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Short Communication

'*O*-Acyl isopeptide method' for peptide synthesis: Solvent effects in the synthesis of Aβ1–42 isopeptide using '*O*-acyl isodipeptide unit'

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Abstract: '*O*-Acyl isopeptide method' is an efficient synthetic method for peptides. We designed '*O*-acyl isodipeptide units', Boc-Ser/Thr(Fmoc-Xaa)-OH, as important building blocks to enable routine use of the *O*-acyl isopeptide method. In the synthesis of an Aβ1–42 isopeptide using *O*-acyl isodipeptide unit Boc–Ser(Fmoc–Gly)–OH, a side reaction, resulting in the deletion of Ser^{26} in the *O*-acyl isopeptide structure, was noticed during coupling of the unit. We observed that the side reaction occurred during the activation step and was solvent-dependent. In DMF or NMP, an intramolecular side reaction, originating from the activated species of the unit, occurred during the activation step. In non-polar solvents such as $CHCl_3$ or CH_2Cl_2, the side reaction was less likely to occur. Using CH_2Cl_2 as solvent in coupling the unit, the target Aβ1–42 isopeptide was synthesized with almost no major side reaction. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: *O*-acyl isodipeptide unit; *O*-acyl isopeptide method; amyloid β peptide; difficult sequence-containing peptide; solvent effect

INTRODUCTION

The synthesis of 'difficult sequence'-containing peptides is one of the most problematic areas in peptide chemistry, and these peptides often have low solid-phase synthetic yield and purity due to their hydrophobic and aggregative properties [1,2]. In 2003, we discovered that the presence of an *O*-acyl instead of *N*-acyl hydroxyamino acid residue within the peptide backbone significantly changed the secondary structure of the native peptide, and thereby decreased the unfavourable nature derived from difficult sequences. This *O*-acyl isopeptide subsequently afforded the corresponding target peptide via an *O*–*N* intramolecular acyl migration reaction. These findings led to the development of an '*O*-acyl isopeptide method' for the synthesis of peptides containing difficult sequences (Figure 1(a)) [3–15]. The method has been successfully applied to efficiently synthesize difficult sequence-containing peptides such as Ac–Val–Val–Ser–Val–Val–NH_2 (**1**) and Alzheimer's disease-related amyloid β peptide (Aβ) 1–42 (**2**) [3–8,11,12,14]. Several other research groups such as Mutter *et al.* [16–20], Carpino *et al.* [21,22], and Börner *et al.* [23–26] have used similar principles, and thus suggesting that the method is widely advantageous for peptide preparation. Recently, we designed and prepared '*O*-acyl isodipeptide units' Boc–Ser/Thr(Fmoc–Xaa)–OH from a two-step solution phase synthesis, as a mean to avoid epimerization-induced esterification on the resin in the synthesis of *O*-acyl isopeptide (Figure 1(b)) [10–12,14,15]. We synthesized forty *O*-acyl isodipeptide units from all naturally coded amino acids [15], and applied the *O*-acyl isopeptide method using these units to successfully synthesize Ac–Val–Val–Thr–Val–Val–NH_2 (**3**) [10] and influenza A virus matrix M1 58–66 (**4**) [15] using manual SPPS protocols. Using the units, fully automated protocols can take advantage of the routine amide bond formation in SPPS of *O*-acyl isopeptides. Other research groups have also synthesized *O*-acyl isopeptides from corresponding units using fully automated protocols [17,22].

In the current study, we synthesized Aβ1–42 isopeptide **5** (26-*O*-acyl isoAβ1–42) employing ABI 431A Peptide Synthesizer with fully automated

Abbreviations: Aβ, amyloid β peptide; DCC, 1,3-dicyclohexylcarbodiimide; DCU, 1,3-dicyclohexylurea; DIPCDI, 1,3-diisopropylcarbodiimide; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; HOOBt, 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine; NMP, *N*-methylpyrrolidone; SPPS, solid-phase peptide synthesis.

