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'*O*-Acyl isopeptide method' for the efficient preparation of amyloid β peptide 1–42 mutants

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Abstract—Novel water-soluble isopeptides of Aβ1–42 mutants, '26-*O*-acyl isoAβ1–42 (26-AIAβ42) mutants', which were efficiently converted to intact Aβ1–42 mutants with no byproduct formation under physiological conditions, were synthesized. These isopeptides provide a new system useful for investigating the biological function of Aβ1–42 mutants.

1. Introduction

Amyloid β peptides (Aβs) are the main proteinaceous component of amyloid plaques found in the brain as a pathognomonic feature of Alzheimer's disease (AD),[1] and have been found to be neurotoxic in vivo and in vitro.[2] Although the predominant forms of Aβ mainly consist of 40- and 42-residue peptides, designated Aβl–40 and Aβl–42, respectively, Aβl–42 is thought to play a more critical role in amyloid formation and the pathogenesis of AD than Aβl–40.[3] In addition, not only wild-type Aβl–42 ($D^{1}AEFRHDSGY^{10}EVHHQKLVF-F^{20}AEDVGSNKGA^{30}IIGLMVGGVV^{40}IA$) observed in AD, but missense mutations inside the Aβ-coding region in the amyloid precursor protein (APP) gene are also well-known. These mutations, known as Flemish-(A21G),[4] Arctic-(E22G),[5] Dutch-(E22Q),[6] Italian-(E22K),[7] and Iowa-type (D23N)[8] are found at positions 21–23 of Aβ. All result in cerebral amyloid angiopathy (CAA) and/or cerebral parenchymal amyloidosis, leading to AD-like diseases. Recently, a Japanese-Tottori-type (D7N) mutation was also reported.[9] Recent studies have discussed several differences among Aβ mutants in amyloid formation, metabolism, and elimination, which are related to the progression of AD-like diseases.[10] Hence, more detailed studies comparing these features among Aβ mutants would afford crucial information for understanding the mechanism of the diseases. A sufficient supply of synthetic Aβ mutants would be key to this research.

Numerous studies have supported the hypothesis that neurotoxicity and the kinetics of Aβ1–42 aggregation are directly related to the assembly state in solution. However, the pathological self-assembly of Aβ1–42 in amyloid plaque formation, a currently unexplained process, is very difficult to demonstrate in vitro due to its uncontrolled polymerization. For example, synthesized Aβ1–42 already contains variable oligomeric forms,[3d,11] as Aβ1–42 undergoes time- and concentration-dependent aggregation in an aqueous TFA–acetonitrile solution used in HPLC purification.[12] Moreover, the Aβ1–42 monomer easily forms an aggregate even in a standard storage solution such as dimethylsulfoxide (DMSO).[13] Uncontrolled self-assembly in an in vitro experiment might cause considerable discrepancy in the biological data.[3d,11] Therefore, this highly agglutinative feature of Aβ1–42 is a significant obstacle for establishing a reliable in vitro biological experiment system to investigate the major causative agents of AD-like diseases.

The highly agglutinative property of Aβl–42 in various media also results in synthetic difficulties with this peptide,[12,14] a so-called 'difficult sequence-containing peptide'.[15] In particular, in conventional reverse-phase HPLC purification of synthesized Aβl–42 with the

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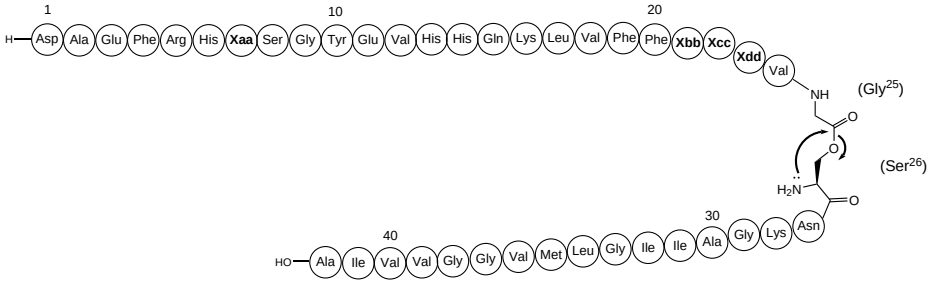
aqueous TFA–acetonitrile system, it is too laborious to remove impurities accumulated during solid-phase peptide synthesis (SPPS) due to its low solubility and broad elution under either acidic or neutral conditions.

An 'in situ' system that prepares an intact monomer Aβ1–42 under physiological experimental conditions while suppressing the spontaneous self-assembly of Aβl–42 under storage conditions would be advantageous in understanding the inherent pathological functions of agglutinative Aβl–42 in AD-like diseases. For this purpose, based on the '*O*-acyl isopeptide method',[16] we developed a novel water-soluble isopeptide of wild-type Aβl–42, '26-*O*-acyl isoAβl–42' (26-AIAβ42, **8**, Fig. 1).[17] This isopeptide exhibited higher water solubility than Aβl–42 (**1**), and *O*–*N* intramolecular acyl migration reaction[18,19] to the original **1** occurred quickly with no side reaction under physiological conditions (pH 7.4), while **8** was stable under storage conditions.

