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O-Acyl isopeptide method: development of an *O*-acyl isodipeptide unit for Boc SPPS and its application to the synthesis of Aβ1-42 isopeptide

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The *O*-acyl isopeptide method was developed for the efficient preparation of difficult sequence-containing peptide. Furthermore, development of the *O*-acyl isodipeptide unit for Fmoc chemistry simplified its synthetic procedure by solid-phase peptide synthesis. Here, we report a novel isodipeptide unit for Boc chemistry, and the unit was successfully applied to the synthesis of amyloid β peptide. Combination of Boc chemistry and the isodipeptide unit would be an effective method for the synthesis of many difficult peptides. Copyright © 2014 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Alzheimer's disease; amyloid β peptide; difficult sequence-containing peptide; *O*-acyl isopeptide; *O*-to-*N* intramolecular acyl migration; SPPS

Introduction

Solid-phase peptide synthesis (SPPS) [1] had been successfully applied to the synthesis of many peptides with many technological improvements; however, a certain kind of peptides is still difficult to prepare. Such peptides are called as difficult sequence-containing peptides, which are exemplified by Alzheimer's disease-related amyloid β peptide 1–42 (Aβ42) [2–4] or diabetes mellitus-related amylin (also known as islet amyloid polypeptide or diabetes-associated peptide) [5,6]. During the chain-assembly of these peptides by SPPS, undesired secondary structures such as β-sheets, which arise from the growing peptide chains on the solid support, lead to folding and aggregating. This aggregation is attributed to intramolecular/intermolecular hydrophobic interactions and/or hydrogen bonding between the resin-bound peptide chains. The aggregation often causes incomplete coupling and deprotection reactions. These aggregative natures of the peptides remain even after acidolysis to obtain a free peptide, hampering final high-performance liquid chromatography (HPLC) purifications [7]. To overcome these situations, the *O*-acyl isopeptide method has been proposed [8–11]. In this method, the target peptide is synthesized in a form of an *O*-acyl isopeptide, which contains an *O*-acyl isopeptide bond instead of the native *N*-acyl peptide bond at a hydroxyl group-containing amino acid residue, for example, Ser or Thr (Scheme 1A). Incorporation of the isopeptide structure drastically changed the secondary structure and the hydrophilicity to increase the efficacy of peptide preparation and isolation/purification. Such an *O*-acyl isopeptide is stable under acidic conditions or as a powder (e.g. a lyophilized trifluoroacetic acid (TFA) salt). The target peptide can be quantitatively obtained by a quick *O*-to-*N* intramolecular acyl migration reaction under neutral conditions. The *O*-acyl isopeptide method has further evolved as a general method in combination with automated Fmoc SPPS and the use of *O*-acyl isodipeptide units (Scheme 1B) [12–14]. Isodipeptide units have enabled routine application of the *O*-acyl isopeptide method by omitting the often difficult esterification reaction on a resin. So far, many difficult sequence-containing peptides could be efficiently synthesized by this method using Fmoc SPPS [15–27].

Toward further application of the *O*-acyl isopeptide method, here, we report for the first time the combination of Boc SPPS and the *O*-acyl isodipeptide unit. Boc chemistry was adopted because it has advantages over Fmoc chemistry in some cases, especially, in the difficult sequence-containing peptide [28]. Moreover, the ester bond within the isopeptide structure is thought to be much more stable under the conditions used for Boc chemistry than those for Fmoc chemistry. As a first example, we designed a novel *O*-acyl isodipeptide unit for Boc SPPS (Scheme 1C), and the unit was successfully applied to the synthesis of the *O*-acyl isopeptide of Aβ42 (isoAβ42) [10,11,29].

