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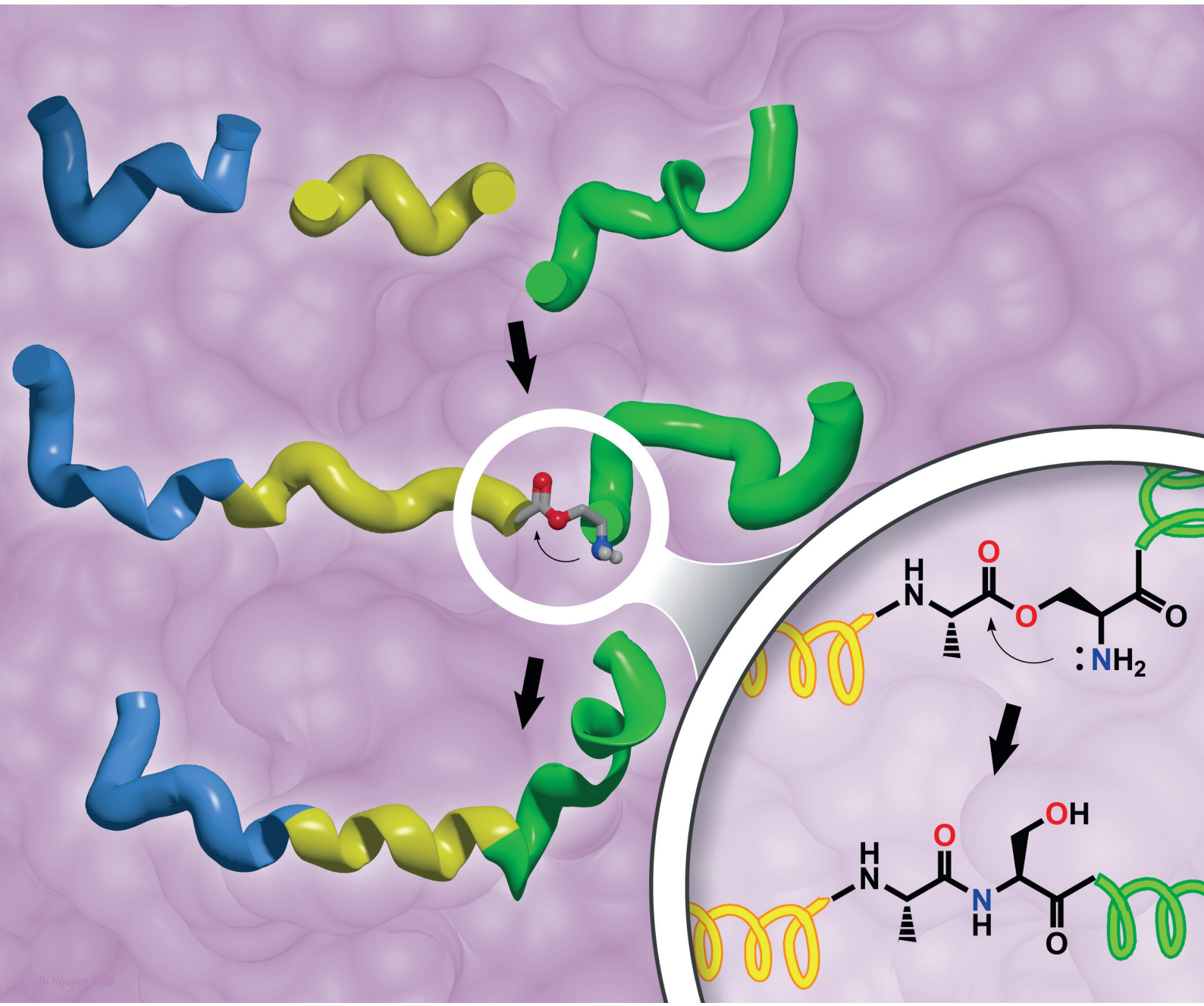

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O-Acyl isopeptide method: efficient synthesis of isopeptide segment and application to racemization-free segment condensation†

Taku Yoshiya, Hiroyuki Kawashima, Youhei Sohma, Tooru Kimura and Yoshiaki Kiso*

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We report the establishment of the *O*-acyl isopeptide method-based racemization-free segment condensation reaction toward future chemical protein synthesis. Peptide segments containing C-terminal *O*-acyl Ser/Thr residues were successfully synthesized by use of a lower nucleophilic base cocktail for Fmoc removal, and then coupled to an amino group of a peptide-resin without side reactions or epimerization. We also succeeded in performing the segment condensation in a sequential manner and in solution phase conditions as well.

Introduction

Peptides and proteins synthesized by chemical means have been widely used in biological studies. Moreover, chemical synthesis of peptides and proteins allows us to incorporate non-native structures into molecules, thereby enlarging our understanding of peptide and protein functions. Stepwise solid phase peptide synthesis is commonly adopted to synthesize ~50 amino acids long with satisfactory outcomes. However, synthesis of longer peptides and proteins using the stepwise method is generally difficult due to a lower reactivity of the constructed peptide-resin. To synthesize longer peptides and proteins, many kinds of convergent synthetic methods have been proposed.[1–3] Among them, "segment condensation",[1,2] in which a side-chain protected peptide carboxylate is coupled with an amino group of another peptide segment to construct a longer peptide, has attracted attention as an important method. However, a fundamental drawback of the segment condensation is that epimerization at the C-terminal residue of an *N*-segment occurs during the condensation reaction with a *C*-segment (Fig. 1A), limiting the *N*-segment to contain either a C-terminal Gly or Pro.[2] This epimerization occurs because, in contrast to urethane-protected amino acids, peptides easily form chirally labile oxazolones upon C-terminal carboxyl activation.

