

The 'O-acyl isopeptide method' for the synthesis of difficult sequence-containing peptides: application to the synthesis of Alzheimer's disease-related amyloid β peptide (A β) 1–42

YOUHEI SOHMA, YOSHIO HAYASHI, MAIKO KIMURA, YOUSUKE CHIYOMORI, ATSUHIKO TANIGUCHI, MASATO SASAKI, TOORU KIMURA and YOSHIAKI KISO*

Department of Medicinal Chemistry, Center for Frontier Research in Medicinal Science, 21st Century COE Program, Kyoto Pharmaceutical University, Yamashina-Ku, Kyoto 607-8412, Japan

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Abstract: An efficient 'O-acyl isopeptide method' for the synthesis of difficult sequence-containing peptides was applied successfully to the synthesis of amyloid β peptide (A β) 1–42 via a water-soluble O-acyl isopeptide of A β 1–42, i.e. '26-O-acyl isoA β 1–42' (**6**). This paper describes the detailed synthesis of A β 1–42 focusing on the importance of resin selection and the analysis of side reactions in the O-acyl isopeptide method. Protected '26-O-acyl isoA β 1–42' peptide resin was synthesized using 2-chlorotriethyl chloride resin with minimum side reactions in comparison with other resins and deprotected crude 26-O-acyl isoA β 1–42 was easily purified by HPLC due to its relatively good purity and narrow elution with reasonable water solubility. This suggests that only one insertion of the isopeptide structure into the sequence of the 42-residue peptide can suppress the unfavourable nature of its difficult sequence. The migration of O-acyl isopeptide to intact A β 1–42 under physiological conditions (pH 7.4) via O–N intramolecular acyl migration reaction was very rapid and no other by-product formation was observed while **6** was stable under storage conditions. These results concluded that our strategy not only overcomes the solubility problem in the synthesis of A β 1–42 and can provide intact A β 1–42 efficiently, but is also applicable in the synthesis of large difficult sequence-containing peptides at least up to 50 amino acids. This synthesis method would provide a biological evaluation system in Alzheimer's disease research, in which 26-O-acyl isoA β 1–42 can be stored in a solubilized form before use and then rapidly produces intact A β 1–42 *in situ* during biological experiments. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: A β 1–42; O-acyl isopeptide method; Alzheimer's disease; Alzheimer's disease research tool; difficult sequence-containing peptide; O–N intramolecular acyl migration reaction

INTRODUCTION

The synthesis of 'difficult sequence'-containing peptides is one of the most problematic areas in peptide chemistry, and such peptides are often obtained with low yield and purity in solid-phase peptide synthesis (SPPS) [1–6]. These difficult sequences are generally hydrophobic and promote aggregation in solvents during synthesis and purification. This aggregation is attributed to intermolecular hydrophobic interaction and hydrogen bond network among resin-bound peptide chains, resulting in the formation of extended secondary structures such as β -sheets [1,2]. The tendency for aggregation depends on the nature of the peptide and side chain protecting groups. In particular, it is known that peptides with sequences containing

Ala, Val, Ile, Asn and Gln residues in high frequency show a strong propensity for difficult sequences.

To solve the synthetic problem of difficult sequence-containing peptides, Sheppard and Johnson *et al.* reported a building block, 2-hydroxy-4-methoxybenzyl (Hmb), a protecting group for the backbone amide nitrogen [3,4]. Mutter *et al.* also introduced building blocks, so-called pseudo-prolines, which are dipeptide derivatives, consisting of Ser/Thr-derived oxazolidines or Cys-derived thiazolidine [5,6]. These special building blocks were designed to disrupt the secondary structure formed by interchain hydrogen bonding. However, to prepare building blocks, prior modifications of Fmoc-amino acids by 2–6 steps of additional solution phase synthesis are required in these approaches, and a strong acid treatment is also required to remove the building blocks. Therefore, the development of novel methods using conventional amino acid derivatives are of great significance in the synthesis of difficult sequence-containing peptides.

Hence, a novel and efficient 'O-acyl isopeptide method' was developed for the synthesis of difficult sequence-containing peptides based on the synthesis of hydrophilic 'O-acyl isopeptides' followed by O–N intramolecular acyl migration reaction (Figure 1A)

Abbreviations: PBS, phosphate buffered saline; Pmc, 2, 2, 5, 7, 8-pentamethylchroman-6-sulfonyl; Pns, phenylnorstatine = (2R, 3S)-3-amino-2-hydroxy-4-phenylbutyric acid; otherwise as in *J. Peptide Sci* 9: 1–8 (2003).

*Correspondence to: Professor Yoshiaki Kiso, Department of Medicinal Chemistry, Center for Frontier Research in Medicinal Science, 21st Century COE Program, Kyoto Pharmaceutical University, Yamashina-Ku, Kyoto 607-8412, Japan; e-mail: kiso@mb.kyoto-phu.ac.jp

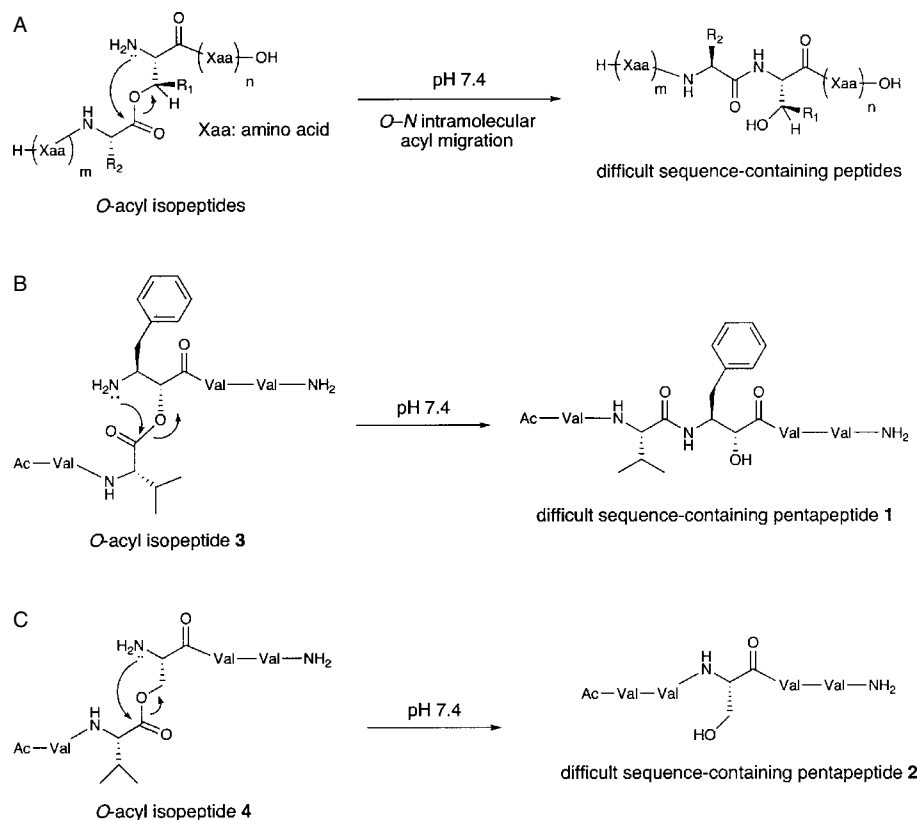


Figure 1 (A) '*O*-Acyl isopeptide method': the synthetic strategy for difficult sequence-containing peptides via the *O*-*N* intramolecular acyl migration reaction of *O*-acyl isopeptide, (B) application of the *O*-acyl isopeptide method for the synthesis of pentapeptide **1**, and (C) application of the *O*-acyl isopeptide method for the synthesis of pentapeptide **2**.

