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Tetrahedron Letters
Tetrahedron Letters 47 (2006) 7905–7909

'*O*-Acyl isopeptide method': racemization-free segment condensation in solid phase peptide synthesis

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Received 8 August 2006; revised 31 August 2006; accepted 1 September 2006
Available online 25 September 2006

Abstract—We disclosed a novel 'racemization-free segment condensation' based on the '*O*-acyl isopeptide method' in which an N-segment including C-terminal *O*-acyl isopeptide structure with urethane-protected Ser/Thr residue was employed for the segment condensation, suggesting that the use of this method contributes to the effective convergent synthesis of long peptides/proteins.

Total chemical synthesis of peptides/proteins is of great significance to understand biological functions. Toward this purpose, many kinds of convergent synthetic methods have been reported.[1] However, a fundamental drawback of convergent synthesis is that racemization at the C-terminal residue of an N-segment occurs during the condensation reaction with the C-segment. In 'segment condensation',[1g–r] which is one of the important methods in convergent synthesis, a large amount of racemization is generally involved. Particularly, in solid phase segment condensation,[1k–r] the lower reactivity causes a higher extent of racemization as compared with solution phase synthesis. That is because, in contrast to urethane-protected amino acids, peptides easily form chirally labile oxazolones upon C-terminal carboxyl activation, limiting the N-segment to contain either a C-terminal Gly or Pro residue.[1j,o,r]

We have recently disclosed a novel '*O*-acyl isopeptide method'[2] in which a native amide bond at a hydroxy-amino acid residue, for example, Ser, was isomerized to an ester bond, followed by an O–N intramolecular acyl migration reaction (Fig. 1A). The method has been successfully applied to efficiently synthesize difficult sequence-containing peptides such as Alzheimer's disease-related amyloid β peptide (Aβ) 1–42.[2c–g,i] Our studies indicated that isomerization of the peptide backbone at only one position in the whole peptide sequence, that is, formation of a single ester bond, significantly changed the unfavorable secondary structure of the difficult sequence-containing peptide, leading to improved coupling and deprotection efficacy during SPPS. Mutter et al.,[3] Carpino et al.,[4] and Börner and co-workers[5] have also confirmed the efficacy of the '*O*-acyl isopeptide method'. Moreover, very recently, we designed a novel '*O*-acyl isodipeptide unit', that is, Boc-Ser/Thr(Fmoc-Xaa)-OH (Fig. 1B).[2h,i] The use of *O*-acyl isodipeptide units, in which the racemization-inducing esterification reaction on resin could be omitted, allows the '*O*-acyl isopeptide method' to fully automated protocols for the synthesis of peptides/proteins.

Herein, we disclosed a novel 'racemization-free segment condensation' based on the '*O*-acyl isopeptide method' (Fig. 2B). We conceived the idea that the N-segment, which possesses a C-terminal *O*-acyl isopeptide structure, could be coupled to the N-terminal amino group of a C-segment without any undesired racemization because the isopeptide structure includes a urethane-protected Ser/Thr residue. Thus, during the activation of the carboxyl group of the isopeptide, the formation of racemization-inducing oxazolones should be remarkably suppressed.

Keywords: *O*-Acyl isodipeptide unit; *O*-Acyl isopeptide method; O–N intramolecular acyl migration; Racemization-free; Segment condensation.
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doi:10.1016/j.tetlet.2006.09.001

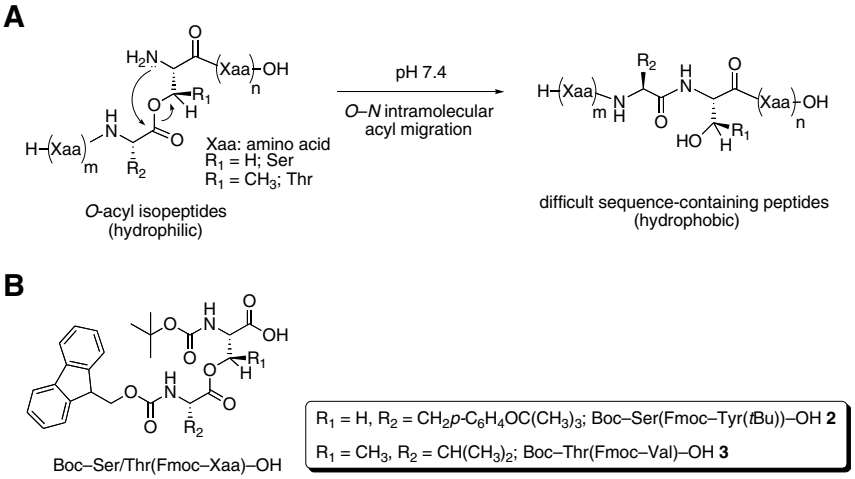


Figure 1. (A) '*O*-Acyl isopeptide method': the synthetic strategy for difficult sequence-containing peptides via the O–N intramolecular acyl migration reaction of *O*-acyl isopeptides; (B) '*O*-acyl isodipeptide units'.