We herein expand the '*O*-acyl isopeptide method' to the synthesis of novel water-soluble *O*-acyl isopeptides (**9**–**14**) of Aβl–42 mutants, such as Japanese-Tottori-(D7N, **2**), Flemish-(A21G, **3**), Arctic-(E22G, **4**), Dutch-(E22Q, **5**), Italian-(E22K, **6**), and Iowa-type (D23N, **7**). These synthesized isopeptides afforded each Aβl–42 mutant under physiological conditions (Fig. 1) via *O*–*N* intramoleculor acyl migration, providing a useful new system for investigating the biological function of Aβl–42 mutants.

2. Chemistry

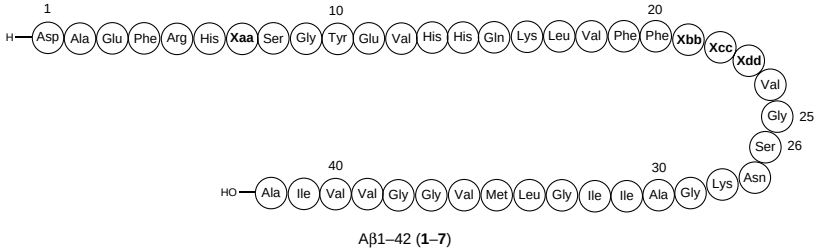
The synthetic scheme of Dutch-type 26-AIAβ42 (E22Q, **12**), based on the *O*-acyl isopeptide method, is depicted in Scheme 1 as a representative example. Fmoc-Ala-*O*-chlorotrityl resin (**15**) was employed according to our previous study.[17c] Fmoc-protected amino acids were sequentially coupled using the DIPCDI (1,3 -diisopropylcarbodiimide)–HOBt (1-hydroxybenzotriazole) method (2 h) after removing each Fmoc group with 20% piperidine–DMF (20 min) to give peptide resin **16**. Then, Boc-Ser-OH was introduced to **16** by the DIPCDI—HOBt



26-*O*-acyl isoAβ1–42 (**8**–**14**)

Entry	Xaa^{7}	Xbb^{21}	Xcc^{22}	Xdd^{23}
8 (wild)	Asp	Ala	Glu	Asp
9 (Japanese-Tottori)	Asn	Ala	Glu	Asp
10 (Flemish)	Asp	Gly	Glu	Asp
11 (Arctic)	Asp	Ala	Gly	Asp

Entry	Xaa^{7}	Xbb^{21}	Xcc^{22}	Xdd^{23}
12 (Dutch)	Asp	Ala	Gln	Asp
13 (Italian)	Asp	Ala	Lys	Asp
14 (Iowa)	Asp	Ala	Glu	Asn

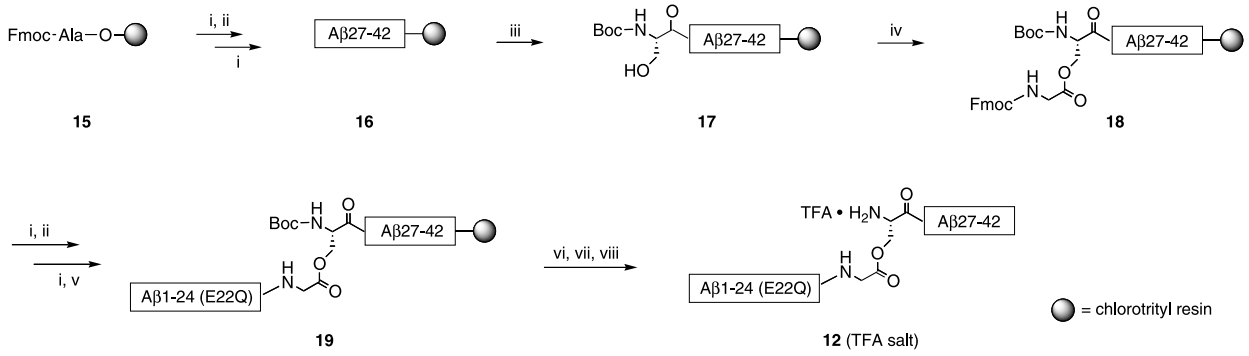


Aβ1–42 (**1**–**7**)

Entry	Xaa^{7}	Xbb^{21}	Xcc^{22}	Xdd^{23}
1 (wild)	Asp	Ala	Glu	Asp
2 (Japanese-Tottori)	Asn	Ala	Glu	Asp
3 (Flemish)	Asp	Gly	Glu	Asp
4 (Arctic)	Asp	Ala	Gly	Asp

Entry	Xaa^{7}	Xbb^{21}	Xcc^{22}	Xdd^{23}
5 (Dutch)	Asp	Ala	Gln	Asp
6 (Italian)	Asp	Ala	Lys	Asp
7 (Iowa)	Asp	Ala	Glu	Asn

Figure 1. '*O*-Acyl isopeptide method' for the efficient preparation of Aβ1–42 (**1**–**7**): the production of Aβ1–42 (**1**–**7**) via *O*–*N* intramolecular acyl migration of 26-*O*-acyl isoAβ1–42 (**8**–**14**).