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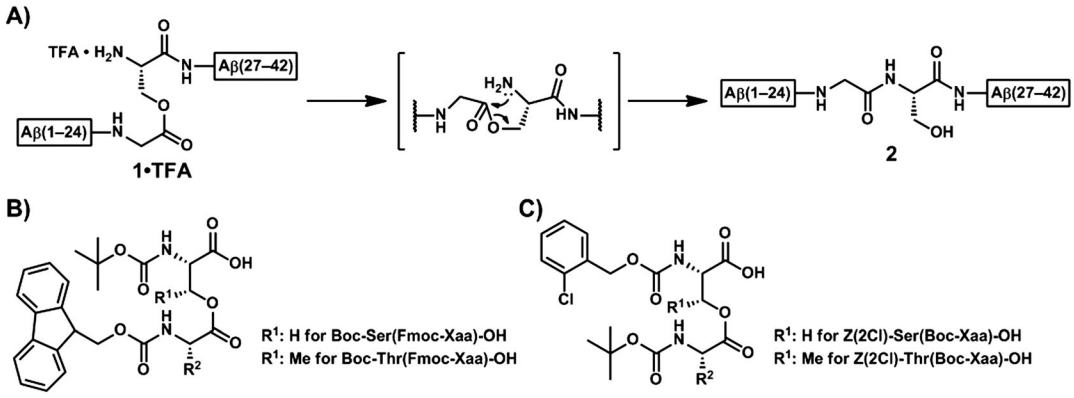

Scheme 1. (A) The *O*-acyl isopeptide method: isopeptide of amyloid β peptide 1–42 (isoAβ42, **1**) releases native Aβ42 via an *O*-to-*N* intramolecular acyl migration reaction under neutral conditions. (B) *O*-Acyl isodipeptide units allow facile application of the *O*-acyl isopeptide method to Fmoc SPPS. (C) General structure of novel-designed isodipeptide unit that could be applied to Boc SPPS.

Materials and Methods

General

All reagents and solvents were obtained from the Peptide Institute, Inc. (Osaka, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), Nacalai Tesque, Inc. (Kyoto, Japan) and Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). Analytical HPLC was performed on a Shimadzu liquid chromatograph LC-6A system (Shimadzu Corporation, Kyoto, Japan) with a ZORBAX ODS column (Agilent Technologies, Inc., Santa Clara, CA, USA) with the following solvent systems: a linear gradient of CH_3CN in H_2O containing 0.1% TFA. Preparative HPLC was performed on a Shimadzu liquid chromatograph LC-8A system (Shimadzu Corporation, Kyoto, Japan) with a DAISOPAK ODS column (DAISO CO., LTD., Osaka, Japan) with the following solvent systems: a linear gradient of CH_3CN in H_2O containing 0.1% TFA. Purity is based on the area percent of the peaks detected at 220 nm. Molecular weights were measured with an electrospray ionization mass spectrometry (ESI MS) using HP1100 LC/MSD (Agilent Technologies, Inc., Santa Clara, CA, USA). ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a JEOL-ECX400 spectrometer (JEOL Ltd., Tokyo, Japan). CAS number of isoAβ42 (**1**) is 753459-14-2, and the following CAS numbers were redundantly assigned: 795302-29-3, 867045-01-0, 867045-10-1, 874200-16-5, 874200-17-6, 934698-64-3, 938186-32-4, 1011252-70-2, 1011252-81-5, 1011253-05-6, 1011253-15-8, 1174420-55-3, 1174420-72-4, 1174420-91-7, 1174421-06-7, 1174421-20-5, 1174422-08-2, 1185727-21-2, 1218906-13-8, 1242162-39-5, 1355481-91-2 and 1361468-33-8. IsoAβ42 (**1**) is commercially available, for example, from the Peptide Institute, Inc. (Osaka, Japan) by the name of 'amyloid β-protein (1–42, *O*-acyl isopeptide)'.

Z(2Cl)-Ser-OPac

Z(2Cl)-ONSu (59.4 g, 209 mmol, 1.1 eq) was added into a stirred suspension of H-Ser-OH (20.0 g, 190 mmol, 1.0 eq) and Et_3N (39.9 ml, 285 mmol, 1.5 eq) in *N,N*-dimethylformamide (DMF)-H_2O (3 : 1, 400 ml), and the mixture was stirred overnight at room temperature. The solution was concentrated *in vacuo*, and the residue was diluted with EtOAc and sat. $NaHCO_3$ aq. The organic layer was removed, and the aqueous layer was acidified with 6 N HCl aq. and saturated with NaCl. Then, EtOAc was added, and the organic layer was washed with brine and dried over $MgSO_4$. After concentration *in vacuo*, Z(2Cl)-Ser-OH was precipitated with hexane and used without further purification (52.8 g, quant.).

