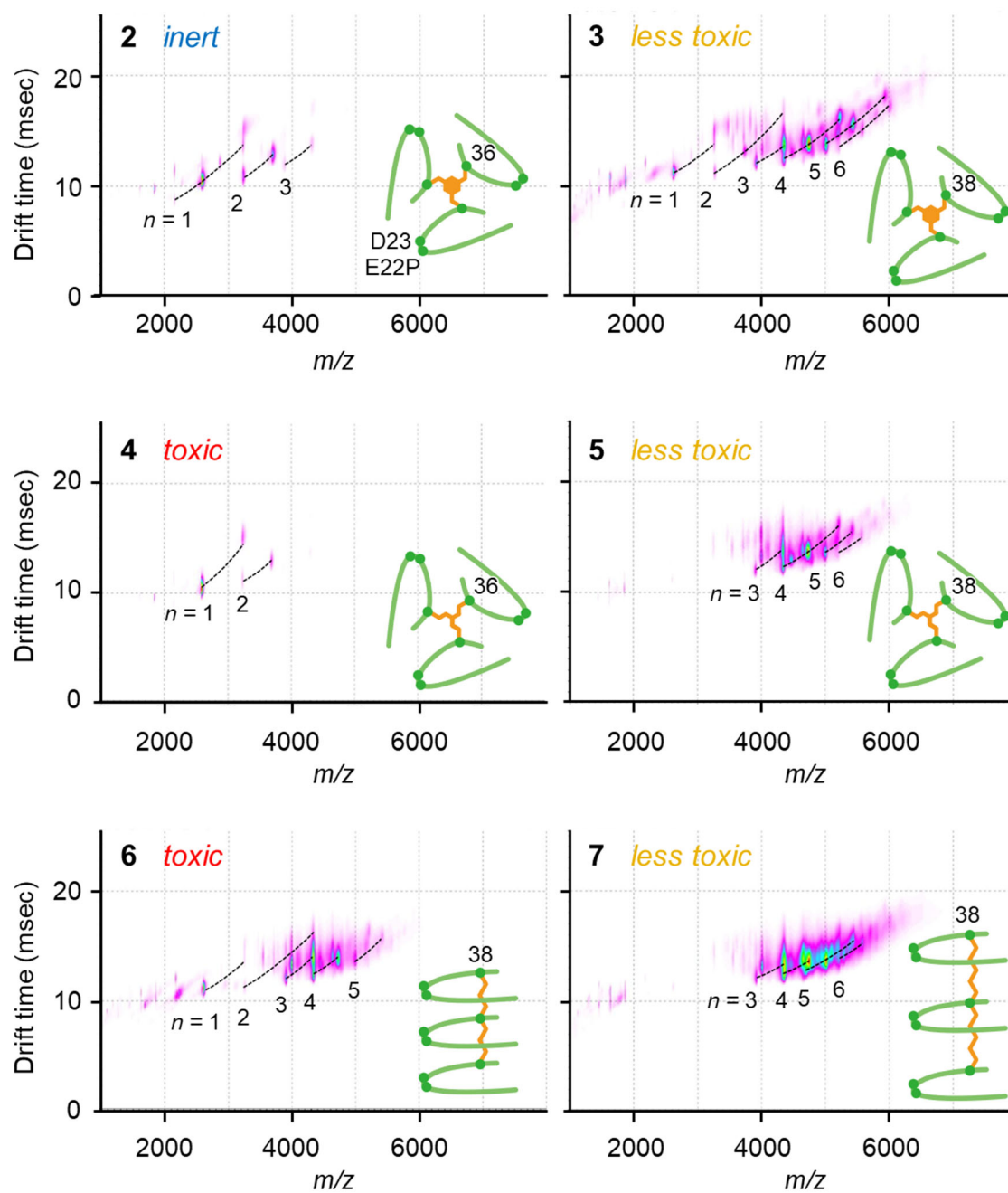


Figure 5. IM-MS of the trimer models 2–7 (8 μ M) after a 4-h incubation at 37 $^{\circ}$ C. “ n ” denotes an integer corresponding to the number of units coexisting depending on their drift time.