Scheme 1. Reagents and conditions: (i) 20% piperidine/DMF, 20 min; (ii) Fmoc-AA-OH (2.5 equiv), DIPCDI (2.5 equiv), HOBt (2.5 equiv), DMF, 2 h; (iii) Boc-Ser-OH (2.5 equiv), DIPCDI (2.5 equiv), HOBt (2.5 equiv), DMF, 2 h; (iv) Fmoc-Gly-OH (15.0 equiv), DIPCDI (15.0 equiv), DMAP (0.3 equiv), CH_2C1_2, 4 h × 2; (v) Boc-Asp (O*t*Bu)-OH (2.5 equiv), DIPCDI (2.5 equiv), HOBt (2.5 equiv), DMF, 2 h; (vi) TFA/*m*-cresol/thioanisole/H_2O (92.5:2.5:2.5:2.5), 90 min; (vii) NH_4I (20 equiv), dimethylsulfide (20 equiv), TFA/H_2O (2:1), 60 min, 0 °C; (viii) preparative HPLC (the linear gradient of CH_3CN in 0.1% aqueous TFA).

method (2 h) to obtain **17**, which was coupled with Fmoc-Gly-OH at the β-hydroxy group of Ser using the DIP-CDI–DMAP method[18b] in CH_2Cl_2 to obtain ester **18**. A protected Dutch-type 26-AIAβ42 resin (**19**) was obtained through the subsequent coupling of amino acid residues using the conventional manner. Finally, Dutch-type 26-AIAβ42 (**12**) was obtained as a major product (Fig. 2A) by treating **19** with TFA/*m*-cresol/thioanisole/H_2O (92.5:2.5:2.5:2.5) for 90 min followed by reduction with NH_4I–dimethylsulfide for 60 min in TFA/H_2O (2:1). Other 26-AIAβ42 mutants (**9**–**11**, **13**, and **14**) were synthesized in a similar manner to that described in **12.**

3. Results and discussion

In the HPLC analysis of crude Dutch-type isopeptide **12** (Fig. 2A), truncated peptide Aβ1–25 (DAEFRHDSG YEVHHQKLVFFAQDVG) was not detected as a by-product, although a small amount (1.8%, HPLC yield) of Aβ26–42 (SNKGAIIGLMVGGVVIA) was observed. This result indicates that the formed ester bond between Gly^{25} and Ser^{26} was almost stable in both 20% piperidine and TFA treatments, comparable to the synthesis of wild-type 26-AIAβ42 (**8**), in which 1.6% of Aβ26–42 was detected in a crude sample.[17] The slight formation of Aβ26–42 observed in crude **12** might be attributed to diketopiperazine formation during Fmoc group deprotection of Val^{24} with 20% piperidine. Additionally, in the previous synthesis of **8** using the *O*-acyl isopeptide method, we confirmed that no side reaction occurred in the Boc-Ser-OH insertion (step (iii) in Scheme 1) or esterification (step (iv) in Scheme 1).[17c] The crude HPLC profiles of other 26-AIAβ42 mutants (**9**–**11**, **13**, and **14**) were similar to that of **12**.

The crude Dutch-type *O*-acyl isopeptide **12** was dissolved in DMSO, applied to preparative HPLC and eluted using 0.1% aqueous TFA–CH_3CN. Since **12** was eluted as a sharp single peak (Figs. 2 and 3A), it was easily purified to give pure **12** (Figs. 2B and 3A) as a TFA salt with a total synthetic yield of 20.0%, calculated from the original loading onto the chlorotrityl resin. In addition, during the synthesis and purification

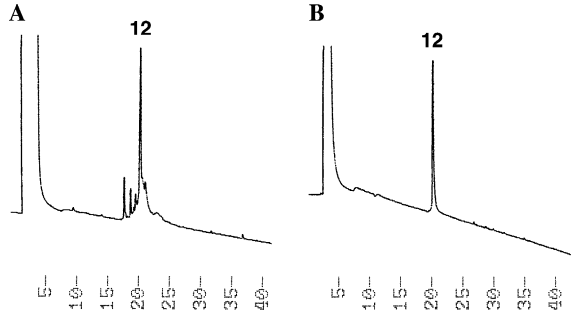


Figure 2. HPLC profiles of (A) crude and (B) purified Dutch-type 26-AIAβ42 (**12**). Analytical HPLC was performed using a C18 reverse-phase column (4.6 × 150 mm; YMC Pack ODS AM302) with a binary solvent system: the linear gradient of CH_3CN (0–100% CH_3CN for 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1} (temperature: 40 °C), detected at 230 nm.