[7,8]. The *O*-*N* intramolecular acyl migration is a well-known reaction in Ser/Thr-containing peptides [9–11]. The *O*-acyl isopeptide method required no special building blocks and markedly improved the synthetic yields of difficult sequence-containing pentapeptides such as Ac-Val-Val-Pns-Val-Val-NH₂ (**1**, Pns: phenylnorstatine, (2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutanoic acid [12–16], Figure 1B) and Ac-Val-Val-Ser-Val-Val-NH₂ (**2**, Figure 1C) [7,8]. For example, in the synthesis of **2** based on this method, *O*-acyl isopeptide **4** was synthesized as a major product with high purity (Figure 2A-b), while a large amount of undesired Fmoc-containing peptide was obtained in a conventional Fmoc-based solid-phase method (Figure 2A-a). This indicated that the branched ester structure improved coupling and deblocking efficacy during SPPS, by suppressing the unfavourable nature of difficult sequence-containing peptides originating from secondary structure formation among the peptide chains on the resin. In addition, *O*-acyl isopeptides with a newly formed amino group attained reasonable H₂O- and MeOH-solubility required in HPLC purification by the formation of salt. Furthermore, from recent research of water-soluble prodrugs [17–24] and *O*-acyl isopeptides [7,8] based on *O*-*N* intramolecular acyl migration, it has been established that the purified *O*-acyl isoform can quantitatively be converted to the

original *N*-acyl form in a short time with no side reaction at pH 7.4 (Figure 2B). These results suggest that the '*O*-acyl isopeptide method' is advantageous for synthesizing small difficult sequence-containing peptides. This study therefore focused on one of the larger difficult sequence-containing peptides, amyloid β peptide (A β) 1–42.

Amyloid β peptides (A β s) are the main proteinaceous component of amyloid plaques found in the brains of Alzheimer's disease (AD) patients [25]. Neuritic plaques, pathognomonic features of AD, contain abundant fibrils formed from A β , which has been found to be neurotoxic *in vivo* and *in vitro* [26]. The predominant forms of A β mainly consist of 40- and 42-residue peptides (designated A β 1–40 and A β 1–42, respectively), which are proteolytically produced from amyloid precursor protein (APP) by enzymatic reactions [27]. Since A β 1–42 is thought to play a more critical role in amyloid formation and the pathogenesis of AD than A β 1–40, many studies using synthetic A β 1–42 have been carried out to clarify the involvement of A β 1–42 in AD [28–32].

However, A β 1–42 is defined as a difficult sequence-containing peptide with a high hydrophobicity and forms aggregates in various media [33–44]. In particular, due to its low solubility and broad elution under acidic or neutral conditions, the conventional

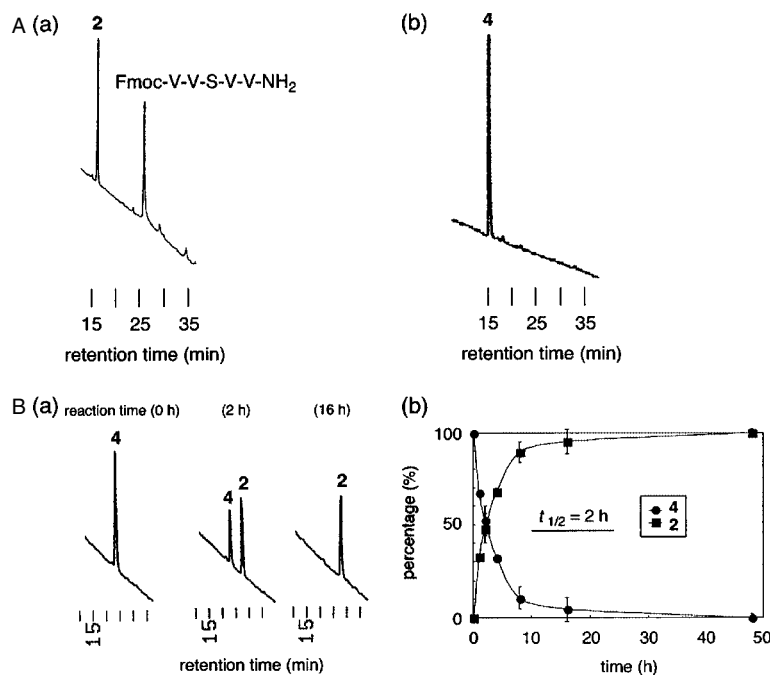


Figure 2 A: HPLC profiles of (a) crude peptide **2** and (b) its *O*-acyl isopeptide **4** (0–100% CH₃CN for 40 min, 230 nm). B: (a) periodical HPLC profiles (0–100% CH₃CN for 40 min, 230 nm) and (b) the graph for conversion of *O*-acyl isopeptide **4** to **2** via the *O*–*N* intramolecular acyl migration in phosphate buffered saline (pH 7.4, 25 °C).

HPLC purification of synthesized A β 1–42 in the aqueous TFA–acetonitrile system is too laborious to remove impurities accumulated during solid-phase peptide synthesis (SPPS). To improve the synthetic difficulty of this peptide, strong acylation reagents such as HATU [36–38,41] and Fmoc-amino acid fluorides [37], the DBU/DMF system for effective Fmoc removal [39], and sulfoxide protection for Met³⁵ to suppress aggregation [44] were employed. For effective HPLC purification, this peptide has also been purified under basic conditions [36,41]. Moreover, A β 1–42 synthesis has been achieved by the segment condensation of fully protected peptide fragments in a solution method employing chloroform–phenol mixed solvent [40].

From the aspect of biological experiments, A β 1–42 is also problematic due to its large extent of aggregation in a standard storage solution such as dimethylsulfoxide (DMSO) [45]. In addition, since this peptide undergoes time- and concentration-dependent aggregation in the acetonitrile–water used for HPLC purification [35], the dry, the purified peptide adopts different structures and aggregation states [46]. However, numerous studies have established that neurotoxicity and the kinetics of aggregation are directly related to the assembly state in solution. Thus, depending on the commercial source, peptide batch, and the aggregation condition, considerable discrepancies might exist in the biological data across different laboratories as well as within the same laboratory over time. Generally, to disaggregate the A β 1–42, the peptide needs to be predissolved in dilute base solution. Therefore, an ‘*in situ*’ system that

could prepare intact monomer A β 1–42 in a soluble form under physiological conditions would be a powerful tool in understanding its inherent pathological function. To create such a system, (1) a novel propeptide possessing high solubility and no aggregate character during HPLC purification and long-term storage as a solution and (2) the capability of intact A β 1–42 production under physiological conditions would be desired.

Based on this background, the idea was conceived that the ‘*O*-acyl isopeptide method’ could be applied to the synthesis of A β 1–42 via a novel water-soluble isopeptide of A β 1–42, i.e. ‘26-*O*-acyl isoA β 1–42 (26-AIA β 42, **6**)’ (Figure 3) [47,48]. This overcomes the problems in the synthesis and storage of A β 1–42. Although there are two Ser residues in A β 1–42 at positions 8 and 26 with the capability of *O*–*N* intramolecular acyl migration, Ser²⁶ was selected for *O*-acylation, since the adjacent Gly²⁵ does not epimerize during ester bond formation (Figure 3). A previous rapid communication [47] reported the efficient synthesis of A β 1–42 by the ‘*O*-acyl isopeptide method’. This paper describes the detailed synthesis of A β 1–42, the importance of resin selection and the analysis of side reactions in the *O*-acyl isopeptide method.

MATERIALS AND METHODS

General

All protected amino acids and resins were purchased from Calbiochem-Novabiochem Japan Ltd (Tokyo). Other chemicals

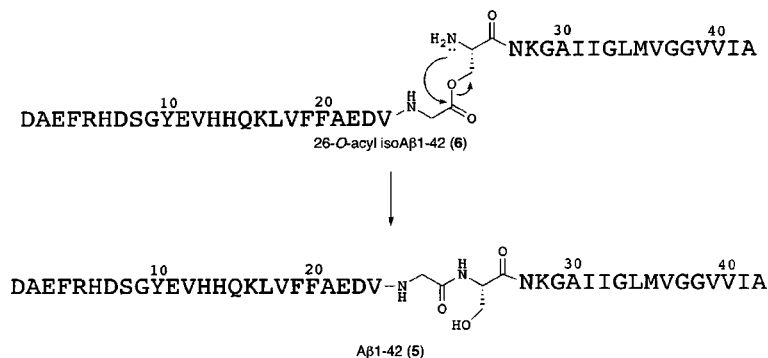


Figure 3 'O-Acyl isopeptide method' for the synthesis of $A\beta$ 1–42 (**5**): The production of $A\beta$ 1–42 (**5**) via the O–N intramolecular acyl migration of 26-O-acyl iso $A\beta$ 1–42 (**6**).

were mainly purchased from commercial suppliers, Wako Pure Chemical Ind. Ltd (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), Aldrich Chemical Co. Inc. (Milwaukee, WI) and Peptide Institute, Inc. (Osaka, Japan) and were used without further purification. MALDI-TOF MASS spectra were recorded on Voyager DE-RP using α -cyano-4-hydroxy cinnamic acid as a matrix. FAB-MS was performed on a Jeol JMS-SX102A spectrometer equipped with the JMA-DA7000 data system. Analytical HPLC was performed using a C18 reverse phase column (4.6 \times 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 $^{\circ}C$), detected at 230 nm. Preparative HPLC was carried out on a C18 reverse phase column (20 \times 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 $^{\circ}C$), detected at 230 nm. Solvents used for HPLC were of HPLC grade.