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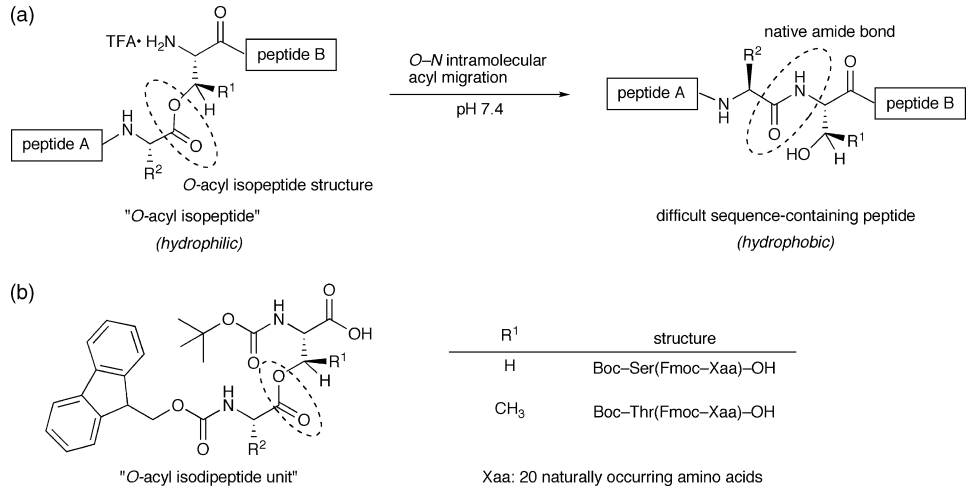
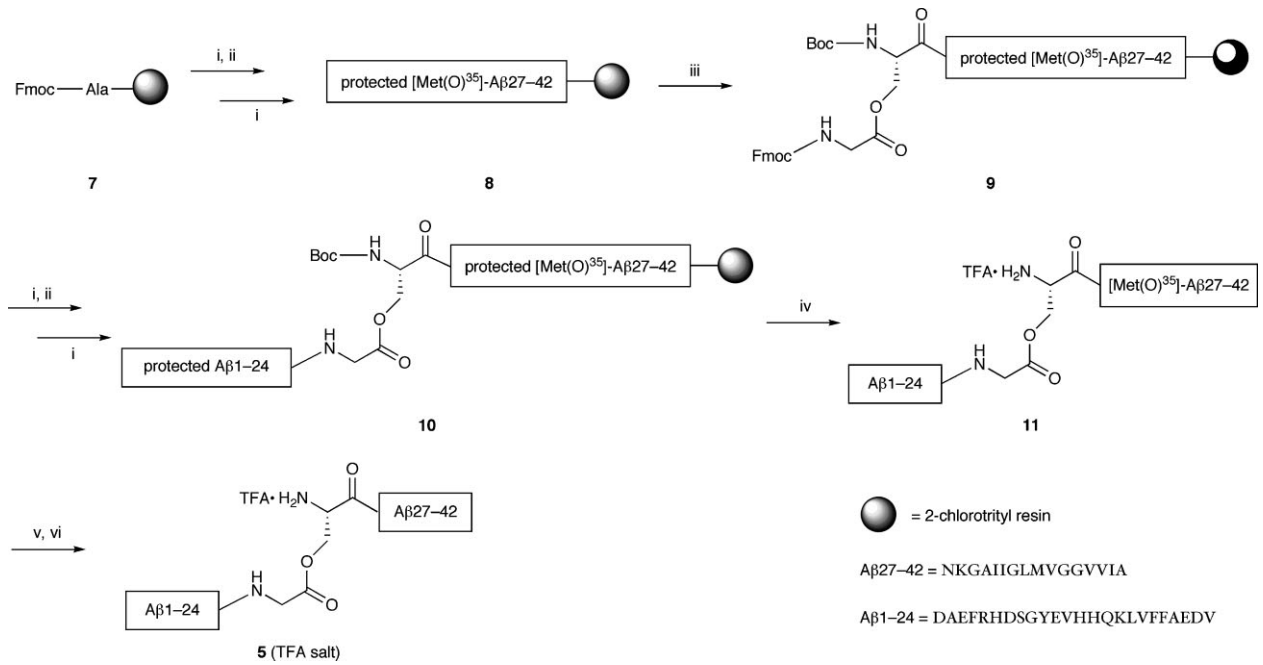