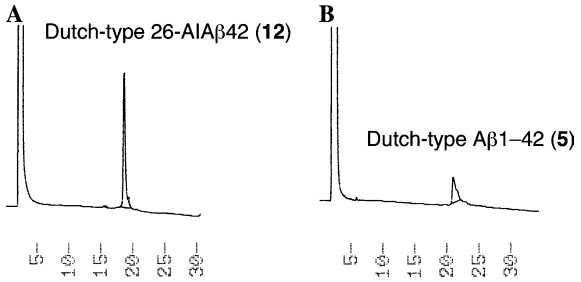


Figure 3. HPLC profiles of pure (A) Dutch-type 26-AIAβ42 (**12**) and (B) Dutch-type Aβ1–42 (**5**). The same quantity (2.3 nmol) of both peptides was applied to the HPLC column as a DMSO solution. Analytical HPLC was performed using a C18 reverse-phase column (4.6 mm × 150 mm; YMC Pack ODS AM302) with a binary solvent system: the linear gradient of CH_3CN (25–55% CH_3CN for 60 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1} (temperature: 40 °C), detected at 230 nm.

of **12**, no conversion to Dutch-type Aβl–42 (**5**) was observed. Similarly, the synthesis of other isopeptides **9**–**11**, **13**, and **14** was performed efficiently with a synthetic yield of 21.3–34.0% (see Section 5). These yields were higher than those obtained in the synthesis of Aβl–42 mutants **2**–**7** by standard Fmoc-based SPPS (see Section 5). Since each **2**–**7** was eluted as a broad

Table 1. Water solubility of 26-AIAβ42 (**8**–**14**) and Aβ1–42 (**1**–**7**)

26-AIAβ42	Aβ1–42	Water solubility		
		26-AIAβ42 (mg mL^{-1})	Aβ1–42 (mg mL^{-1})	ratio[b]
8[a]	**1**[a](wild-type)	15.0	0.14	107
9	**2** (Japanese-Tottori-type)	4.4	0.15	29.3
10	**3** (Flemish-type)	18.3	0.95	19.3
11	**4** (Arctic-type)	5.1	0.40	12.8
12	**5** (Dutch-type)	14.5	0.93	15.6
13	**6** (Italian-type)	16.2	8.7	1.9
14	**7** (Iowa-type)	8.1	2.2	3.7

[a] Data from Ref. 17.
[b] Ratio = solubility of 26-AIAβ42/solubility of Aβ1–42.

peak in preparative scale HPLC purification, it was laborious to separate the impurities and evaluate the purity as wild-type Aβl–42 (**1**).[14,17]

The water solubility of mutant 26-AIAβ42s (TFA salt) was 15.0 mg mL^{-1} (**8**),[17] 4.4 mg mL^{-1} (**9**), 18.3 mg mL^{-1} (**10**), 5.1 mg mL^{-1} (**11**), 14.5 mg mL^{-1} (**12**), 16.2 mg mL^{-1} (**13**), and 8.1 mg mL^{-1} (**14**), higher than those in the corresponding mutant Aβl–42s (**1**: 0.14,[17] **2**: 0.15, **3**: 0.95, **4**: 0.40, **5**: 0.93, **6**: 8.7, and **7**: 2.2 mg mL^{-1}) (Table 1). The water solubility ratios of isopeptides compared to the corresponding Aβl–42s were in a relatively wide range (1.9- to 107-fold), but the observed water solubility of each isopeptide was similar over the range of 4.4–18.3 mg mL^{-1} (Table 1). This observation corresponds to our previous study regarding '*O*–*N* intramolecular acyl/acyloxy migration'-type water-soluble prodrugs of taxoids.[19i] It is suggested that the solubility of isopeptides is related much more to the isopeptide structure than to the amino acid substitution in each mutant, while the solubility of each Aβl–42 is highly dependent on the nature of the mutated amino acid. In addition, the HPLC analysis of Dutch-type isopeptide **12** exhibited quite a sharp peak even in the slow gradient system (25–55% CH_3CN, 60 min, Fig. 3A), while the corresponding Aβl–42 **5** was eluted as a broad peak under the same elution conditions (Fig. 3B) as wild-type Aβl–42 **1**.[14,17] These results indicate that the highly insoluble and agglutinative nature of Aβl–42 based on its secondary structure was suppressed by only one insertion of the isopeptide structure with a branched ester bond. A similar result was demonstrated in the case of wild-type Aβl–42 **1** in our previous study.[17] Therefore, the *O*-acyl isopeptide method is a common strategy for increasing water-solubility. Recent reports by Carpino et al.[14l] and Mutter et al.[20] have supported our data that *O*-acyl isopeptide structures have attractive solubilizing efficacy.