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 eq) in DMF (1.5–2 ml) in the presence of 1,3-diisopropylcarbodiimide (DIPCDI, 2.5 eq) and 1-hydroxybenzotriazole (HOBT, 2.5 eq) with a reaction time of 2 h at room temperature. The resins were then washed with DMF (\times 5) and the completeness of each coupling was verified by the Kaiser test. *N* α -Fmoc deprotection was carried out by treatment with piperidine (20% v/v in DMF) (2 ml, 1 min \times 1 and 20 min \times 1), followed by washing with DMF (1.5 ml, \times 10) and chloroform (1.5 ml, \times 5). If necessary, the coupling and deprotection cycles were repeated. After the peptide-resins were washed with methanol (1.5 ml, \times 5) and dried for at least 2 h *in vacuo*, the peptides were 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 $^{\circ}C$ followed by centrifugation at 3000 rpm for 5 min (\times 3). The resultant peptides were dissolved or suspended with water and lyophilized for at least 12 h. The crude products were purified by preparative reversed-phase HPLC with 0.1% aqueous TFA– CH_3CN system as an eluant, immediately frozen at –78 $^{\circ}C$, and lyophilized

at least 12 h. Purified peptides were stored dry at –20 $^{\circ}C$ until use.

Ac-Val-Val-Pns-Val-Val-NH₂ (**1**, by the conventional method).

The peptide **1** was synthesized on Rink amide aminomethyl (AM) resin (200 mg, 0.148 mmol) according to the general Fmoc-based solid-phase procedure described. After the resin was washed with DMF (1.5 ml, \times 5), Fmoc-Val-OH (125.6 mg, 0.37 mmol) and Fmoc-Pns-OH (185.4 mg, 0.44 mmol) were coupled in the presence of DIPCDI (57.9 μ l, 0.37 mmol) and HOBT (56.7 mg, 0.37 mmol) in DMF (1.5 ml) for 2 h according to the sequence. The Fmoc-group was removed by 20% piperidine/DMF. *N*-Acetylation was carried out with acetic anhydride (20.9 μ l, 0.222 mmol) in the presence of TEA (20.7 μ l, 0.148 mmol) for 2 h. The peptide was cleaved from the resin using TFA (5 ml) in the presence of thioanisole (136.3 μ l), *m*-cresol (136.3 μ l) and distilled water (136.3 μ l) for 90 min at room temperature, concentrated *in vacuo*, washed with diethyl ether, centrifuged, suspended with water and lyophilized to give the crude peptide (64.6 mg). Subsequently, this crude peptide (20 mg) was saturated in DMSO (1–2 ml), filtered using a 0.46 μ m filter unit, and immediately injected into preparative HPLC with a 0.1% aqueous TFA– CH_3CN system. The peak fractions were collected and immediately lyophilized to afford the desired peptide **1** as a white amorphous powder. Yield: 2.0 mg (6.9%); HRMS (FAB): calcd. for $C_{32}H_{53}N_6O_7(M+H)^+$: 633.3976, found: 633.3982; HPLC analysis at 230 nm: purity was higher than 98%.

Ac-Val-Val-Pns-Val-Val-NH₂ (**1**, by the O-acyl isopeptide method).

After preparation of the H-Val-Val-NH-resin (Rink amide AM resin, 200 mg, 0.126 mmol) in the same manner described in the synthesis of **1** using the conventional method, Boc-Pns-OH (111.6 mg, 0.378 mmol) was coupled in the presence of DIPCDI (59.2 μ l, 0.378 mmol) and HOBT (57.9 mg, 0.378 mmol) in DMF (1.5 ml). Subsequent coupling with Fmoc-Val-OH (128.3 mg, 0.378 mmol) to the α -hydroxy group of Pns was performed using the DIPCDI (59.2 μ l, 0.378 mmol)–DMAP (3.1 mg, 0.0252 mmol) method in CH_2Cl_2 (1.5 ml) for 16 h (\times 2), followed by the coupling of another Val residue, *N*-acetylation using Ac_2O (14.3 μ l, 0.15 mmol)–TEA (17.5 μ l, 0.126 mmol), TFA (4.4 ml)–thioanisole (117.2 μ l)–*m*-cresol (117.2 μ l)–distilled water (117.2 μ l) treatment for 90 min at room temperature, concentration *in vacuo*, diethyl ether wash, centrifugation, suspension with water and lyophilization to give the crude O-acyl isopeptide **3** (51.3 mg).

Subsequently, this crude peptide (20 mg) was dissolved in methanol (500 μ l), filtered using a 0.46 μ m filter unit, and immediately injected into preparative HPLC with a 0.1% aqueous TFA-CH₃CN system. The peak fractions were collected and immediately lyophilized, affording the desired *O*-acyl isopeptide **3** as a white amorphous powder (18.7 mg, 58.3%). HRMS (FAB): calcd for C₃₂H₅₃N₆O₇(M + H)⁺: 633.3976, found: 633.3979; HPLC analysis at 230 nm: purity was higher than 99%.

Purified *O*-acyl isopeptide **3** was then dissolved in phosphate-buffered saline (PBS, pH 7.4) at room temperature (1 mg ml⁻¹) and stirred for 5 min at room temperature. The resultant precipitate was centrifuged and washed with water and methanol followed by drying *in vacuo* to give **1** as a white powder. Yield: 18.5 mg (57.6%); HRMS (FAB): calcd. for C₃₂H₅₃N₆O₇(M + H)⁺: 633.3976, found: 633.3983; HPLC analysis at 230 nm: purity was higher than 99%.

Ac-Val-Val-Ser-Val-Val-NH₂ (2, by the conventional method). Peptide **2** was synthesized from Rink amide AM resin (200 mg, 0.126 mmol) and Fmoc-Ser-OH (120.8 mg, 0.315 mmol) in a similar manner to that described for peptide **1** in the conventional method. Yield: 4.1 mg (6.0%); HRMS (FAB): calcd. for C₂₅H₄₇N₆O₇(M + H)⁺: 543.3506, found: 543.3510; HPLC analysis at 230 nm: purity was higher than 95%.

Ac-Val-Val-Ser-Val-Val-NH₂ (2, by the *O*-acyl isopeptide method). Peptide **2** was synthesized from Rink amide AM resin (200 mg, 0.126 mmol) and Boc-Ser-OH (65.2 mg, 0.315 mmol) in a similar manner to that described for peptide **1** in the *O*-acyl isopeptide method. *O*-Acyl isopeptide **4**: Yield: 27.7 mg (40.6%); HRMS (FAB): calcd. for C₂₅H₄₇N₆O₇(M + H)⁺: 543.3506, found: 543.3509; HPLC analysis at 230 nm: purity was higher than 99%. **2**: Yield: 27.7 mg (40.6%); HRMS (FAB): calcd. for C₃₂H₅₃N₆O₇(M + H)⁺: 543.3506, found: 543.3499; HPLC analysis at 230 nm: purity was higher than 96%.