Figure 1 (a) *O*-Acyl isopeptide method: Synthetic strategy for difficult sequence-containing peptides via the *O*–*N* intramolecular acyl migration reaction of *O*-acyl isopeptides, and (b) general structure of *O*-acyl isodipeptide units.



Scheme 1 Synthetic scheme of Aβ1–42 isopeptide **5** (26-*O*-acyl isoAβ1–42) using *O*-acyl isodipeptide unit **6**. *Reagents/conditions*: fully automated protocol (i) (ii) (iii) ABI 431A Peptide Synthesizer according to the protocols of DCC-HOBt method in NMP (Fmoc chemistry, standard scale): manual protocol (i) 20% piperidine/DMF, 20 min; (ii) Fmoc-AA-OH (2.5 eq), DIPCDI (2.5 eq), HOBt (2.5 eq), DMF, 2 h; (iii) Boc-Ser(Fmoc-Gly)-OH (**6**, 2.5 eq), DIPCDI (2.5 eq), HOBt (2.5 eq), DMF or CH_2Cl_2, 2 h; (iv) TFA–*m*-cresol–thioanisole–H_2O (92.5 : 2.5 : 2.5 : 2.5), 90 min; (v) NH_4I (40 eq), dimethylsulfide (40 eq), TFA:H_2O (2 : 1), 0 °C, 60 min; (vi) preparative HPLC (the linear gradient of CH_3CN in 0.1% aq TFA).

protocols using Boc–Ser(Fmoc–Gly)–OH (**6**), in which a native Gly^{25}–Ser^{26} bond was modified to a β-ester bond in the *O*-acyl isopeptide structure (Scheme 1). During the synthesis, after Fmoc deprotection, the peptide-resin was coupled with the corresponding HOBt ester of an Fmoc amino acid or unit **6**, which was 'pre-activated' with DCC and HOBt in NMP, to obtain peptide-resin **10**. Interestingly, after TFA treatment, in HPLC and MS analysis of the crude peptide, production of des-(Ser^{26})- [$Met(O)^{35}$]-Aβ1–42 (**12**) was observed instead of desired [$Met(O)^{35}$]-26-*O*-acyl isoAβ1–42 (**11**) (Figure 2(a)). From this observation, we explored a side reaction of unit **6**, that led to the deletion of Ser^{26} in the *O*-acyl isopeptide structure. Herein, we report the elucidation and suppression of this side reaction by solvent effects.

In our previous studies using *O*-acyl isodipeptide units, isopeptides of **3** and **4** were synthesized with no side reaction by manual protocols in which the coupling