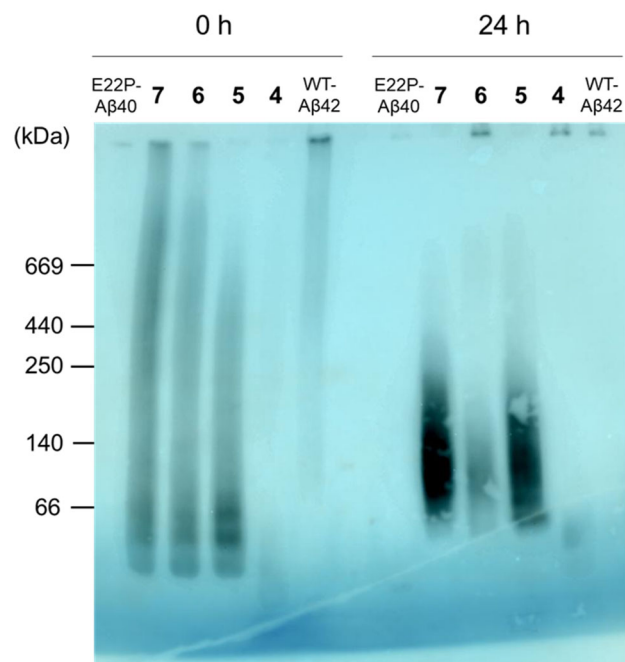
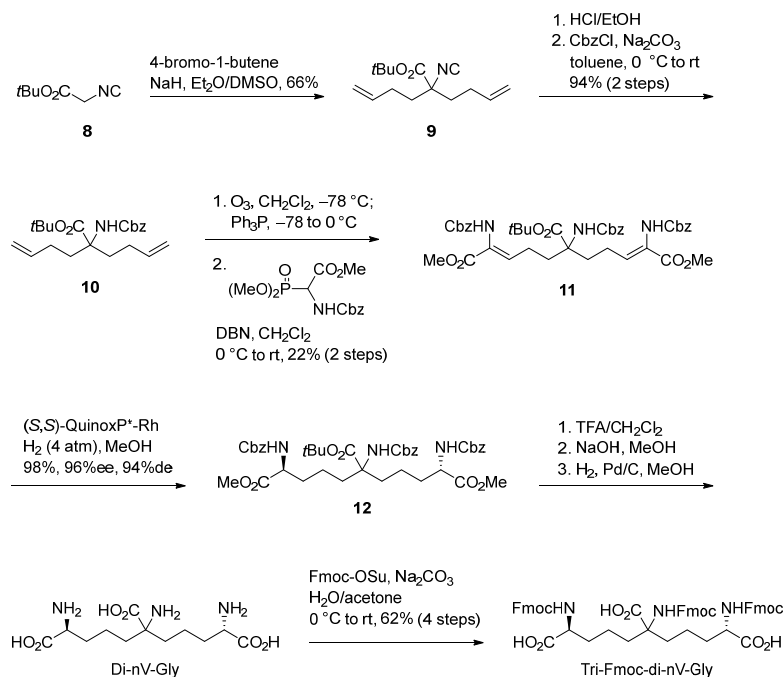


Figure 6. Non-denaturing PAGE of the trimer models 4–7 (0.67 μ M) along with that of WT-A β 42 and E22P-A β 40 (2 μ M) after a 24-h incubation at 37 $^{\circ}$ C.



Scheme 1. Synthesis of tri-Fmoc- α,α -di-L-norvalyl-L-glycine. DMSO: dimethyl sulfoxide, Cbz: benzyloxycarbonyl, DBN: 1,5-diazabicyclo[4.3.0]non-5-ene, (S,S)-QuinoxP*-Rh: (S,S)-2,3-bis(*tert*-butylmethylphosphino)quinoxaline(1,5-cyclooctadiene)rhodium(I)tetrafluoroborate, TFA: trifluoroacetic acid, Fmoc-OSu: *N*-(9-fluorenylmethoxy-carbonyloxy)succinimide.