Amyloid β peptide A β 1-42 (5, by the conventional method). The chlorotriyl 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 DIPEA (26.2 μ l, 0.15 mmol) in 1,2-dichloroethane (1.5 ml). After washing with DMF (1.5 ml, \times 5), capping was performed with MeOH (200 μ l) in the presence of DIPEA (52.5 μ l, 0.3 mmol) in DMF for 20 min. After washing with DMF (\times 5), DMF-H₂O (1:1, \times 5), CHCl₃ (\times 2) and MeOH (\times 2) followed by drying *in vacuo*, the loading ratio was determined (0.1 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 (0.25 mmol) were manually coupled in the presence of DIPCDI (36.4 μ l, 0.25 mmol) and HOBt (35.6 mg, 0.25 mmol) for 2 h in DMF (1.5 ml) after the removal of each Fmoc group by 20% piperidine-DMF for 20 min (resin: 465.4 mg). The resulting protected peptide-resin (128.9 mg) was treated with TFA (2.5 ml)-*m*-cresol (64.5 μ l)-thioanisole (64.5 μ l)-H₂O (64.5 μ l) for 90 min, concentrated *in vacuo*, washed with diethyl ether, centrifuged, suspended with water and lyophilized to give the crude peptide **5** (53.6 mg). This peptide (20 mg) was dissolved in TFA (2 ml)-H₂O (1 ml) in the presence of NH₄I (12 mg, 0.08 mmol)

and dimethylsulfide (6 μ l, 0.08 mmol) and stood for 60 min at 0 °C. After concentration *in vacuo*, the crude peptide was dissolved in hexafluoroisopropanol, filtered using a 0.46 μ m filter unit, applied to preparative HPLC, and eluted using 0.1% aqueous TFA-CH₃CN. The peak fractions were collected and immediately lyophilized to afford the desired peptide **5** as a white amorphous powder. Yield: 3.6 mg (7.2%); MALDI-MS (TOF): M_{calc}: 4514.04; M + H_{found}: 4515.48; HPLC analysis at 230 nm: purity was >94%; the retention time on HPLC (0-100% CH₃CN for 40 min, 230 nm) of synthesized **5** was identical to that of commercially available A β 1-42 (Peptide Institute, Inc., Osaka, Japan).

Amyloid β peptide A β 1-42 (5, in *O*-acyl isopeptide method). After protected A β 27-42-resin (chlorotriyl chloride resin, 0.1 mmol) was synthesized in the same manner as described in the synthesis of A β 1-42 in the conventional method, Boc-Ser-OH (48.1 mg, 0.25 mmol) was coupled by the DIPCDI (36.4 μ l, 0.25 mmol)-HOBt (35.6 mg, 0.25 mmol) method for 2 h in DMF (1.5 ml). Coupling with Fmoc-Gly-OH (82.9 mg, 0.3 mmol) was performed using the DIPCDI (43.7 μ l, 0.3 mmol)-DMAP (2.3 mg, 0.02 mmol) method in CH₂Cl₂ (1.5 ml) for 16 h (\times 2). Subsequent amino acid residues were coupled after the removal of each Fmoc group using 20% piperidine for 20 min (resin: 536.9 mg). Resulting protected peptide-resin (176.5 mg) was treated with TFA (3.4 ml)-*m*-cresol (88.3 μ l)-thioanisole (88.3 μ l)-H₂O (88.3 μ l) for 90 min, concentrated *in vacuo*, washed with diethyl ether, centrifuged, suspended with water and lyophilized to give the crude *O*-acyl isopeptide **6** (97.3 mg). This peptide **6** (40 mg) was dissolved in TFA (4 ml)-H₂O (2 ml) in the presence of NH₄I (21.8 mg, 0.17 mmol) and dimethylsulfide (11 μ l, 0.17 mmol) and stood for 60 min at 0 °C. After concentration *in vacuo*, the crude peptide was dissolved in hexafluoroisopropanol, filtered using a 0.46 μ m filter unit, applied to preparative HPLC, and eluted using a 0.1% aqueous TFA-CH₃CN. The desired fractions were collected and immediately lyophilized to afford peptide **6** as a white amorphous powder. Yield: 22.9 mg (33.6%); MALDI-MS (TOF): M_{calc}: 4514.04; M + H_{found}: 4515.26; HPLC analysis at 230 nm: purity was >96%.

The purified **6** was dissolved in H₂O and stirred for 48 h at room temperature followed by lyophilization to yield **5** quantitatively as a TFA salt. MALDI-MS (TOF): M_{calc}: 4514.04; M + H_{found}: 4515.48; HPLC analysis at 230 nm: purity was >95%; the retention time on HPLC (0-100% CH₃CN for 40 min, 230 nm) of synthesized **5** was identical to that of commercially available A β 1-42 (Peptide Institute, Inc., Osaka, Japan).

26-O-Ser-A β 26-42 (15). After protected A β 26-42-resin (**9**, chlorotriyl chloride resin, 0.046 mmol) was synthesized in the same manner as described in the synthesis of **6**, Fmoc-Ser(*t*Bu)-OH (52.9 mg, 0.138 mmol) was coupled using DIPCDI (25.7 μ l, 0.138 mmol) and DMAP (1.3 mg, 0.0092 mmol) in CH₂Cl₂ (1.5 ml) for 16 h (\times 2). The peptide-resin was treated with 20% piperidine-DMF for 20 min and TFA (1.79 ml)-*m*-cresol (45.4 μ l)-thioanisole (45.4 μ l)-H₂O (45.4 μ l) for 90 min, followed by concentration *in vacuo*, diethyl ether washing, centrifugation, suspension with water and lyophilization to give the crude peptide **15** (26.1 mg). This peptide **15** (10 mg) was dissolved in TFA (1 ml)-H₂O (0.5 ml) in the presence of NH₄I (5.45 mg, 0.043 mmol) and dimethylsulfide (11 μ l, 0.043 mmol) and stood for 60 min at 0 °C. After concentration,

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-CH₃CN. The desired fractions were collected and immediately lyophilized to afford the titled peptide **15** as a white amorphous powder. MALDI-MS (TOF): M_{calc} : 1686.03; $M + H_{\text{found}}$: 1687.04.

Water-, Methanol- and DMSO-Solubility

Peptides **1–6** were saturated in distilled water, methanol or DMSO and shaken at room temperature. The saturated solutions were passed through a centrifugal filter (0.23 μm or 0.46 μm filter unit, Ultrafree[®]-MC, Millipore). The filtrate was analysed by RP-HPLC to determine the solubility.

Stability of O-Acyl Isopeptides **2** and **4** in Phosphate Buffered Saline (PBS, pH 7.4)

To 495 μl of PBS (pH 7.4) were added 4 μl of DMSO and 1 μl of a solution including **2** or **4** (10 mM in DMSO), and the mixture was stirred at room temperature. At the desired time points, 500 μl of DMSO was added to the samples and 500 μl of the mixture was directly analysed by RP-HPLC. HPLC was performed using a C18 (4.6 \times 150 mm; YMC Pack ODS AM302) reverse-phase column with a binary solvent system: linear gradient of CH₃CN (40%–100%, 30 min) in 0.1% aqueous TFA at a flow rate of 0.9 ml min⁻¹, detected at UV 230 nm.

Stability of O-Acyl IsoA β 1–42 (**6**) in Buffers

The conversion profiles at various pHs of **6** were determined in buffered saline in a manner similar to that described for the stability studies of **2** and **4**. To 494–497 μl of buffered saline (pH 7.4: PBS, pH 4.9: PBS, pH 3.5: acetate buffer) was added 3–6 μl of solution including **6** (1 mM in DMSO), and the mixture was incubated at 25 °C or 37 °C in a water bath. At the desired time points 500 μl of hexafluoroisopropanol was added to the samples and 500 μl of the mixture was directly analysed by RP-HPLC.

RESULTS AND DISCUSSION

In the synthesis of 26-AIA β 42 (**6**) based on the O-acyl isopeptide method (Scheme 1), Fmoc-Ala-O-chlorotrityl resin (**7**) was employed and Fmoc-protected amino acids were sequentially coupled using the DIPCDI-HOBt method (2 h) after the removal of each Fmoc group with 20% piperidine-DMF (20 min) to give peptide-resin **8**. After Boc-Ser-OH was introduced to **8** by the DIPCDI-HOBt method (2 h), the obtained **9** was coupled with Fmoc-Gly-OH at the β -hydroxy group of Ser using the DIPCDI-DMAP method in CH₂Cl₂ to obtain ester **10**. 26-AIA β 42-resin (**13**) was obtained through the further coupling of additional amino acid residues using the conventional manner. Finally, 26-AIA β 42 (**6**) was obtained as a major product (Figure 4A) by treatment with TFA-*m*-cresol-thioanisole-H₂O (92.5 : 2.5 : 2.5 : 2.5) for 90 min