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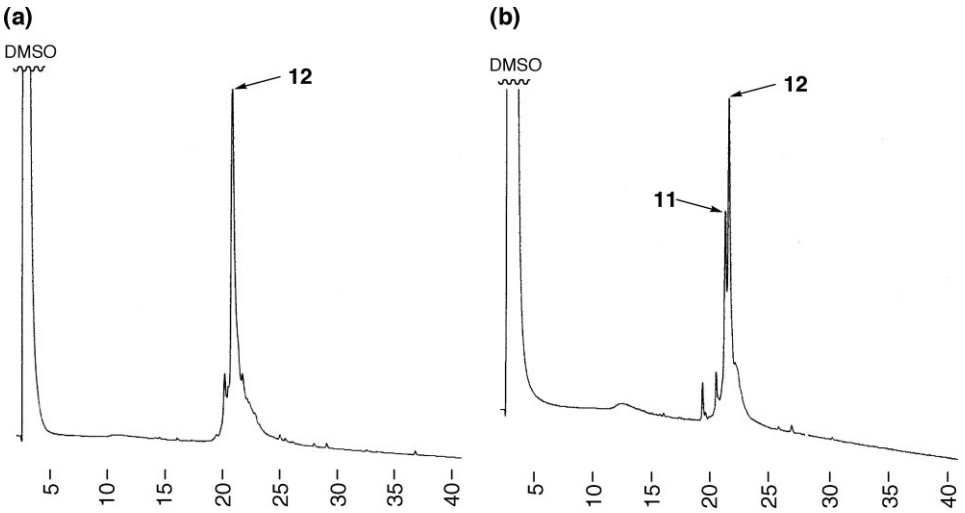
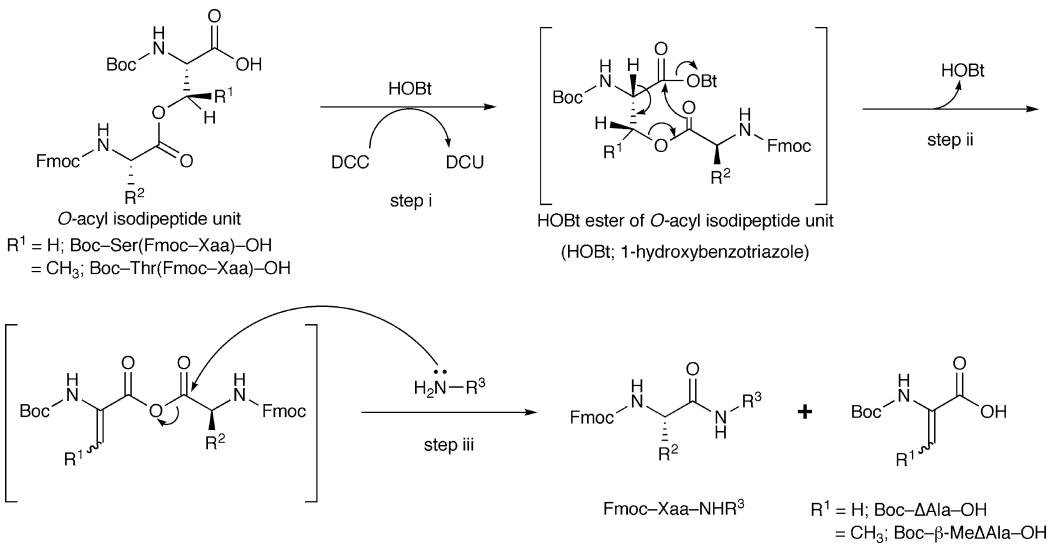


Figure 2 HPLC profiles of the crude peptide after TFA treatment in the synthesis of Aβ1–42 isopeptide **5** using *O*-acyl isodipeptide unit **6** with (a) fully automated protocols, in which **6** was coupled using DCC-HOBt method in NMP, and (b) manual protocols, in which **6** was coupled using DIPCDI-HOBt method in DMF. **11**: [Met(O)^{35}]-26-*O*-acyl isoAβ1–42, **12**: des-(Ser^{26})-[Met(O)^{35}]-Aβ1–42. Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS AM302) with binary solvent system: a linear gradient of CH_3CN (0–100% CH_3CN, 40 min) in 0.1% aq TFA at a flow rate of 0.9 ml min^{-1} (40 °C), detected at 230 nm.

steps were carried out using DIPCDI-HOBt method in DMF without pre-activation [10,15], and so, we decided to synthesize Aβ1–42 isopeptide **5** by the same manual protocols (Scheme 1). In HPLC and MS analysis of a crude intermediate peptide, although **11** was also observed, a major product was **12** (Figure 2(b)). We considered that, because the peptide ([Met(O)^{35}]-Aβ27-42 in **8**) coupled with the unit in synthesizing **5** is longer than that used in synthesizing isopeptides of **3** and **4** and also a difficult sequence, leading to a slow coupling rate of the HOBt ester of unit **6**, a side reaction associated with the activated species might have occurred. We also performed the reaction using automated protocols and noted that the idea that the activated species initiated the side reaction is supported by the observation that more by-products appeared in automated protocols with pre-activation than in manual protocols without pre-activation (Figure 2).