On the other hand, as shown in Figures 4 and 5B, Dutch-type 26-AIAβ42 **12** was quantitatively converted to the corresponding Aβl–42 **5** in phosphate-buffered saline (PBS, pH 7.4) at 37 °C with a half-life of ~1 min with no side reaction such as hydrolysis of the ester bond, and migration was completed after 30 min. An HPLC retention time (0–100% CH_3CN for 40 min, 230 nm) of the newly appeared peak (Fig. 4) was identical to that of an independently synthesized Dutch-type Aβl–42 **5** by a conventional method. Moreover, the

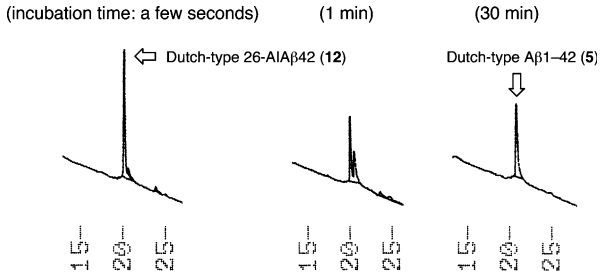


Figure 4. HPLC profiles of the conversion of Dutch-type 26-AIAβ42 (**12**) to corresponding Aβ1–42 (**5**) in PBS (pH 7.4) at 37 °C. Analytical HPLC was performed using a C18 reverse-phase column (4.6 mm × 150 mm; YMC Pack ODS AM302) with a binary solvent system: the linear gradient of CH_3CN (0–100% CH_3CN for 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1} (temperature: 40 °C), detected at 230 nm.

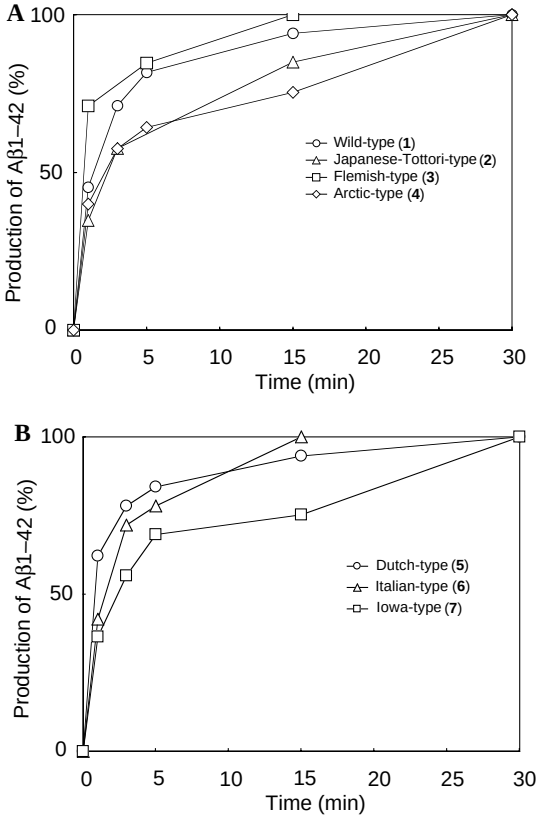


Figure 5. Time course of Aβ1–42 production in (A) wild-, Japanese-Tottori-, Flemish-, and Arctic-type isopeptides (B) Dutch-, Italian-, and Iowa-type isopeptides in PBS (pH 7.4) at 37 °C.

mass spectrometry analysis of this peak was identical to **5** (M_{calcd}: 4513.1; $M+H_{found}$: 4514.0). The rapid migration in **12** may be attributed to the lower steric hindrance of the Gly^{25} residue. In contrast, the TFA salt of **12** was stable at 4 °C for at least 1 month in either the solid state or DMSO solution. Moreover, other mutant isopeptides (**9**–**11**, **13**, and **14**) were quantitatively converted to each Aβl–42 mutant (for **2**; M_{calcd}: 4513.1; $M+H_{found}$ 4514.1; for **3**: M_{calcd}: 4500.0; $M+H_{found}$: 4501.4; for **4**: M_{calcd}: 4442.0; $M+H_{found}$: 4443.0; for **6**: M_{calcd}: 4513.1; $M+H_{found}$: 4513.9; for **7**: M_{calcd}: 4513.1; $M+H_{found}$: 4514.2) at pH 7.4 (37 °C) in a short time with no significant difference in the half-life values (Fig. 5), indicating that amino acid substitutions at positions 21, 22, and 23, which are relatively far from position 26, do not significantly affect the migration rate at Gly^{25}-Ser^{26}. These rapid migrations under physiological conditions would enable the production of intact monomer Aβl–42 mutants 'in situ' to investigate the inherent biological functions of Aβl–42 mutants in AD-like diseases.