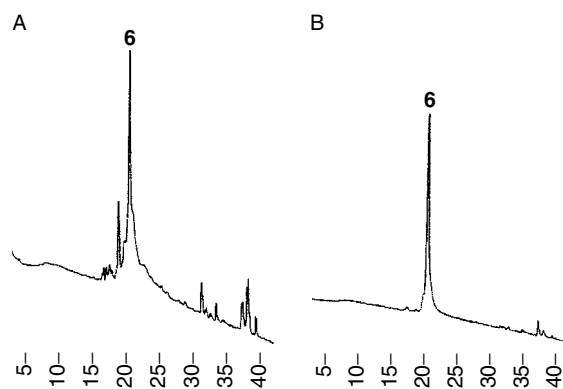


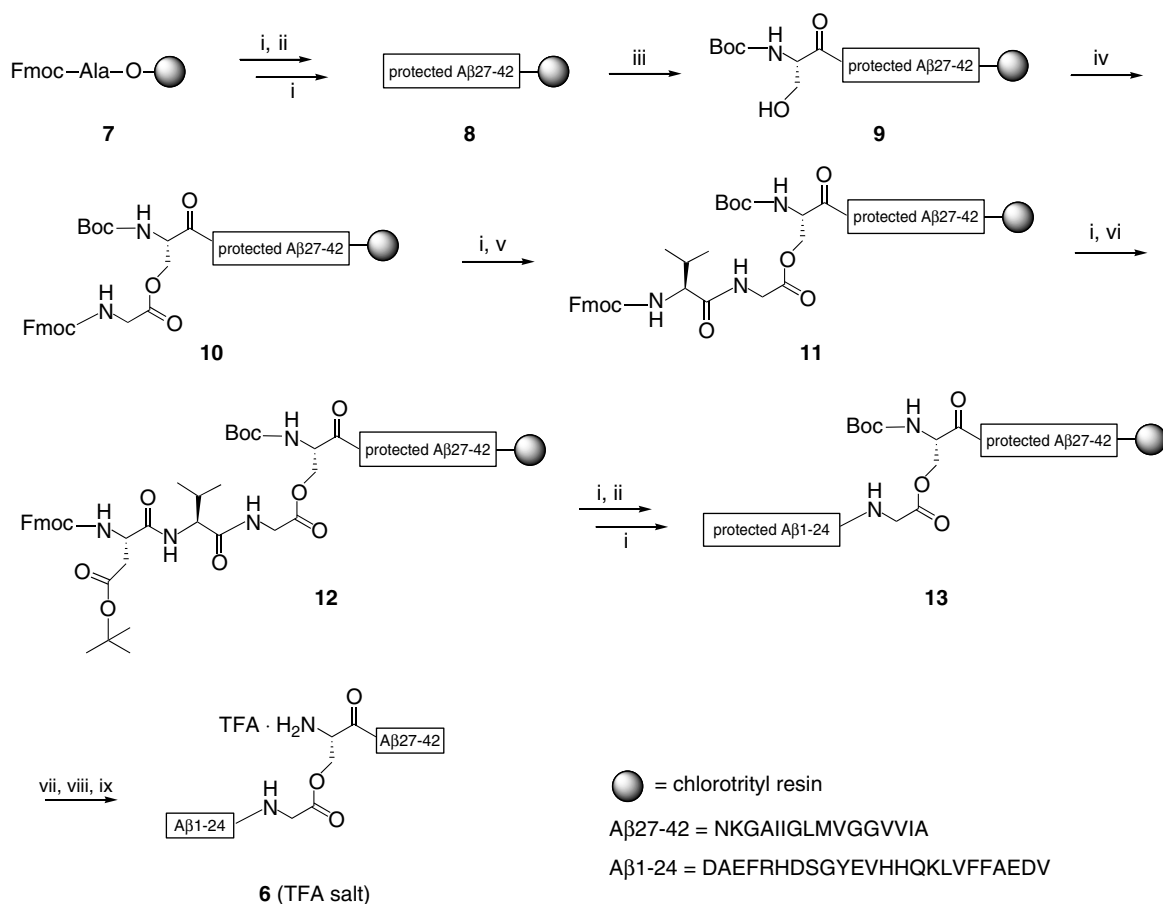
Figure 4 HPLC profiles of (A) crude and (B) purified 26-AIA β 42 (**6**). Analytical HPLC was performed using a C18 reverse-phase column (4.6 \times 150 mm; YMC Pack ODS AM302) with a binary solvent system: the linear gradient of CH₃CN (0–100% CH₃CN for 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 ml min⁻¹ (temperature: 40 °C), detected at 230 nm.

followed by reduction with NH₄I–dimethylsulfide for 60 min in TFA:H₂O (2 : 1).

To examine whether the coupling of Boc-Ser-OH with peptide-resin **8** (Scheme 1, step iii) gives a by-product (**14**, Figure 5A) which has an additional insertion of Boc-Ser-OH onto the β -hydroxy group of Ser²⁶, an expected by-product **15**, which is a deprotected-cleaved product of **14**, was independently prepared (see Materials and Methods), and compared with the sample deprotected from peptide-resin **9** with a TFA-thioanisole system. However, no by-product **15** was detected in this sample by HPLC analysis, indicating that this side reaction with the formation of **14** can be neglected in the coupling step with Boc-Ser-OH based on the DIPCDI-HOBt method in DMF for 2 h (Figure 5A).

It was confirmed that the esterification of the β -hydroxy group of Ser²⁶ in **9** with Fmoc-Gly-OH completed on the solid support, since the major product deprotected from peptide-resin **10** by TFA corresponds to O-acyl isoA β 25–42 by HPLC analysis (data not shown) [TOF-MS: M_{calc} : 1878.24; $M + H_{\text{found}}$: 1879.29], and A β 26–42, which corresponds to the unreacted component, was not detected. In addition, another possible by-product **16** with Fmoc-Gly-Gly sequence caused by elimination of the Fmoc group of **10** by DMAP during the coupling reaction was not detected in the TOF-MS analysis of a deprotected sample of peptide-resin **10** (Figure 5B).

In the HPLC analysis of crude **6** (Figure 4A), A β 1–25 (DAEFRHDSGYEVHHQKLVFFAEDVG) was not detected as a by-product, although a very small amount (1.6%, HPLC yield) of A β 26–42 (SNKGAIIGLMVGGVIA) was observed. This indicates that the formed ester bond between Gly and Ser was almost stable in both 20% piperidine and TFA treatment. However, this slight A β 26–42 formation in crude **6** might be



Scheme 1 Reagents/conditions: (i) 20% piperidine/DMF, 20 min; (ii) Fmoc-AA-OH (2.5 eq), DIPCDCI (2.5 eq), HOBT (2.5 eq), DMF, 2 h; (iii) Boc-Ser-OH (2.5 eq), DIPCDCI (2.5 eq), HOBT (2.5 eq), DMF, 2 h; (iv) Fmoc-Gly-OH (3.0 eq), DIPCDCI (3.0 eq), DMAP (0.2 eq), CH₂Cl₂, 16 h × 2; (v) Fmoc-Val-OH (2.5 eq), DIPCDCI (2.5 eq), HOBT (2.5 eq), DMF, 2 h; (vi) Fmoc-Asp(OtBu)-OH (2.5 eq), DIPCDCI (2.5 eq), HOBT (2.5 eq), DMF, 2 h; (vii) TFA-*m*-cresol-thioanisole-H₂O (92.5:2.5:2.5:2.5), 90 min; (viii) NH₄I (20 eq), dimethylsulfide (20 eq), TFA:H₂O (2:1), 60 min, 0°C; (ix) preparative HPLC (the linear gradient of CH₃CN in 0.1% aqueous TFA).

attributed to the production of **9** by the formation of diketopiperazine during 20% piperidine treatment (20 min) of peptide-resin **11** (Scheme 1). This finding does not correspond to the case of Ac-Val-Val-Ser-Val-Val-NH₂ (**2**) [8] since diketopiperazine formation did not occur in a similar elongation of the peptide chain from the primary hydroxy group of Ser in **4**, suggesting that the less steric-hindered Gly²⁵ in **11** promoted the cyclization reaction. Therefore, if there are plural Ser or Thr residues in the difficult sequence, the position for converting isopeptide structure is better selected based on the structure of two amino acids residues at the *N*-terminal side next to the isopeptide site (Ser/Thr) to minimize diketopiperazine formation.