We set up a hypothesis that the side reaction of the activated *O*-acyl isodipeptide unit occurred as shown in Scheme 2. Namely, after the unit is activated as an HOBt ester (step i), the HOBt ester reacts intramolecularly, forming a mixed anhydride of Fmoc–Xaa–OH and Boc–ΔAla–OH (step ii). Then, an amino group would react with the carbonyl carbon of Xaa in the mixed anhydride rather than that of ΔAla, which possesses a less reactive enone conjugated unsaturated system (step iii).



Scheme 2 Hypothesis for side reaction during activation step of *O*-acyl isodipeptide unit.

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To demonstrate our hypothesis in a simpler solution phase model experiment, Boc–Ser(Fmoc–Gly)–OH (**6**) was pre-activated with DCC (2.0 eq) and HOBt (2.0 eq) in NMP for 2 h, and then reacted with benzylamine (2.2 eq). In HPLC and MS analysis of the crude compound, side reaction products Fmoc–Gly–NHBzl (**13**) and Boc–ΔAla–NHBzl (**14**) were observed and nearly none of the commonly expected product Boc–Ser(Fmoc–Gly)–NHBzl (**15**) was observed. In HPLC analysis, **13** had a yield of 80% calculated from amount of starting unit **6**. Moreover, in ^{1}H-NMR analysis of a mixture from a different experiment in which unit **6** was pre-activated with DIPCDI (2.0 eq) and HOBt (2.0 eq) in DMF-d_7 for 2 h, two particular signals derived from E- and Z-olefin protons of ΔAla derivatives (δ 5.79, 5.51) appeared. These results of HPLC, MS, and NMR suggest that unit **6** reacted to Fmoc–Gly–OH and Boc–ΔAla–OH derivatives almost quantitatively in DMF or NMP during the 2 h pre-activation.

Moreover, we observed similar side reactions for Boc–Ser(Fmoc–Ile)–OH (**16**), Boc–Thr(Fmoc–Gly)–OH (**17**), and Boc–Thr(Fmoc–Ile)–OH (**18**). Each unit was pre-activated with DIPCDI (2.0 eq) and HOBt (2.0 eq) in DMF for 2 h, and reacted with benzylamine (2.0 eq), respectively. In all cases, significant amounts of corresponding Fmoc–Gly/Ile–NHBzl (**19, 20**) and Boc–ΔAla/β-MeΔAla–NHBzl (**21, 22**) were detected in HPLC and MS analysis of the corresponding crude solution. These results indicate that *O*-acyl isodipeptide units **16**–**18** are susceptible to the side reaction during the activation step regardless of the bulkiness of the amino acids.

Surprisingly, in ^{1}H-NMR analysis of a mixture in which unit **6** was pre-activated in $CDCl_3$ instead of DMF-d_7, no signal derived from olefin protons of ΔAla derivatives was detected. This result suggests that the HOBt ester of **6** is less prone to the side reaction in more non-polar solvents such as $CHCl_3$ or CH_2Cl_2 than DMF or NMP, and the side reaction could be suppressed by choice of solvent.