Phenacyl bromide (37.8 g, 190 mmol, 1.0 eq) was added into a stirred solution of Z(2Cl)-Ser-OH (52.8 g, 190 mmol) and Et_3N (29.3 ml, 209 mmol, 1.1 eq) in DMF (400 ml), and the mixture was stirred overnight. After precipitation with 0.1 N HCl aq., the precipitate was isolated and dissolved in $CHCl_3$. The solution was washed with H_2O and concentrated *in vacuo*. Crystallization with Et_2O afforded the title compound as a white powder (61.2 g, 156 mmol, 82%). HPLC analysis at 220 nm: purity was higher than 99%; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.99–7.90 (m, 2H), 7.78 (d, J = 8.4 Hz, 1H), 7.72–7.63 (m, 1H), 7.60–7.42 (m, 4H), 7.40–7.30 (m, 2H), 5.57 (d, J = 16.8 Hz, 1H), 5.50 (d, J = 17.2 Hz, 1H), 5.12 (s, 2H), 5.04–4.97 (m, 1H), 4.37–4.28 (m, 1H), 3.88–3.65 (m, 2H); ESI MS: calcd $(M+H)^+$: 392.1, found: 392.1.

Z(2Cl)-Ser(Boc-Gly)-OH (5)

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC HCl, 7.38 g, 38.5 mmol, 1.1 eq) was added into a stirred solution of Boc-Gly-OH (6.74 g, 38.5 mmol, 1.1 eq), Z(2Cl)-Ser-OPac (13.7 g, 35.0 mmol, 1.0 eq) and 4-dimethylaminopyridine (DMAP, 855 mg, 7.0 mmol, 0.2 eq) in DMF (100 ml) on an ice bath, and the mixture was stirred for 4 h at room temperature. Next, EtOAc and H_2O were added. The organic layer was separated and washed successively with sat. $NaHCO_3$ aq., 1 N HCl aq. and brine. The organic solution was dried over $MgSO_4$ and concentrated *in vacuo*. After precipitation with hexane, Z(2Cl)-Ser(Boc-Gly)-OPac was obtained. Next, Z(2Cl)-Ser(Boc-Gly)-OPac was dissolved in AcOH (250 ml) on an oil bath at 40 °C. Zn powder (45.8 g, 700 mmol, 20 eq) was suspended, and the mixture was stirred vigorously for 1 h at 40 °C. The insoluble material was filtered off, and the solution was concentrated *in vacuo*. The residue was dissolved in EtOAc and washed successively with 0.5 N HCl aq. and H_2O. The organic layer was concentrated *in vacuo*, and the residue was solidified with hexane and diisopropyl ether (IPE). After recrystallization with EtOAc and IPE, the title compound was obtained as a white powder (12.6 g, 83%). HPLC analysis at 220 nm: purity was higher than 99%; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.81 (d, J = 8.0 Hz, 1H), 7.52–7.30 (m, 4H), 7.19 (t, J = 6.0 Hz, 1H), 5.12 (s, 2H), 4.42–4.28 (m, 2H), 4.24–4.15 (m, 1H), 3.70 (dd, J = 18, 6.0 Hz, 1H), 3.63 (dd, J = 18, 6.0 Hz, 1H), 1.42–1.26 (m, 9H); ESI MS: calcd $(M+Na)^+$: 453.1, found: 453.1.