The crude *O*-acyl isopeptide **6** was dissolved in hexafluoroisopropanol, applied to preparative HPLC and eluted using 0.1% aqueous TFA-CH₃CN. Since **6** was eluted as a narrow single peak (Figures 4 and 6A), it was easily purified using preparative scale HPLC to give pure **6** (Figures 4B and 6A) as a TFA salt with a total isolated yield of 33.6%, calculated from the original loading onto the chlorotriptyl resin. This yield was higher

than that obtained in the synthesis of **5** by standard Fmoc-based SPPS (7.2%). Since **5** was eluted as a broad peak (Figure 6B) in HPLC analysis and purification, it was difficult to evaluate the purity and laborious to isolate **5** from impurities as reported [33–44]. In addition, in the synthesis of **6**, no conversion to **5** was observed.

As a resin selection in the synthetic condition setting, 0.16 mmol/g of preloaded Fmoc-Ala-NovaSyn[®]TGA-resin consisting of Tentagel [49] and the TFA-labile linker [50] was also employed (0.0288 mmol) with a similar synthetic procedure as described in the use of chlorotriptyl resin for the synthesis of 26-AIAβ42 (**6**), since this type of resin has been well used for the synthesis of Aβ1–42 due to its high swelling characteristic [37–39,41,43]. However, in the use of TGA resin, a large amount of undesired Aβ1–25 and Aβ26–42 (20%–30%, HPLC yield, respectively) was detected on analytical HPLC and MALDI-MS (TOF) (Aβ1–25: M_{calc} : 2833.11 M + H_{found}: 2934.11, Aβ26–42, M_{calc} : 1598.95; M + H_{found}: 1599.28) in the resultant crude **6**, indicating that

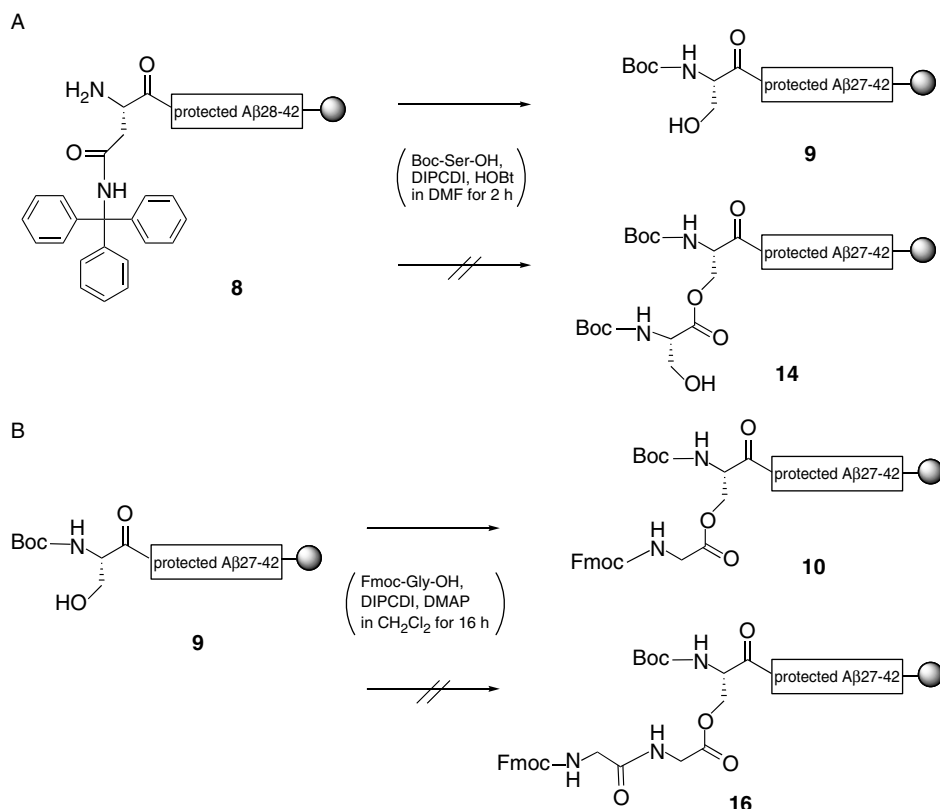


Figure 5 Examination whether a side reaction occurred in the reaction of (A) Boc-Ser-OH insertion to **8** (Scheme 1, step iii) and (B) esterification of **9** (Scheme 1, step iv).

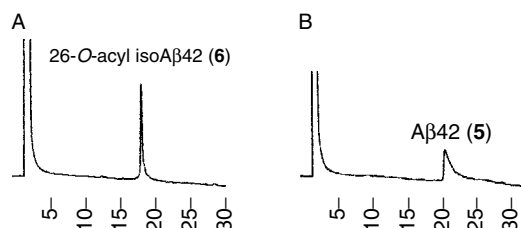


Figure 6 HPLC profiles of pure (A) 26-AIAβ42 (**6**) and (B) Aβ1-42 (**5**). The same quantity (2.3 nmol) of both peptides was applied to the HPLC column as DMSO solution. Analytical HPLC was performed using a C18 reverse-phase column (4.6 × 150 mm; YMC Pack ODS AM302) with a binary solvent system: the linear gradient of CH₃CN (25%–55% CH₃CN 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.

the ester bond between Gly²⁵ and Ser²⁶ was significantly cleaved by the treatment of TFA-*m*-cresol-thioanisole-H₂O (92.5 : 2.5 : 2.5 : 2.5) in the final deprotection. Significant ester bond cleavage occurred in the use of TGA resin, but not more acid-sensitive chlorotriyl resin in the same TFA-cocktail condition suggests that undesired ester bond cleavage mainly occurred in the resin-bound *O*-acyl isopeptides, although the precise reason remains unclear. Consequently, extremely acid-labile 2-chlorotriyl chloride resin is more efficient in the synthesis of *O*-acyl

isopeptides to avoid acid-mediated ester bond cleavage. In the case of Wang resin (0.8 mmol/g), peptide chain elongation was difficult in the 7 to 8 residues from the C-terminal.

The water solubility of **6** (TFA salt) was 15 mg ml⁻¹, 100-fold higher than that of Aβ1-42 (**5**, 0.14 mg ml⁻¹). Interestingly, as a slight modification of the peptide chain by inserting one ester bond drastically increased the solubility of the insoluble original peptide with 42 residues, this suggests that *O*-acyl isopeptides totally destroy the secondary structures responsible for the insolubility of the original peptide. In addition, the HPLC analysis of **6** exhibited quite a sharp peak even in the slow gradient system (25%–55% CH₃CN, 60 min, Figure 6A), while **5** was eluted as a broad peak in the same elution condition (Figure 6B) as reported [33–44]. These results also support that the high assembly characteristic of Aβ1-42 was suppressed by only one insertion of the isopeptide structure. Recent solution-state NMR studies of the Aβ1-40 and Aβ1-42 by Zagorski *et al.* [32] indicated that the Ser²⁶ residue comprises turn- or bendlike structures that bring two β-sheets in contact and hydrogen bonding among peptide chains, which is associated with β-aggregation. As it was demonstrated that *O*-acyl isopeptide could suppress the unfavourable nature of difficult sequence-containing pentapeptides in the previous study [7,8], this result in the synthesis of **6**

indicates that this method is a powerful strategy for increasing the solubility of even larger peptides. Recent reports by Carpino *et al.* [51] and Mutter *et al.* [52] have also supported our hypothesis that *O*-acyl isopeptide structures possess attractive solubilizing efficacy.

As shown in Figure 7A, purified **6** was quantitatively converted to A β 1–42 (**5**) at room temperature in PBS (pH 7.4) with a half-life of 2.6 min and with no side reaction such as hydrolysis of the ester bond. This faster migration may be attributed to the less steric hindrance of the Gly²⁵ residue. In addition, in PBS (pH 7.4) at 37°C, this migration was very rapid with a half-life of approximately 1 min and with no side reaction, and migration was completed after 30 min. On the other hand, the TFA salt of **6** was stable at 4°C for at least 1 month in either a solid state or a DMSO solution. Moreover, as shown in Figure 7B, slower migration was observed at pH 4.9 (PBS) with a half-life of 3 h and no migration at pH 3.5 (acetate buffer) after incubation for 3 h at room temperature. This rapid migration under physiological conditions enables the production of an intact monomer A β 1–42, *in situ* to investigate the inherent biological function of A β 1–42 in AD. The conversion of **6** (TFA salt) in water for 48 h at room temperature followed by lyophilization yielded A β 1–42 (**5**) quantitatively as TFA salt with >95% purity.