We confirmed the effect of solvent on SPPS by coupling Boc–Ser(Fmoc–Gly)–OH (**6**) with protected [Met(O)^{35}]-Aβ27–42-resin **8**. Unit **6** (2.5 eq) was coupled to **8** with DIPCDI (2.5 eq) and HOBt (2.5 eq) in either DMF or CH_2Cl_2. Each peptide-resin was treated with a TFA cocktail and the obtained crude peptide was analyzed by HPLC (Figure 3). As expected, HPLC yield of desired 25-*N*-Fmoc-[Met(O)^{35}]-26-*O*-acyl isoAβ25–42 (**23**) in the crude peptide prepared using CH_2Cl_2 was remarkably higher than that of the DMF conditions. In MS analysis, undesired 25-*N*-Fmoc-des-(Ser^{26})-[Met(O)^{35}]-Aβ25–42 (**24**) was observed in the crude peptide prepared using DMF, while **24** was not detected when CH_2Cl_2 was used instead. These results suggested that less polar CH_2Cl_2 was superior to DMF from the viewpoint of suppression of the side reaction. Additionally, further experiments revealed that no significant difference was observed between additives HOBt, HOAt [27], and HOObt [28] in the side reaction and coupling of the unit.

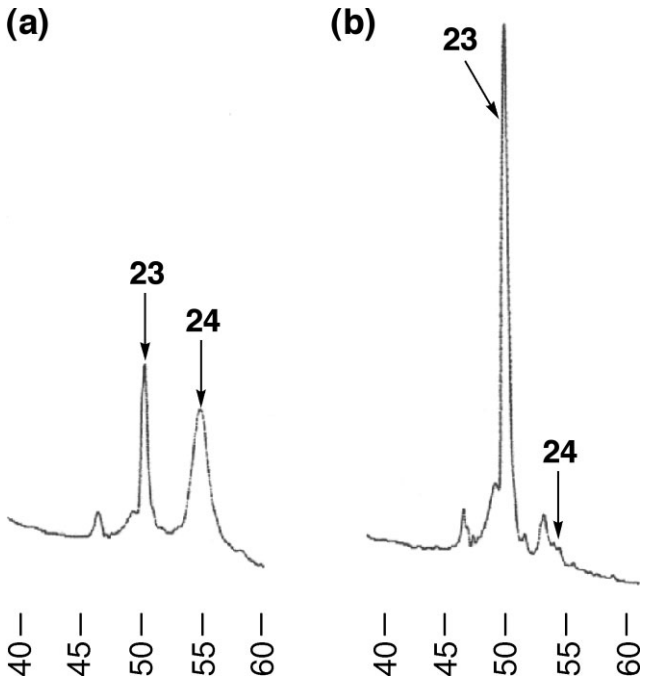

Figure 3 HPLC profiles of crude peptide treated with TFA cocktails after coupling of *O*-acyl isodipeptide unit **6** with protected [Met(O)^{35}]-Aβ27–42-resin **8** by DIPCDI-HOBt method in (a) DMF and (b) CH_2Cl_2. **23**: 25-*N*-Fmoc-[Met(O)^{35}]-26-*O*-acyl isoAβ25–42, **24**: 25-*N*-Fmoc-des-(Ser^{26})-[Met(O)^{35}]-Aβ25–42. Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS AM302) with binary solvent system: a linear gradient of CH_3CN (15–55% CH_3CN, 80 min) in 0.1% aq TFA at a flow rate of 0.9 ml min^{-1} (40 °C), detected at 230 nm.

On the basis of these results, we synthesized Aβ1–42 isopeptide **5** by the *O*-acyl isopeptide method using unit **6** and manual protocols (Scheme 1). After the synthesis of protected [Met(O)^{35}]-Aβ27–42-resin **8** (2-chlorotrityl resin, 0.393 mmol/g, 0.079 mmol peptide) [7], unit **6** (2.5 eq) was coupled with **8** by DIPCDI-HOBt (2.5 eq) method in CH_2Cl_2 for 2 h. After coupling additional amino acid residues using conventional manners to obtain protected [Met(O)^{35}]-26-*O*-acyl isoAβ1–42-resin **10** (462.2 mg), a portion of **10** (50.0 mg) was treated with a TFA cocktail to obtain a crude peptide (25.7 mg). In HPLC and MS analysis of the crude peptide, desired [Met(O)^{35}]-26-*O*-acyl isoAβ1–42 (**11**) was mainly observed and undesired des-(Ser^{26})-[Met(O)^{35}]-Aβ1–42 (**12**) was not detected (Figure 4). The HPLC profile and yield of this crude peptide were almost similar to those of the crude peptides using the *O*-acyl isopeptide method without the unit, as described in previously studies [7]. Thus, in the synthesis using unit **6** which was coupled in CH_2Cl_2 (Figure 4), **11** was obtained more efficiently than in DMF (Figure 2(b)). Aβ1–42 isopeptide **5** was prepared from **11** as reported [5–9].