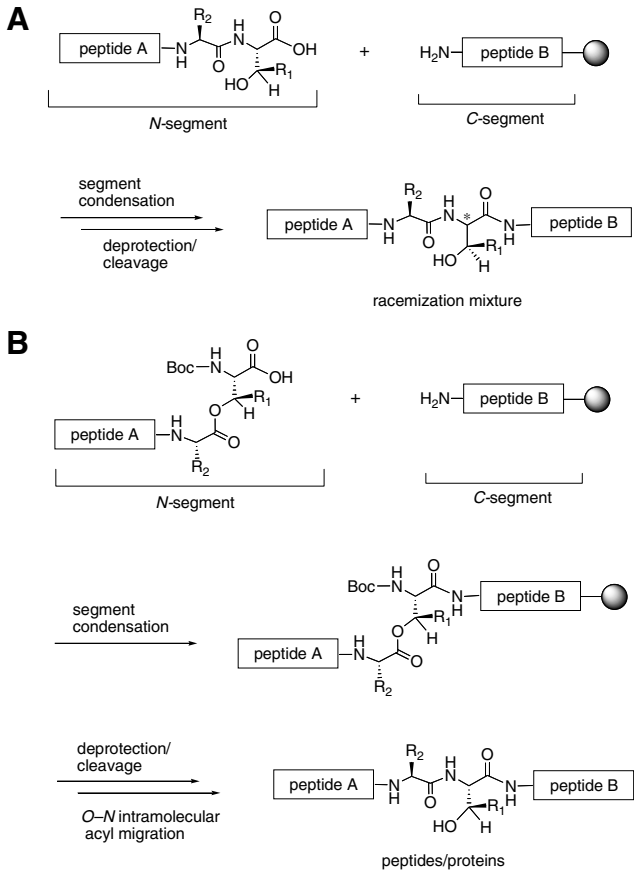


Figure 2. (A) A standard segment condensation; (B) a novel 'racemization-free segment condensation' based on the '*O*-acyl isopeptide method'.

To evaluate this hypothesis, we first selected Fmoc-Tyr-Ser-Phe-OH (**1**) as a model. As a comparative study, **1** was synthesized by the standard segment condensation method.[6] Fmoc-Tyr(*t*Bu)-Ser(*t*Bu)-OH was coupled to H-Phe-O-resin (2-chlorotrityl resin) using the DIPCDI(1,3-diisopropylcarbodiimide, 2.5 equiv)–HOBt(1-hydroxybenzotriazole, 2.5 equiv) method to obtain Fmoc-Tyr(*t*Bu)-Ser(*t*Bu)-Phe-O-resin. After the protected peptide resin was deprotected with TFA, the resulting crude **1** was analyzed by HPLC. As a result, 3.0% of Fmoc-Tyr-D-Ser-Phe-OH was detected in crude **1** (Fig. 3A), which was confirmed by an independent synthesis of the D-Ser derivative. This result indicated that racemization at the activated Ser residue occurred during segment condensation.

On the other hand, in segment condensation based on the '*O*-acyl isopeptide method',[7] *O*-acyl isodipeptide unit, Boc-Ser(Fmoc-Tyr(*t*Bu))-OH[8] (**2**, Fig. 1B) was coupled to H-Phe-O-resin using the DIPCDI(2.5 equiv)–HOBt(2.5 equiv) method to obtain Boc-Ser-(Fmoc-Tyr(*t*Bu))-Phe-O-resin. After deprotection with TFA, the obtained isopeptide H-Ser(Fmoc-Tyr)-Phe-OH·TFA was treated with phosphate buffer (pH 7.4) to induce an O–N intramolecular acyl migration to afford **1**. In HPLC analysis of crude **1**, no detectable racemized compound Fmoc-Tyr-D-Ser-Phe-OH was observed (Fig. 3B), indicating that the *O*-acyl isodipep-