Isopeptide of Aβ42 (isoAβ42, 1)

The isopeptide-resin was synthesized using an ABI 430A peptide synthesizer (Applied Biosystems, Inc., Foster City, CA, USA) on a

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Boc-Ala-phenylacetamidomethyl (Boc-Ala-PAM) resin (0.17 g, 0.12 mmol) by Boc chemistry. The functional groups of the side chains were protected as follows: Bzl for Thr and Ser, cHex for Asp and Glu, Z(2Br) for Tyr, Tos for Arg, Bom for His, and Z(2Cl) for Lys. Deprotection of N^{α}-Boc group was performed using 50% TFA/CH_2Cl_2. After neutralization with *N,N*-diisopropylethylamine, the Boc amino acid (8 eq) was coupled with *N,N'*-dicyclohexylcarbodiimide (DCC, 8 eq) and HOBt (8 eq) in *N*-methylpyrrolidone (1 h). Double coupling was applied if necessary. The acetyl capping was performed using Ac_2O-HOBt after each coupling step. After Aβ(27–42) was constructed on the resin, the isodipeptide unit **5** (2.7 eq) was coupled with *N,N'*-diisopropylcarbodiimide (DIPCDI, 2.7 eq) and HOBt (2.7 eq) at room temperature for 3.5 h in CH_2Cl_2. Subsequently, the automated Boc SPPS was carried out to obtain the protected isoAβ42-resin. The resin was then treated with HF (16 ml) containing *p*-cresol (4 ml), 2-mercaptopyridine (5 eq) and methoxyamine hydrochloride (6 eq) for 60 min at −5 °C [30]. After the mixture had been concentrated *in vacuo*, the title compound was precipitated with ether. The crude isopeptide was purified by HPLC (H_2O-MeCN system containing 0.1% TFA) to afford the title compound as a white amorphous powder of a TFA salt (36.2 mg, 6%). HPLC analysis at 220 nm: purity was 97%; ESI MS: calcd $(M + 3H)^{3+}$: 1505.4, found: 1505.4.

Results

Preparation of the *O*-acyl Isodipeptide Unit

As shown in Scheme 2, we synthesized isoAβ42 (**1**) by Boc SPPS using a novel isodipeptide unit Z(2Cl)-Ser(Boc-Gly)-OH (**5**). Isodipeptide unit **5** was synthesized from Z(2Cl)-Ser-OPac (**3**). First, **3** was coupled with Boc-Gly-OH by DIPCDI in the presence of DMAP to afford Z(2Cl)-Ser(Boc-Gly)-OPac (**4**). After deprotection of Pac of **4** by Zn, isodipeptide unit **5** was obtained in a high purity without need for column chromatography.

Synthesis of isoAβ42 Using the Isodipeptide Unit

Isodipeptide unit **5** was then subjected to Boc SPPS. Protected peptide-resin **7** was constructed on PAM resin by a standard DCC-HOBt method. Next, **5** was incorporated to **7** by the DIPCDI-HOBt method in CH_2Cl_2. Subsequent peptide chain elongation and final deprotection by HF afforded a crude isoAβ42 (**1**) of high purity (Figure 1A). Des-Ser peptide, which is derived from the known side reaction during dipeptide unit coupling (Scheme 3) [14], was not observed by HPLC and ESI MS analyses of the crude mixture. Additionally, ester hydrolysis products, such as Aβ(1–25), were not observed in these analyses. After HPLC purification, isoAβ42 (**1**) was readily obtained; thanks to its hydrophilic nature at the yield of 6% based on the resin-loaded Ala (Figure 1B).

Stability of Isopeptide Bond Under Acidic/Basic Conditions

We estimated the stability of isopeptide bond (ester bond) in acidic or basic conditions using *O*-acyl isodipeptide unit **5**. The stabilities of **5** in 50% TFA/$CHCl_3$ or 20% piperidine/DMF are monitored by reversed-phase-HPLC and summarized in Figure 2. The results were roughly quantified using an area percent of