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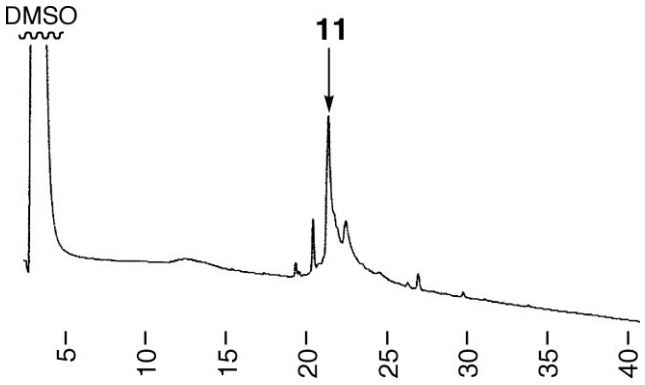

Figure 4 HPLC profile of crude [Met(O)^{35}]-26-*O*-acyl isoAβ1–42 (**11**) using *O*-acyl isodipeptide unit **6** which was coupled using DIPCDI-HOBt method in CH_2Cl_2. Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS AM302) with binary solvent system: a linear gradient of CH_3CN (0–100% CH_3CN, 40 min) in 0.1% aq TFA at a flow rate of 0.9 ml min^{-1} (40 °C), detected at 230 nm.

In summary, a side reaction, which resulted in the deletion of Ser^{26} in the *O*-acyl isopeptide structure, was observed during the synthesis of Aβ1–42 isopeptide **5** using *O*-acyl isodipeptide unit **6**. In solution phase model experiments, we observed that unit **6** formed Fmoc-Gly-OH and Boc-ΔAla-OH derivatives during the activation step, and that similar side reactions also occurred in other kinds of units. Moreover, we optimized the coupling conditions to suppress the side reaction. Non-polar solvent CH_2Cl_2 was superior to DMF in suppressing the side reaction. On the basis of this result, using CH_2Cl_2 as solvent for the coupling of unit **6**, we synthesized Aβ1–42 isopeptide **5** with nearly no side reaction associated with the Ser^{26} deletion. By choice of solvent, **5** would be efficiently synthesized using fully automated protocols. With the expansion of the use of the *O*-acyl isodipeptide units, *O*-acyl isopeptide method can be applied to wide ranges of peptide chemistry.

EXPERIMENTAL

General experimental details for the synthesis of *O*-acyl isodipeptide units [10,15] and the SPPS of Aβ1–42 isopeptide **5** [5–9] were as previously reported.

Fully Automated Synthesis Using Boc–Ser(Fmoc–Gly)–OH (6)

After synthesizing Fmoc–Ala-resin **7** (2-chlorotrityl resin, 0.249 mmol/g, 0.174 mmol peptide) as reported [7], fully automated synthesis was carried out using ABI 431A Peptide Synthesizer according to the protocols for DCC-HOBt method in NMP (Fmoc chemistry, standard scale). Unit **6** was carried out under the same conditions for Fmoc amino acids. After the final deprotection step, peptide-resin **10** was then washed with MeOH, dried for at least 2 h *in vacuo*, treated with a TFA cocktail, washed with diethyl ether, suspended in water and lyophilized as reported [5–9].