4. Conclusion

The '*O*-acyl isopeptide method' was successful in the efficient preparation of 26-*O*-acyl isopeptides of six Aβl–42 mutants. Namely, the isopeptides (1) suppressed the unfavorable nature of Aβl–42 mutants with only one insertion of the isopeptide structure into the whole sequence of the 42-residue peptide, and (2) could migrate to the corresponding Aβl–42 mutants in a short time with no side reaction under physiological conditions (pH 7.4), while being stable under storage conditions. It is noteworthy that this method does not cause any negative effects by water-solubilizing auxiliaries in the biological experiment system, since no additional auxiliaries are released during conversion to Aβl–42. These results suggest that this method provides a new system in AD-related research, in which 26-*O*-acyl isoAβl–42 (26-AIAβ42) can be stored in a solubilized form and rapidly produce intact Aβl–42 in situ during biological experiments, which is useful for investigating the biological function of Aβ1–42 mutants.

5. Experimental

5.1. General

All protected amino acids and resins were purchased from Calbiochem–Novabiochem Japan Ltd (Tokyo). Other chemicals were mainly purchased from commercial suppliers, Wako Pure Chemical Ind. Ltd (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), and Aldrich Chemical Co. Inc. (Milwaukee, WI) and were used without further purification. MALDI-TOF MASS spectra were recorded on Voyager DE-RP using α-cyano-4-hydroxycinnamic acid as a matrix. Analytical HPLC was performed using a C18 reverse-phase column (4.6 mm × 150 mm; YMC Pack ODS AM302) with a binary solvent system: a linear gradient of CH_3CN in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1} (temperature: 40 °C), detected at 230 nm. Preparative HPLC was carried out on a C18 reverse-phase column (20 mm × 250 mm; YMC Pack ODS SH343-5) with a binary solvent system: a linear gradient of CH_3CN in 0.1% aqueous TFA at a flow rate of 5.0 mL min^{-1} (temperature: 40 °C), detected at 230 nm. Solvents used for HPLC were of HPLC grade.

5.2. Solid-phase peptide synthesis

The Fmoc-amino acid side-chain protections were selected as follows: *t*Bu (Asp, Glu, Ser, Thr, Tyr), Boc (Lys), Pmc (Arg), Trt (Asn, Gln, His). Generally, the peptide chains were assembled by the sequential coupling of activated N^{α}-Fmoc-amino acid (2.5 equiv) in DMF (1.5–2 mL) in the presence of DIPCDI (2.5 equiv) and HOBt (2.5 equiv) with a reaction time of 2 h at room temperature. The resins were then washed with DMF (1.5 mL, 5×) and the completeness of each coupling was verified by the Keiser test. N^{α}-Fmoc deprotection was carried out by treatment with piperidine (20% v/v in DMF) (2 mL, 1 min × 1 and 20 min × 1), followed by washing with DMF (1.5 mL, 10×) and chloroform (1.5 mL, 5×). If necessary, these coupling and deprotection cycles were repeated. After complete elongation of the peptide chains, the peptide resins were washed with methanol (1.5 mL, 5×) and dried for at least 2 h in vacuo. The peptides were then cleaved from the resin with TFA in the presence of thioanisole, *m*-cresol, and distilled water (92.5:2.5:2.5:2.5) for 90 min at room temperature, concentrated in vacuo, and precipitated with diethyl ether (4–8 mL) at 0 °C followed by centrifugation at 3000 rpm for 5 min (3×). The resultant peptides were dissolved or suspended in water and lyophilized for at least 12 h. The peptides were reduced using NH_4I–dimethylsulfide in TFA/H_2O (2:1) for 60 min at 0 °C, followed by concentration in vacuo. The crude products were purified by preparative reverse-phase HPLC with a 0.1% aqueous TFA–CH_3CN system as an eluant, immediately frozen at −78 °C and lyophilized for at least 12 h. Purified peptides were stored dry at −20 °C until use.