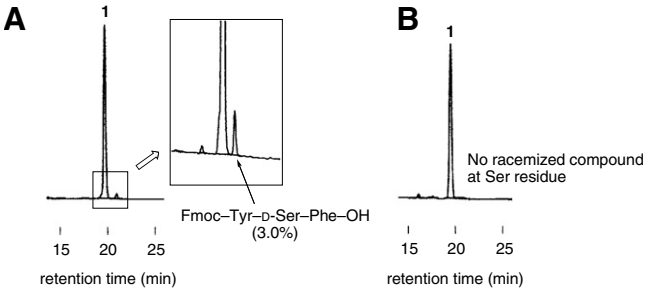


Figure 3. HPLC profiles of crude peptide Fmoc-Tyr-Ser-Phe-OH (**1**) synthesized using (A) the standard segment condensation and (B) '*O*-acyl isopeptide method'-based segment condensation. Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS AM302) with a binary solvent system: a linear gradient of CH_3CN (35–55% CH_3CN, 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1} (40 °C), detected at 230 nm.

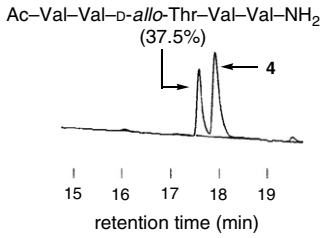


Figure 4. HPLC profile of crude **4** synthesized using a standard segment condensation. Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS AM302) with a binary solvent system: a linear gradient of CH_3CN (0–100% CH_3CN, 40 mm) in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1} (40 °C), detected at 230 nm.

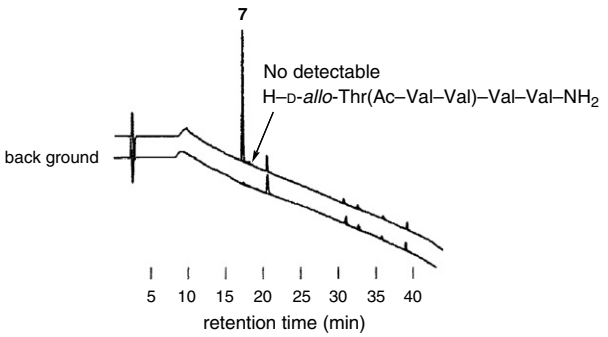
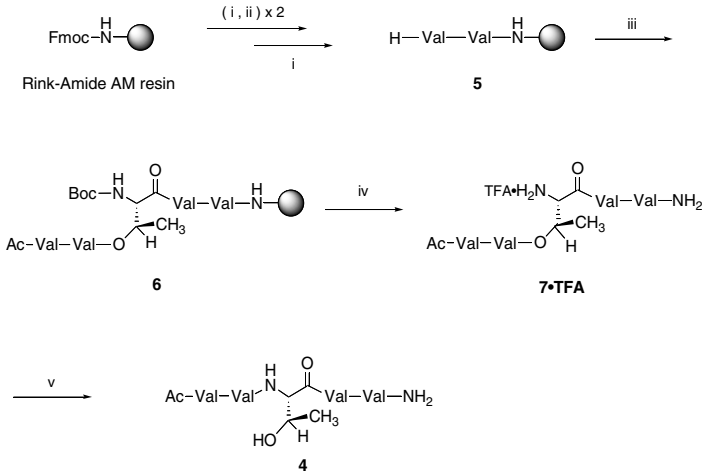


Figure 5. HPLC profile of crude isopeptide **7** (Rt = 17.0 min) synthesized using the '*O*-acyl isopeptide method'-based segment condensation. The retention time of H-D-*allo*-Thr(Ac-Val-Val)-Val-Val-NH_2, which was synthesized independently, was 17.8 min. Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS AM302) with a binary solvent system: a linear gradient of CH_3CN (0–100% CH_3CN, 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1} (40 °C), detected at 230 nm.

tide unit could be introduced to the amino group on the resin without any racemization at the activated Ser residue in the isopeptide structure.