Examination of Side Reaction of Boc–Ser(Fmoc–Gly)–OH (6) by HPLC and MS

Unit **6** (40 mg, 0.0826 mmol) was added to a mixture of 1 mol/l DCC in NMP (165.1 µl, 0.165 mmol) and 1 mol/l HOBt in NMP (165.1 µl, 0.165 mmol) at rt and the mixture was shaken for 2 h. To the mixture was added benzylamine (19.8 µl, 0.182 mmol) and shaken overnight at rt. After a filtration of DCU, the filtrate was diluted with EtOAc (15 ml), washed with 5% $NaHCO_3$ (5 ml), 10% citric acid (5 ml), and brine (5 ml), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude compound was dissolved in MeOH and analyzed by RP-HPLC and ESI-MS.

Quantitative Determination of Formed Fmoc–Gly–NHBzl (13) by HPLC

Unit **6** (20 mg, 0.0413 mmol) was reacted in a similar manner described above, and then Boc-Gly-NHBzl (**25**) (54.5 mg, 0.206 mmol) was added to the reaction mixture. After the mixture was treated in a similar manner described above, the quantity of **13** was calculated using **25** as internal standard.

Examination of Side Reaction of Boc–Ser(Fmoc–Gly)–OH (6) in DMF-d_7 by ^{1}H-NMR

To a solution of unit **6** (18.8 mg, 0.0388 mmol) in DMF-d_7 (156.0 µl) were added DIPCDI (6.0 µl, 0.0387 mmol) and HOBt (6.0 mg, 0.0392 mmol) at rt and the mixture was shaken for 2 h. A part (66.0 µl) of the mixture was diluted with DMF-d_7 (684.0 µl), filtered and analyzed by ^{1}H-NMR.

Comparison of Side Reaction of Boc–Ser(Fmoc–Ile)–OH (16), Boc–Thr(Fmoc–Gly)–OH (17) and Boc–Thr(Fmoc–Ile)–OH (18)

To a solution of unit **16**, **17** or **18** (5.4, 5.0 or 5.5 mg, respectively; 0.010 mmol) in DMF (40 µL) were added DIPCDI (3.1 µl, 0.020 mmol) and 0.5 mol/l HOBt in DMF (40 µl, 0.020 mmol) at rt and each mixture was shaken for 2 h. To each mixture was added benzylamine (2.0 µl, 0.020 mmol) and each mixture was shaken overnight at rt. A part (0.8 µl) of each mixture was diluted with DMF (99.2 µl) and analyzed by RP-HPLC.

Examination of Side Reaction of Boc–Ser(Fmoc–Gly)–OH (6) in $CDCl_3$ by ^{1}H-NMR

Unit **6** was treated in a manner similar to that described for the examination of side reaction of **6** in DMF-d_7 by ^{1}H-NMR, using $CDCl_3$ instead of DMF-d_7.

Investigation of Coupling Conditions in SPPS

To obtain protected [Met(O)^{35}]-Aβ27–42-resin **8**, protected 27-*N*-Fmoc-[Met(O)^{35}]-Aβ27–42-resin (2-chlorotrityl resin, 0.367

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mmol/g, 0.0734 mmol peptide) was synthesized using conventional SPPS, washed with MeOH (1.5 ml, ×5), dried for at least 2 h *in vacuo* (yield of the peptide-resin: 285.6 mg), divided into each 40 mg of the peptide-resin (0.367 mmol/g, 0.0103 mmol peptide), shaken in DMF for 1.5 h, and the Fmoc group was removed as reported [5–9]. Unit **6** (2.5 eq) was coupled with **8** in DMF or CH_2Cl_2 (500 µl) in the presence of DIPCDI (2.5 eq) and HOBt, HOAt, or HOOBt (2.5 eq, respectively) and shaken for 2 h at rt. Each peptide-resin was then washed with DMF (1.5 ml, ×5) and MeOH (1.5 ml, ×5), and dried for at least 2 h *in vacuo*. After TFA treatment, diethyl ether wash, water suspension and lyophilization as reported [5–9], each crude product was analyzed by RP-HPLC and MALDI-TOF MS.

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