This result demonstrates that this '*O*-acyl isopeptide method' is applicable for the synthesis of large peptides. In particular, it is noteworthy that only one insertion of the isopeptide structure into the sequence of 42-residue

peptide can suppress the unfavourable nature of its difficult sequence. Therefore, the '*O*-acyl isopeptide method' can be applied to larger difficult sequence-containing peptides than A β 1–42 as a general method. In addition, rapid migration of *O*-acyl isopeptides to intact A β 1–42 under physiological conditions (pH 7.4) was observed while it was stable under storage conditions. Hence, our strategy not only overcomes the solubility problem in the synthesis of A β 1–42, but also provides a novel tool for the biological evaluation system in AD research, in which 26-*O*-acyl isoA β 1–42 can be stored in a solubilized form before use and rapidly produces intact monomer A β 1–42 *in situ* during biological experiments.

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REFERENCES

1. Kent SBH. Chemical synthesis of peptides and proteins. *Annu. Rev. Biochem.* 1988; **57**: 957–990.
2. Tam JP, Lu YA. Coupling difficulty associated with interchain clustering and phase transition in solid phase peptide synthesis. *J. Am. Chem. Soc.* 1995; **117**: 12 058–12 063.
3. Johnson T, Quibell M, Owen D, Sheppard RC. A reversible protecting group for the amide bond in peptides. Use in the synthesis of 'difficult sequences'. *J. Chem. Soc. Chem. Commun.* 1993; 369–372.
4. For a review, see: Sheppard R. The fluorenylmethoxycarbonyl group in solid phase synthesis. *J. Peptide Sci.* 2003; **9**: 545–552.
5. Wöhr T, Wahl F, Nefzi A, Rohwedder B, Sato T, Sun X, Mutter M. Pseudo-prolines as a solubilizing, structure-disrupting protection technique in peptide synthesis. *J. Am. Chem. Soc.* 1996; **118**: 9218–9227.
6. Guichou J-F, Patiny L, Mutter M. Pseudo-prolines (ψ Pro): Direct insertion of ψ Pro systems into cysteine containing peptides. *Tetrahedron Lett.* 2002; **43**: 4389–4390.
7. Sohma Y, Sasaki M, Ziora Z, Takahashi N, Kimura T, Hayashi Y, Kiso Y. A novel approach for the hydrophobic peptides synthesis and purification through *O*-*N* intramolecular acyl migration reaction. *Peptides, Peptide Revolution: Genomics, Proteomics & Therapeutics*. Kluwer Academic: Netherlands, 2003; 67–68.
8. Sohma Y, Sasaki M, Hayashi Y, Kimura T, Kiso Y. Novel and efficient synthesis of difficult sequence-containing peptides through *O*-*N* intramolecular acyl migration reaction of *O*-acyl isopeptides. *Chem. Commun.* 2004; 124–125.
9. Stewart JM. Protection of the hydroxyl group in peptide synthesis. In *The Peptides*. Gross E, Meienhofer J (eds). Academic Press: NY, 1981; **3**: 169–201.
10. Tamamura H, Kato T, Otaka A, Fujii N. Synthesis of potent β -secretase inhibitors containing a hydroxyethylamine dipeptide

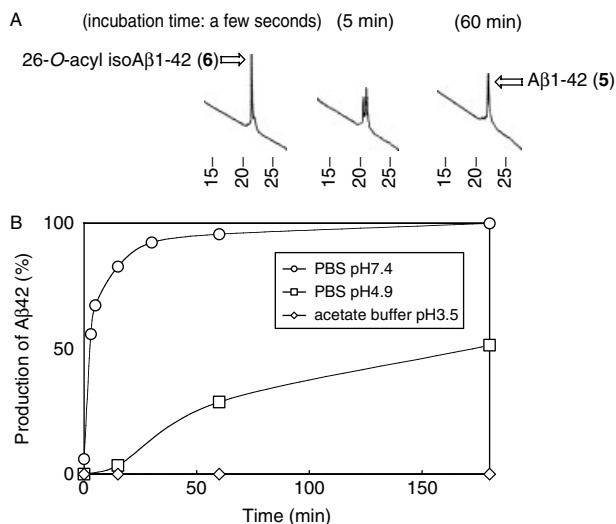


Figure 7 (A) HPLC profiles of the conversion of 26-AIA β 42 (**6**) to A β 1–42 (**5**) via *O*-*N* intramolecular acyl migration in PBS (pH 7.4) at 25°C and (B) a graph of the production of A β 1–42 (**5**) in various pH conditions at 25°C. Analytical HPLC was performed using a C18 reverse-phase column (4.6 × 150 mm; YMC Pack ODS AM302) with a binary solvent system: the linear gradient of CH₃CN (0–100% CH₃CN 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.