To further elucidate the efficacy of this '*O*-acyl isopeptide method'-based segment condensation, pentapeptide Ac-Val-Val-Thr-Val-Val-NH_2[2h,i] (**4**) was adopted. In the condensation of Ac-Val-Val-Thr(*t*Bu)-OH with H-Val-Val-NH-resin (as a standard segment condensation),[9] a large amount of racemization (37.5%) at the activated Thr residue occurred during the DIPCDI–HOBt segment condensation (Fig. 4), which was confirmed by an independent synthesis of Ac-Val-Val-D-*allo*-Thr-Val-Val-NH_2. In contrast, in the '*O*-acyl isopeptide method'-based segment condensation (Scheme 1),[10] N-segment Boc-Thr(Ac-Val-Val)-OH,[11] which was synthesized using *O*-acyl isodipeptide unit Boc-Thr(Fmoc-Val)-OH **3** (Fig. 1B),[2h] was coupled to C-segment H-Val-Val-NH-resin (**5**) to obtain isopeptide resin **6**. The DIPCDI(2.5 equiv)–HOBt(2.5 equiv) method in DMF (2 h) was employed for segment condensation, in which N-segment Boc-Thr(Ac-Val-Val)-OH was readily solubilized. The completeness of the coupling was verified by the Keiser test. After TFA treatment, *O*-acyl isopeptide **7**·TFA was obtained with an isolated yield of 69%. As shown in Figure 5, HPLC analysis of crude **7** exhibited a high purity of the desired product without any byproduct derived from racemization at Thr, which was confirmed by an independent synthesis of H-D-*allo*-Thr(Ac-Val-Val)-Val-Val-NH_2. Moreover, the use of an N-segment with a C-terminal isopeptide did not lead to any additional side reaction. Isopeptide **7** was converted to **4** in phosphate buffered saline at pH 7.4.[2h] These results reveal that a protected *O*-acyl isopeptide with a C-terminal Boc-Thr residue could be introduced to the peptide resin without any racemization at the activated Thr residue, in contrast to the standard method using Ac-Val-Val-Thr(*t*Bu)-OH that involved a significant amount of racemization during condensation to the solid support.

In summary, we herein developed a novel 'racemization-free segment condensation' based on the '*O*-acyl isopeptide method' with the successful synthesis of small peptides. This method allows the use of an N-segment



Scheme 1. Reagents and conditions: (i) 20% piperidine/DMF, 20 min; (ii) Fmoc-Val-OH (2.5 equiv), DIPCDI (1,3-diisopropylcarbodiimide, 2.5 equiv), HOBt (2.5 equiv), DMF, 2 h; (iii) Boc-Thr(Ac-Val-Val)-OH (2.5 equiv), DIPCDI (2.5 equiv), HOBt (2.5 equiv), DMF, 2 h; (iv) TFA–*m*-cresol–thioanisole–H_2O (92.5:2.5:2.5:2.5), 90 min; (v) phosphate buffered saline, pH 7.4, 25 °C.

possessing a C-terminal Ser/Thr residue for segment condensation, without any racemization, as a result of the C-terminal *O*-acyl isopeptide structure with a urethane-protected Ser/Thr residue. Thus, in the synthesis of long peptides/proteins, racemization-free segment condensation becomes possible at not only the C-terminal Gly/Pro but also Ser/Thr residues of the N-segment. Additionally, final deprotected peptides/proteins synthesized using the '*O*-acyl isopeptide method'-based segment condensation are effectively purified by HPLC, because a simple isomerization to an *O*-acyl isopeptide remarkably and temporarily changes the physicochemical properties of the native peptide, and an O–N intramolecular acyl migration triggers the native amide bond formation under physiological conditions.[2] Examples of such studies include membrane peptides/proteins that are difficult to handle in various conditions because of their high self-assembling characters.

Acknowledgments

This research was supported in part by the 'Academic Frontier' Project for Private Universities: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology) from the Japanese Government, and The 21st Century COE Program from MEXT. Y.S. is grateful for the Research Fellowships of JSPS for Young Scientists. We are grateful to Ms. K. Oda for mass spectra measurements. We thank Dr. J.-T. Nguyen and Dr. Y. Hamada for his help in preparing the manuscript.

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6. Protected peptide Fmoc-Tyr(*t*Bu)-Ser(*t*Bu)-OH (2.5 equiv) was coupled to H-Phe-O-resin (2-chlorotrityl resin, 0.055 mmol) in the presence of DIPCDI (2.5 equiv) and HOBt (2.5 equiv) in DMF for 2 h. The peptide was cleaved from the resin using TFA–thioanisole–*m*-cresol–H_2O (92.5:2.5:2.5:2.5) for 90 min at rt, concentrated in vacuo, washed with Et_2O, centrifuged, suspended with water, and lyophilized to give the crude Fmoc-Tyr-Ser-Phe-OH **1**. ESI-MS: calcd for $(M+Na)^+$: 660.2, found: 660.0. The retention time on HPLC (0–100% CH_3CN for 40 min, 230 nm) of synthesized product was identical to that of **1** which was synthesized independently by the standard Fmoc-based SPPS.
7. *O*-Acyl isodipeptide unit, Boc-Ser(Fmoc-Tyr(*t*Bu))-OH[8] **2** (2.5 equiv) was coupled to H-Phe-O-resin (2-chlorotrityl resin, 0.058 mmol) in the presence of DIPCDI (2.5 equiv) and HOBt (2.5 equiv) in DMF for 2 h. The crude H-Ser(Fmoc-Tyr)-Phe-OH was obtained in a same deprotection manner described in Ref. 6. The isopeptide was dissolved in phosphate buffer and stirred for 6 h at rt to give the crude **1**. ESI-MS: calcd for $(M+H)^+$: 638.2, found: 638.0. The retention time on HPLC (0–100% CH_3CN for 40 min, 230 nm) of synthesized product was identical to that of **1** which was synthesized independently by the standard Fmoc-based SPPS.
8. EDC·HCl (623 mg, 3.25 mmol) was added to a stirring solution of Boc-Ser-OBzl[12] (400 mg, 1.35 mmol), Fmoc-Tyr(*t*Bu)-OH (1.5 g, 3.25 mmol), and DMAP (16.6 mg, 0.136 mmol) in dry $CHCl_3$ (40 mL) at 0 °C. The mixture was slowly warmed to rt over 2 h, stirred additionally for