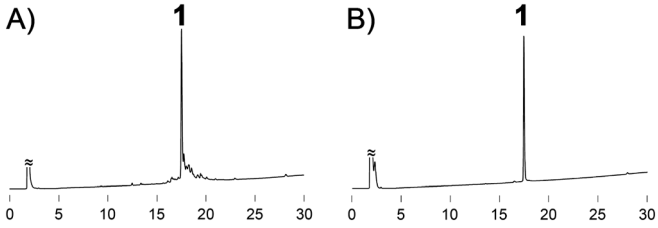
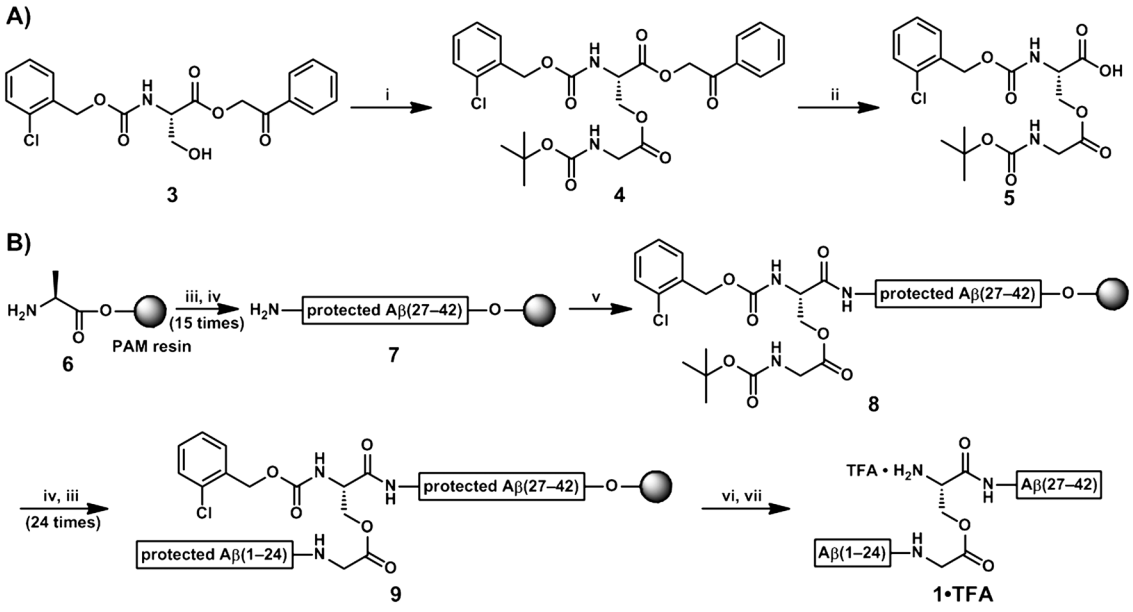
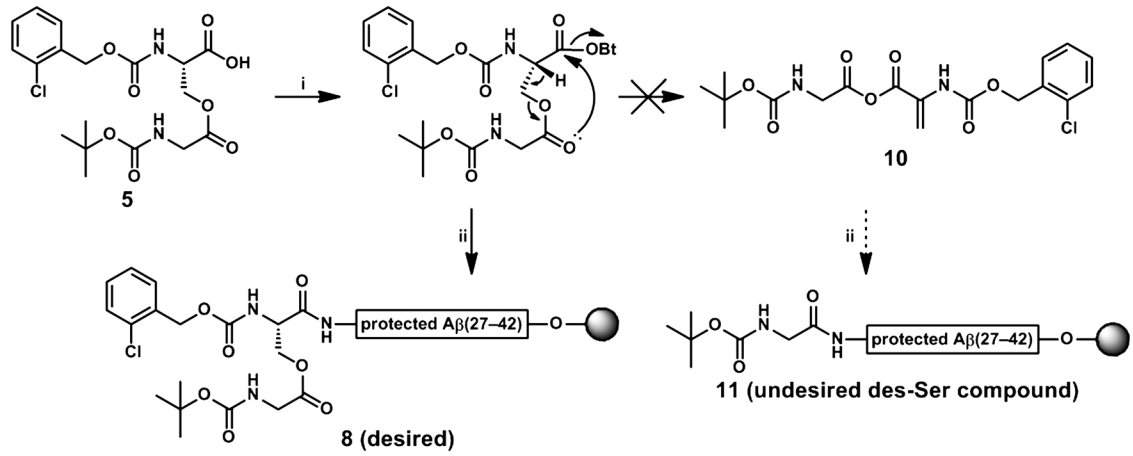


Figure 1. High-performance liquid chromatography (HPLC) profiles of (A) crude isoAβ42 (**1**) and (B) purified **1**. Analytical HPLC was performed with a binary solvent system: a linear gradient of CH_3CN (10–60% CH_3CN, 25 min) containing 0.1% TFA at a flow rate of 1 ml min^{-1} (50 °C) detected at 220 nm.