- isostere and their structure-activity relationship studies. *Org. Biomol. Chem.* 2003; **1**: 2468–2473.
11. Mouls L, Subra G, Enjalbal C, Martinez J, Aubagnac J-L. O-N-Acyl migration in N-terminal serine-containing peptides: mass spectrometric elucidation and subsequent development of site-directed acylation protocols. *Tetrahedron Lett.* 2004; **45**: 1173–1178.
 12. Nishizawa R, Saino T, Takita T, Suda H, Aoyagi T, Umezawa H. Synthesis and structure-activity relations of bestatin analogs, inhibitors of aminopeptidase B. *J. Med. Chem.* 1977; **20**: 510–515.
 13. Iizuka K, Kamijo T, Harada H, Akahane K, Kubota T, Umeyama H, Ishida T, Kiso Y. Orally potent human renin inhibitors derived from angiotensinogen transition state: design, synthesis, and mode of interaction. *J. Med. Chem.* 1990; **33**: 2707–2714.
 14. Mimoto T, Imai J, Tanaka S, Hattori N, Takahashi O, Kisanuki S, Nagano Y, Shintani M, Hayashi H, Sakikawa H, Akaji K, Kiso Y. Rational design and synthesis of a novel class of active site-targeted HIV protease inhibitors containing a hydroxymethylcarbonyl isostere. Use of phenylnorstatine or allophenylnorstatine as a transition-state mimic. *Chem. Pharm. Bull.* 1991; **39**: 2465–2467.
 15. Nezami A, Luque I, Kimura T, Kiso Y, Freire E. Identification and characterization of allophenylnorstatine-based inhibitors of plasmepsin II, an antimalarial target. *Biochemistry* 2002; **41**: 2273–2280.
 16. Kimura T, Shuto D, Hamada Y, Igawa N, Kasai S, Liu P, Hidaka K, Hamada T, Hayashi Y, Kiso Y. Design and synthesis of highly active Alzheimer's β -secretase (BACE1) inhibitors, KMI-420 and KMI-429, with enhanced chemical stability. *Bioorg. Med. Chem. Lett.* 2005; **15**: 211–215.
 17. Kimura T, Ohtake J, Nakata S, Enomoto H, Moriwaki H, Akaji K, Kiso Y. Synthesis of prodrugs of HIV protease inhibitors. *Peptide Chemistry 1994*, Ohno M. (ed.) Protein Research Foundation: Osaka, 1995; 157–160.
 18. Oliyai R, Stella VJ. Structural factors affecting the kinetics of O,N-acyl transfer in potential O-peptide prodrugs. *Bioorg. Med. Chem. Lett.* 1995; **5**: 2735–2740.
 19. Kiso Y, Kimura T, Ohtake J, Nakata S, Enomoto H, Moriwaki H, Nakatani M, Akaji K. 'O \rightarrow N intramolecular acyl migration' - type prodrugs of tripeptide inhibitors of HIV protease. *Peptides: Chemistry, Structure and Biology*, Mayflower Scientific: England, 1996; 157–159.
 20. Hamada Y, Ohtake J, Sohma Y, Kimura T, Hayashi Y, Kiso Y. New water-soluble prodrugs of HIV protease inhibitors based on O \rightarrow N intramolecular acyl migration. *Bioorg. Med. Chem.* 2002; **10**: 4155–4167.
 21. Hamada Y, Matsumoto H, Kimura T, Hayashi Y, Kiso Y. Effect of the acyl groups on O \rightarrow N acyl migration in the water-soluble prodrugs of HIV-1 protease inhibitor. *Bioorg. Med. Chem. Lett.* 2003; **13**: 2727–2730.
 22. Hayashi Y, Skwarczynski M, Hamada Y, Sohma Y, Kimura T, Kiso Y. A novel approach of water-soluble paclitaxel prodrug with no auxiliary and no byproduct: design and synthesis of isotaxel. *J. Med. Chem.* 2003; **46**: 3782–3784.
 23. Skwarczynski M, Sohma Y, Kimura M, Hayashi Y, Kimura T, Kiso Y. O-N Intramolecular acyl migration strategy in water-soluble prodrugs of taxoids. *Bioorg. Med. Chem. Lett.* 2003; **13**: 4441–4444.
 24. Hamada Y, Matsumoto H, Yamaguchi S, Kimura T, Hayashi Y, Kiso Y. Water-soluble prodrugs of dipeptide HIV protease inhibitors based on O \rightarrow N intramolecular acyl migration: Design, synthesis and kinetic study. *Bioorg. Med. Chem.* 2004; **12**: 159–170.
 25. Selkoe DJ. Translating cell biology into therapeutic advances in Alzheimer's disease. *Nature* 1999; **399**: A23–A31.
 26. Geula C, Wu CK, Saroff D, Lorenzo A, Yuan ML, Yankner BA. Aging renders the brain vulnerable to amyloid β -protein neurotoxicity. *Nature Med.* 1998; **4**: 827–831.
 27. Sinha S, Lieberburg I. Cellular mechanisms of beta-amyloid production and secretion. *Proc. Natl Acad. Sci. USA* 1999; **96**: 11 049–11 053.
 28. Iwatsubo T, Odaka A, Suzuki N, Mizusawa H, Nukina N, Ihara Y. Visualization of A β 42(43) and A β 40 in senile plaques with end-specific A β monoclonals: evidence that an initially deposited species is A β 42(43). *Neuron* 1994; **13**: 45–53.
 29. Younkin SG. Evidence that A β 42 is the real culprit in Alzheimer's disease. *Ann. Neurol.* 1995; **37**: 287–288.
 30. Dahlgren KN, Manelli AM, Stine WB Jr, Baker LK, Krafft GA, LaDu MJ. Oligomeric and fibrillar species of amyloid- β peptides differentially affect neuronal viability. *J. Biol. Chem.* 2002; **277**: 32 046–32 053.
 31. Bitan G, Tarus B, Vollers SS, Lashuel HA, Condron MM, Straub JE, Teplow DB. A molecular switch in amyloid assembly: Met35 and amyloid β -protein oligomerization. *J. Am. Chem. Soc.* 2003; **125**: 15 359–15 365.
 32. Hou L, Shao H, Zhang Y, Li H, Menon NK, Neuhaus EB, Brewer JM, Byeon I-J L, Ray DG, Vitek MP, Iwashita T, Makula RA, Przybyla AB, Zagorski MG. Solution NMR studies of the A β (1–40) and A β (1–42) peptides establish that the Met35 oxidation state affects the mechanism of amyloid formation. *J. Am. Chem. Soc.* 2004; **126**: 1992–2005.
 33. Burdick D, Soreghan B, Kwon M, Kosmoski J, Knauer M, Henschen A, Yates J, Cotman C, Glabe C. Assembly and aggregation properties of synthetic Alzheimer's A β amyloid peptide analogs. *J. Biol. Chem.* 1992; **267**: 546–554.
 34. Quibell M, Turnell WG, Johnson T. Improved preparation of β -amyloid(1–43): structural insight leading to optimized positioning of N-(2-hydroxy-4-methoxybenzyl) (Hmb) backbone amide protection. *J. Chem. Soc., Perkin Trans. I* 1995; 2019–2024.
 35. Shen C-L, Murphy RM. Solvent effects on self-assembly of β -amyloid peptide. *Biophys. J.* 1995; **69**: 640–651.
 36. Fukuda H, Shimizu T, Nakajima M, Mori H, Shirasawa T. Synthesis, aggregation, and neurotoxicity of the Alzheimer's A β 1–42 amyloid peptide and its isoaspartyl isomers. *Bioorg. Med. Chem. Lett.* 1999; **9**: 953–956.
 37. Milton SCF, Milton RCD, Kates SA, Glabe C. Improved synthesis and purification of Alzheimer's A β 1–42 and analogs. *Lett. Peptide Sci.* 1999; **6**: 151–156.
 38. El-Agnaf OMA, Mahil DS, Patel BP, Austen BM. Oligomerization and toxicity of β -amyloid-42 implicated in Alzheimer's disease. *Biochem. Biophys. Res. Commun.* 2000; **273**: 1003–1007.
 39. Tickler AK, Barrow CJ, Wade JD. Improved preparation of amyloid- β peptides using DBU as N^α-Fmoc deprotection reagent. *J. Peptide Sci.* 2001; **7**: 488–494.
 40. Inui T, Bódi J, Nishio H, Nishiuchi Y, Kimura T. Synthesis of amyloid β -peptides in solution employing chloroform-phenol mixed solvent for facile segment condensation of sparingly soluble protected peptides. *Lett. Peptide Sci.* 2002; **8**: 319–330.
 41. Murakami K, Irie K, Morimoto A, Ohgashi H, Shindo M, Nagao M, Shimizu T, Shirasawa T. Neurotoxicity and physicochemical properties of A β mutant peptides from cerebral amyloid angiopathy: implication for the pathogenesis of cerebral amyloid angiopathy and Alzheimer's disease. *J. Biol. Chem.* 2003; **278**: 46 179–46 187.
 42. Tickler AK, Clippingdale AB, Wade JD. Amyloid- β as a 'difficult sequence' in solid phase peptide synthesis. *Protein Peptide Lett.* 2004; **11**: 377–384.
 43. D'Ursi AM, Armenante MR, Guerrini R, Salvadori S, Sorrentino G, Picone D. Solution structure of amyloid β -peptide (25–35) in different media. *J. Med. Chem.* 2004; **47**: 4231–4238.
 44. Kim YS, Moss JA, Janda KD. Biological tuning of synthetic tactics in solid-phase synthesis: Application to A β (1–42). *J. Org. Chem.* 2004; **69**: 7776–7778.
 45. Stine WB Jr, Dahlgren KN, Krafft GA, Ladu MJ. *In vitro* characterization of conditions for amyloid- β peptide oligomerization and fibrillogenesis. *J. Biol. Chem.* 2003; **278**: 11 612–11 622.
 46. Soto C, Castano EM, Kumar RA, Beavis RC, Frangione B. Fibrillogenesis of synthetic amyloid-beta peptides is dependent on their initial secondary structure. *Neurosci. Lett.* 1995; **200**: 105–108.

47. Sohma Y, Sasaki M, Hayashi Y, Kimura T, Kiso Y. Design and synthesis of a novel water-soluble A β 1–42 isopeptide: an efficient strategy for the preparation of Alzheimer's disease-related peptide, A β 1–42, via O–N intramolecular acyl migration reaction. *Tetrahedron Lett.* 2004; **45**: 5965–5968.
48. Sohma Y, Hayashi Y, Skwarczynski M, Hamada Y, Sasaki M, Kimura T, Kiso Y. O–N Intramolecular acyl migration reaction in the development of prodrugs and the synthesis of difficult sequence-containing bioactive peptides. *Biopolymers* 2004; **76**: 344–356.
49. Bayer E. Protein synthesis. *Angew. Chem. Int. Ed.* 1991; **30**: 113–129.
50. Sheppard RC, Williams BJ. Acid-labile resin linkage agents for use in solid phase peptide synthesis. *Int. J. Peptide Protein Res.* 1982; **20**: 451–454.
51. Carpino LA, Krause E, Sferdean CD, Schuemann M, Fabian H, Bienert M, Beyermann M. Synthesis of 'difficult' peptide sequences: application of a depsipeptide technique to the Jung-Redemann 10- and 26-mers and the amyloid peptide A β (1–42). *Tetrahedron Lett.* 2004; **45**: 7519–7523.
52. Mutter M, Chandravarkar A, Boyat C, Lopez J, Santos SD, Mandal B, Mimna R, Murat K, Patiny L, Saucedo L, Tuhscherer G. Switch peptides in statu nascendi: induction of conformational transitions relevant to degenerative diseases. *Angew. Chem. Int. Ed.* 2004; **43**: 4172–4178.