16 h, diluted with AcOEt, and washed successively with water, 1 M HCl, water, a saturated $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and the solvent was removed in vacuo. The resulting oil was purified by silica gel column chromatography (AcOEt–hexane 1:3.5) to yield Boc-Ser(Fmoc-Tyr(*t*Bu))-OBzl (889 mg, 1.23 mmol, 91%). After that, Pd/C was added (87 mg) to the stirring solution of the Boc-Ser(Fmoc-Tyr(*t*Bu))-OBzl (856 mg, 1.18 mmol) in AcOEt (36 mL), and the reaction mixture was vigorously stirred under a hydrogen atmosphere for 16 h. The catalyst was filtered off through Celite. The solvent was removed in vacuo and the crude product was filtered via silica gel, at first with AcOEt–hexane 1:2.5 and then the final product was washed out by methanol to give pure **2** (708 mg, 1.09 mmol, 93%). HRMS (FAB): calcd for $C_{36}H_{42}N_2O_9Na$ $(M+Na)^+$: 669.2788, found: 669.2783; HPLC analysis at 230 nm: purity was higher than 95%; NMR (CD_3OD, 300 MHz): δ 7.77 (d, $J = 7.4$ Hz, 2H), 7.63–7.60 (m, 2H), 7.38 (t, $J = 7.1$ Hz, 2H), 7.32–7.27 (m, 2H), 7.13 (d, $J = 8.3$ Hz, 2H), 6.85 (d, $J = 8.4$ Hz, 2H), 4.84–4.09 (m, 7H), 3.25–3.15 (m, 1H), 2.91–2.83 (m, 1H), 1.41 (s, 9H), 1.22 (s, 9H).

9. The protected peptide Ac-Val-Val-Thr(*t*Bu)-OH (2.5 equiv) was coupled to H-Val-Val-NH-resin (Rink-amide AM resin, 0.065 mmol) in the presence of DIPCDI (2.5 equiv) and HOBt (2.5 equiv) in DMF for 2 h. Crude **4** was obtained in a similar deprotection manner described in Ref. 6. ESI-MS: calcd for $(M+Na)^+$: 579.4, found: 579.3. The retention time on HPLC (0–100% CH_3CN for 40 min, 230 nm) of the synthesized product was identical to that of **4** which was synthesized previously.[2h]
10. After the preparation of the H-Val-Val-NH-resin (Rink-amide AM resin, 0.009 mmol), Boc-Thr(Ac-Val-Val)-OH[11] (2.5 equiv) was coupled in the presence of DIPCDI (2.5 equiv) and HOBt (2.5 equiv) in DMF for 2 h at rt. The crude *O*-acyl isopeptide **7**·TFA was obtained in a similar deprotection manner described in Ref. 6. Yield: 69% (calculated from the original loading of Rink-amide AM resin). HRMS (FAB): calcd for $C_{26}H_{45}N_6O_7$ $(M+H)^+$: 557.3663, found: 557.3656; HPLC analysis at 230 nm: purity was higher than 95%. The retention time on HPLC (0–100% CH_3CN for 40 min, 230 nm) of the synthesized product was identical to that of **7** which was synthesized previously.[2h]
11. After *O*-acyl isodipeptide unit, Boc-Thr(Fmoc-Val)-OH[2h] **3** was loaded to 2-chlorotrityl resin, subsequent coupling with Fmoc-Val-OH, N-acetylation using Ac_2O, 0.1% TFA treatment, and HPLC purification gave pure Boc-Thr(Ac-Val-Val)-OH. ESI-MS: calcd for $(M+Na)^+$: 482.3, found: 482.1.
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