Scheme 2. Synthesis of *O*-acyl isodipeptide unit **5** and isoAβ42 (**1**). Reagents and conditions: (i) Boc-Gly-OH, EDC HCl, DMAP, DMF; (ii) Zn, AcOH; (iii) Boc-Xaa-OH, DCC, HOBt, NMP; (iv) 50% TFA in CH_2Cl_2; (v) **5**, DIPCDI, HOBt, CH_2Cl_2; (vi) HF, *p*-cresol; and (vii) HPLC purification.



Scheme 3. Reported side reaction of isodipeptide unit coupling affording des-Ser compound **11**. No such side reaction was observed in the synthesis of isoAβ42 (**1**). Reagents and conditions: (i) DIPCDI, HOBt and (ii) peptide-resin **7**.

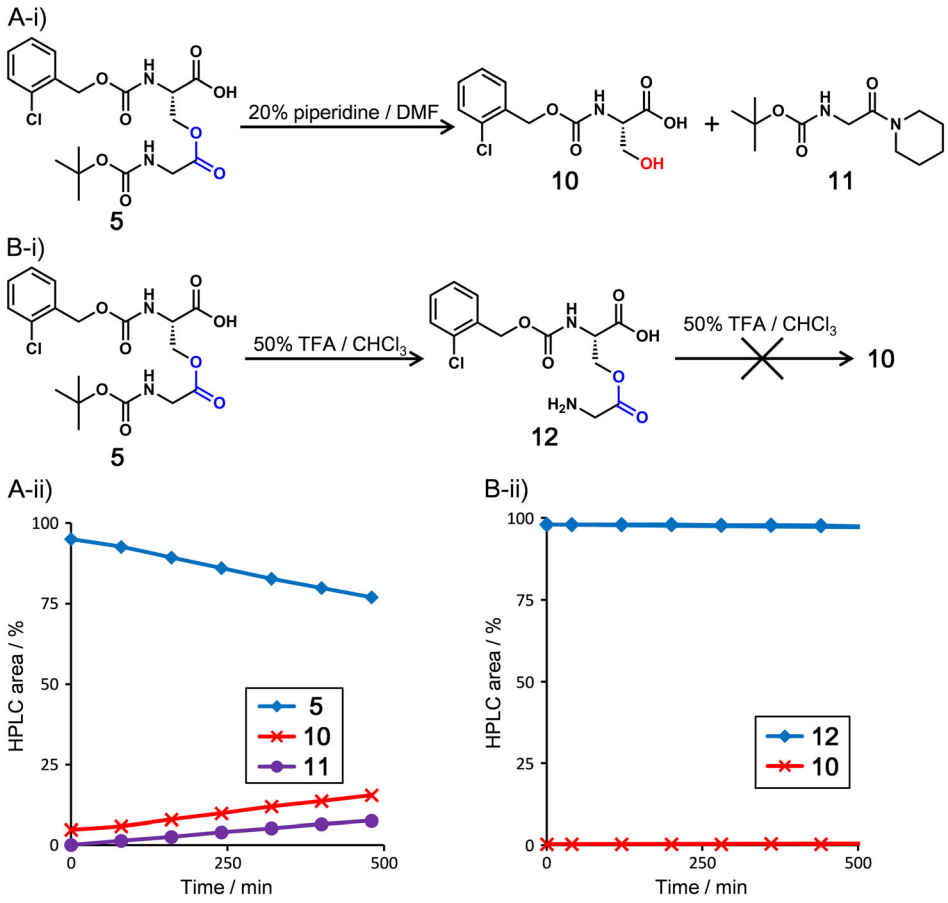


Figure 2. Stability of the isodipeptide unit **5** under basic/acidic conditions at room temperature: (A-i, A-ii) The isodipeptide unit **5** was decomposed to Z(2Cl)-Ser-OH (**10**) and Boc-Gly-piperidide (**11**) in 20% piperidine/DMF. (B-i, B-ii) By contrast, isodipeptide **12** derived from the isodipeptide unit **5** was stable in 50% TFA-$CHCl_3$.

absorbance of each compound at 220 nm. The isopeptide bond was stable in 50% TFA/$CHCl_3$ over 500 min at room temperature, while N^{α}-Boc group was deprotected to afford **12**. On the other hand, approximately 25% decomposition was observed over 500 min at room temperature in 20% piperidine/DMF to afford Z(2Cl)-Ser-OH (**10**) and Boc-Gly-piperidide (**11**). The other products were not detected.