5.3. Amyloid β peptide (Aβ)1–42 (E22Q, 5) by '*O*-acyl isopeptide method'

(1) 26-*O*-Acyl isoAβ1–42(E22Q, **12**)

After protected Aβ27–42-resin (chlorotrityl chloride resin, 0.1 mmol) was synthesized by the conventional method (see: conventional method for synthesizing **5**), Boc-Ser-OH (49.2 mg, 0.24 mmol) was coupled by the DIPCDI (37.1 μL, 0.24 mmol)–HOBt (32.1 mg, 0.24 mmol) method for 2 h in DMF (1.5 mL). Fmoc-Gly-OH (423.5 mg, 1.4 mmol) was coupled using the DIPCDI (223 μL, 1.4 mmol)–DMAP (3.5 mg, 0.03 mmol) method in CH_2Cl_2 (1.5 mL) for 4 h[18b] (2×). Subsequent amino acid residues were coupled after removing each Fmoc group using 20% piperidine for 20 min (resin: 521.2 mg). The resulting protected peptide resin was treated with TFA (10.3 mL)–*m*-cresol (261 μL)–thioanisole (261 μL)–H_2O (261 μL) for 90 min at rt, concentrated in vacuo, washed with diethyl ether, centrifuged, suspended in water, and lyophilized to give the crude *O*-acyl

isopeptide **12** (267.4 mg). This peptide **12** (10 mg) was dissolved in TFA (1 mL)–H_2O (0.5 mL) in the presence of NH_4I (6 mg, 0.04 mmol) and dimethylsulfide (3 μL, 0.04 mmol) and stood for 60 min at 0 °C. After concentration in vacuo, the crude peptide was dissolved in DMSO, filtered using a 0.46 μm filter unit, applied to preparative HPLC, and eluted using a 0.1% aqueous TFA–CH_3CN. The desired fractions were collected and immediately lyophilized to afford peptide **12** as a white amorphous powder. Yield: 3.4 mg (20.0%); MALDI-MS (TOF): M_{calcd}: 4513.1; $M+H_{found}$: 4514.4; HPLC analysis at 230 nm: purity was >95%.

(2) The purified **12** was dissolved in PBS (pH 7.4) and stirred for 30 min at 37 °C to produce **5** quantitatively (determined by analytical HPLC). MALDI-MS (TOF): M_{calcd}: 4513.1; $M+H_{found}$: 4514.0; HPLC analysis at 230 nm: purity was >95%. The retention time on HPLC (0–100% CH_3CN for 40 min, 230 nm) of **5** was identical to that of Dutch-type Aβl–42 synthesized independently by the conventional method.

5.4. Aβ1–42 (D7N, 2), (A21G, 3), (E22G, 4), (E22K, 6), (D23N, 7) by '*O*-acyl isopeptide method'

(1) 26-*O*-Acyl isoAβ1–42 (D7N, **9**), (A21G, **10**), (E22G, **11**), (E22K, **13**), (D23N, **14**)

26-*O*-Acyl isoAβ1–42 (**9**–**11**, **13**, and **14**) were synthesized in a similar manner to **12**. Chemical data for **9**: yield: 26.2%; MALDI-MS (TOF): M_{calcd}: 4513.1; $M+H_{found}$: 4514.5; HPLC analysis at 230 nm: purity was higher than 95%; for **10**: yield: 26.4%; MALDI-MS (TOF): M_{calcd}: 4500.0; $M+H_{found}$: 4501.3; HPLC analysis at 230 nm: purity was higher than 95%; for **11**: yield: 34.0%; MALDI-MS (TOF): M_{calcd}: 4442.0; $M+H_{found}$: 4442.9; HPLC analysis at 230 nm: purity was higher than 95%; for **13**: yield: 24.6%; MALDI-MS (TOF): M_{calcd}: 4513.1; $M+H_{found}$: 4514.6; HPLC analysis at 230 nm: purity was higher than 95%; for **14**: yield: 21.3%; MALDI-MS (TOF): M_{calcd}: 4513.1; $M+H_{found}$: 4514.5; HPLC analysis at 230 nm: purity was higher than 95%.

(2) The purified **9**–**11**, **13**, and **14** were dissolved in PBS (pH 7.4) and stirred for 30–60 min at 37 °C to produce **2**–**4**, **6**, and **7** quantitatively (determined by analytical HPLC). MALDI-MS (TOF): for **2**: M_{calcd}: 4513.1; $M+H_{found}$: 4514.1; for **3**: M_{calcd}: 4500.0; $M+H_{found}$: 4501.37; for **4**: M_{calcd}: 4441.98; $M+H_{found}$: 4443.0; for **6**: M_{calcd}: 4513.1; $M+H_{found}$: 4513.9; for **7**: M_{calcd}: 4513.1; $M+H_{found}$: 4514.2; HPLC analysis of **2**–**4**, **6**, and **7** at 230 nm: purity was >95%. The retention time on HPLC (0–100% CH_3CN for 40 min, 230 nm) of formed **2**–**4**, **6**, and **7** was identical to that of each independently synthesized Aβl–42 mutant by the conventional manner.

5.5. Amyloid β peptide (Aβ)1–42 (E22Q, 5) by the conventional method

The chlorotrityl chloride resin (200 mg, 0.3 mmol) and Fmoc-Ala-OH (49.4 mg, 0.15 mmol) were taken to the manual solid-phase reactor under an argon atmosphere and stirred for 2.5 h in the presence of *N*,*N*-diisopropylethylamine (DIPEA, 26.2 μL, 0.15 mmol) in 1,2-dichloroethane (1.5 mL). After washing with DMF (1.5 mL, 5×), capping was performed with MeOH (200 μL) in the presence of DIPEA (52.5 μL, 0.3 mmol) in DMF for 20 min. After washing with DMF (1.5 mL, 5×), DMF–H_2O (1:1, 1.5 mL, 5×), $CHCl_3$ (1.5 mL, 2×), and MeOH (1.5 mL, 2×) followed by drying in vacuo, the loading ratio was determined (0.04 mmol) photometrically from the amount of Fmoc chromophore liberated upon treatment with 50% piperidine–DMF for 30 min at 37 °C. The sequential Fmoc-protected amino acids (0.09 mmol) were manually coupled in the presence of DIPCDI (14.1 μL, 0.09 mmol) and HOBt (12.2 mg, 0.09 mmol) for 2 h in DMF (1.5 mL) after the removal of each Fmoc group by 20% piperidine–DMF for 20 min (resin: 362 mg). The resulting protected peptide resin was treated with TFA (7.1 mL)–*m*-cresol (181 μL)–thioanisole (181 μL)–H_2O (181 μL) for 90 min at rt, concentrated in vacuo, washed with diethyl ether, centrifuged, suspended in water, and lyophilized to give the crude peptide **5** (141.2 mg). This peptide (10 mg) was dissolved in TFA (1 mL)–H_2O (0.5 mL) in the presence of NH_4I (6 mg, 0.04 mmol), and dimethylsulfide (3 μL, 0.04 mmol), and stood for 60 min at 0 °C. After concentration in vacuo, the crude peptide was dissolved in DMSO, filtered using a 0.46 μm filter unit, applied to preparative HPLC, and eluted using 0.1% aqueous TFA–CH_3CN. The peak fractions were collected and immediately lyophilized to afford the desired peptide **5** as a white amorphous powder. Yield: 0.8 mg (7.2%); MALDI-MS (TOF): M_{calcd}: 4513.0; $M+H_{found}$: 4514.2; HPLC analysis at 230 nm: purity was >94%.

5.6. Aβ1–42 (D7N, 2), (A21G, 3), (E22G, 4), (E22K, 6), (D23N, 7) by the conventional method

Peptides **2**–**4**, **6**, and **7** were synthesized in a similar manner to peptide **5**. Chemical data for **2**: yield: 8.1%; MALDI-MS (TOF): M_{calcd}: 4513.1; $M+H_{found}$: 4514.0; HPLC analysis at 230 nm: purity was higher than 94%; for **3**: yield: 0.5%; MALDI-MS (TOF): M_{calcd}: 4500.0; $M+H_{found}$: 4501.4; HPLC analysis at 230 nm: purity was higher than 95%; for **4**: yield: 10.1%; MALDI-MS (TOF): M_{calcd}: 4442.0; $M+H_{found}$: 4442.9; HPLC analysis at 230 nm: purity was higher than 95%; for **6**: yield: 22.7%; MALDI-MS (TOF): M_{calcd}: 4513.1; $M+H_{found}$: 4514.2; HPLC analysis at 230 nm: purity was higher than 95%; for **7**: yield: 6.7%; MALDI-MS (TOF): M_{calcd}: 4513.1; $M+H_{found}$: 4513.9; HPLC analysis at 230 nm: purity was higher than 95%. The elution profiles of Aβl–42 mutants in a slow gradient system (25–55% CH_3CN for 60 min) were ill-defined; however, TOF-MS indicated that only a single species was present. Glabe and coworkers[14a] and Johnson and coworkers[14b] have commented upon the chromatographic behavior of Aβ, suggesting that the ill-defined profiles are intrinsic properties of the peptides.

5.7. Water solubility

Peptides were saturated in distilled water and shaken at room temperature. The saturated solutions were passed through a centrifugal filter (0.46 μm filter unit,

Ultrafree®-MC, Millipore). The filtrate was analyzed by RP-HPLC to determine its solubility.

5.8. Stability of *O*-acyl isopeptides 9–14 in phosphate-buffered saline (pH 7.4)

To 495 μL of PBS (pH 7.4) were added 5 μL of a solution including each *O*-acyl isopeptide (1 mM in DMSO), and the mixture was incubated at 37 °C in a water bath. At the desired time points, 500 μL of 1,1,1,3,3,3-hexafluoro-2-propanol was added to the samples and 500 μL of the mixture was directly analyzed by RP-HPLC. HPLC was performed using a C18 (4.6 × 150 mm; YMC Pack ODS AM302) reverse-phase column with a binary solvent system: linear gradient of CH_3CN (0–100%, 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1}, detected at UV 230 nm.

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