Discussion

We synthesized isoAβ42 (**1**) by Boc SPPS using a novel isodipeptide unit Z(2Cl)-Ser(Boc-Gly)-OH (**5**) (Scheme 2). Use of the isodipeptide unit can allow omission of an esterification reaction on a solid support during the synthesis of *O*-acyl isopeptides. Such an on-resin esterification reaction is often hampered by the

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lower reactivity of a hydroxyl group on the peptide resin. Thus far, no isodipeptide unit for Boc SPPS had been reported, while we have developed 40 kinds of isodipeptide units composed from all naturally coded amino acids and Ser/Thr for Fmoc SPPS [13]. Boc SPPS is known to be more suitable for the synthesis of a difficult sequence-containing peptide than that of Fmoc SPPS [28]. Moreover, the ester bond within the isopeptide structure is recognized to be more stable during chain elongation performed by Boc chemistry compared with that by Fmoc chemistry, because no basic treatments are needed in Boc SPPS. Thus far, one paper has reported the Boc SPPS of isoAβ42 without use of the isodipeptide unit [31].

First, we confirmed the difference of applicability of isopeptide bond during Boc SPPS and Fmoc SPPS. One of advantages of Boc SPPS is the use of acidic conditions for the deprotection of the temporal N^{α}-amino protective groups. Thus, we simply confirmed the stability of *O*-acyl isodipeptide unit **5** in 50% TFA/$CHCl_3$ or 20% piperidine/DMF, which are typical conditions for the deprotection reactions in Boc or Fmoc chemistry, respectively. Isodipeptide unit **5** was efficiently obtained from Z(2Cl)-Ser-OPac (**3**) and Boc-Gly-OH at a high purity without the need for column chromatography. As shown in Figure 2, the ester bond within **5** was stable in 50% TFA/$CHCl_3$ over 500 min at room temperature. On the other hand, approximately 25% decomposition was monitored in 20% piperidine/DMF over 500 min at room temperature. These results implied the advantage of the use of Boc chemistry for the *O*-acyl isopeptide method. It should be noted that this may represent a susceptible example to piperidine as the compound **5** is the smallest isopeptide structure composed of Gly and Ser. Generally, the isopeptide can be fairly synthesized by Fmoc SPPS, although caution should be taken for Fmoc deprotection with a peptide containing the isopeptide structure by shortening the duration of piperidine treatment or using a less basic reagent than piperidine [32,33].

Then, we applied the *O*-acyl dipeptide unit **5** for the synthesis of isoAβ42 (**1**) to confirm the applicability of the isodipeptide units. When activating an isodipeptide unit for the coupling reaction, a side reaction resulting in deletion of Ser was reported in Fmoc SPPS (Scheme 3) [14]. To prevent this side reaction, the use of a less polar solvent (e.g. $CHCl_3$) has been recommended over a polar solvent (e.g. DMF). Thus, we adopted CH_2Cl_2 for the corresponding coupling reaction. As a result, such a side product was not detected by HPLC and ESI MS in the crude mixture. Additionally, no side products associated with decomposition of the ester bond were observed in the crude mixture.

Conclusions

We synthesized the isopeptide of Aβ42 'isoAβ42 (**1**)' by Boc SPPS with the novel isodipeptide unit. This isodipeptide unit could be readily prepared and applied to Boc SPPS without any difficulties. During the preparation of the isopeptide, side reactions associated with the *O*-acyl isopeptide method were not observed. Judging from these results, many difficult sequence-containing peptides could be synthesized by the *O*-acyl isopeptide method with the isodipeptide unit applicable for not only Fmoc chemistry but also Boc chemistry.

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