We developed an "*O*-acyl isopeptide method"[4] for the synthesis of peptides containing a difficult sequence. The presence of an *O*-acyl instead of a native *N*-acyl residue at a hydroxyamino acid residue (*e.g.* Ser, Thr) in the peptide backbone drastically changed the secondary structure of the native peptide, and the

Department of Medicinal Chemistry, Division of Medicinal Chemical Sciences, 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; Fax: +81 75 591 9900; Tel: +81 75 595 4635

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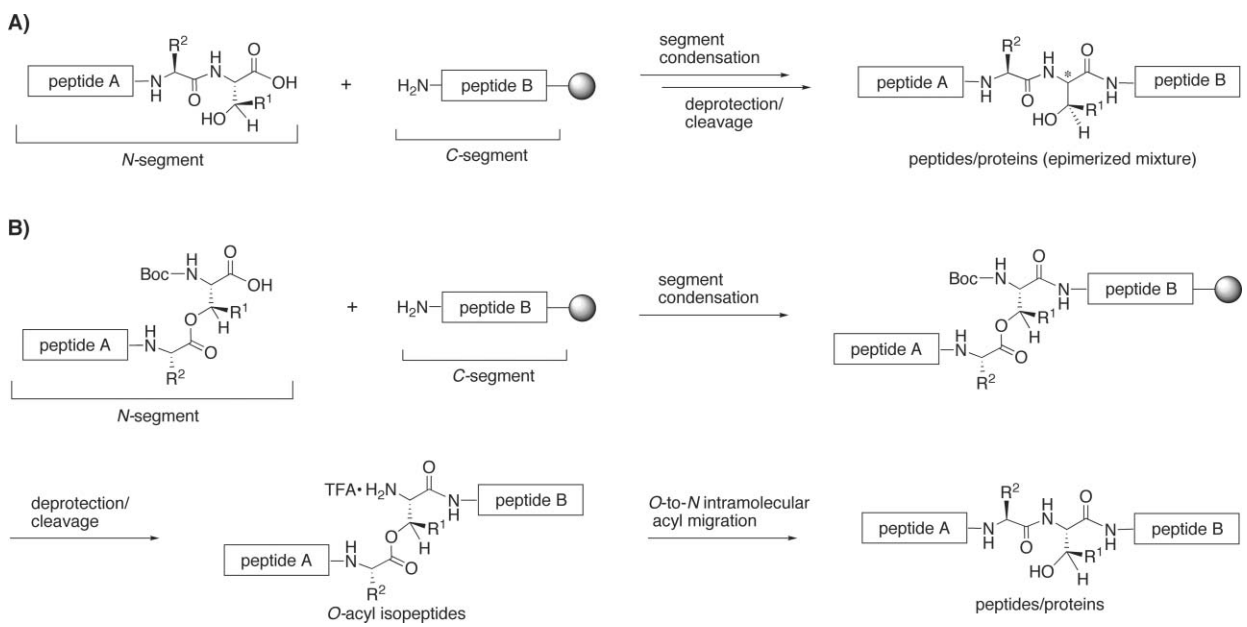


Fig. 1 (A) Conventional segment condensation, (B) *O*-acyl isopeptide method-based segment condensation.

target peptide was generated *via* an *O*-to-*N* intramolecular acyl migration. An application of the method to Alzheimer's disease-related Aβ1–42 revealed that the *O*-acyl isopeptide of Aβ1–42 could be effectively synthesized and stored without spontaneous self-assembly.[5] The intact monomer of Aβ1–42 could then be obtained from the isopeptide under physiological experimental conditions. The *O*-acyl isopeptide method has further evolved as a general method for peptide synthesis with the development of "*O*-acyl isodipeptide units" (Fig. 2).[6] Isodipeptide units have enabled routine use of the *O*-acyl isopeptide method by avoiding the often difficult esterification reaction on resin. So far, Mutter *et al.*,[7] Bienert *et al.*,[8] the Merck KGaA group,[9] Börner *et al.*,[10] Otaka *et al.*,[11] Martinez *et al.*,[12] Brik *et al.*[13] and Muir *et al.*[14] have also reported using the *O*-acyl isopeptide method. We also expanded the concept to the "*S*-acyl isopeptide method".[15]

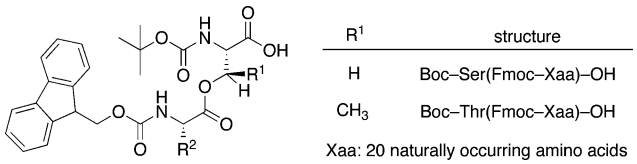


Fig. 2 *O*-Acyl isodipeptide unit.

Under these contexts, we have developed the "racemization-free segment condensation"[16] based on the *O*-acyl isopeptide method (Fig. 1B). The idea was that an *N*-segment with a C-terminal isopeptide structure (*O*-acyl Ser/Thr residue) can be coupled to an N-terminal amino group of the *C*-segment without any epimerization, because the amino group of C-terminal isopeptide part is protected by a urethane-type protective group. Activation of the carboxyl group of the isopeptide was thus expected to suppress the formation of racemization-inducing oxazolone. We have succeeded synthesizing model pentapeptide Ac–Val–Val–Thr–Val–Val–NH_2 to prove the concept.[16] An *N*-segment, Boc–Thr(Ac–Val–Val)–OH, was successfully coupled to a H–Val–Val–NH-resin without epimerization. This result suggested that, toward the synthesis of longer peptides or proteins, effective segment condensation would be possible for C-terminal Ser/Thr residues in addition to Gly/Pro. After the disclosure, Bienert *et al.*[17] and the Merck KGaA group[18] also reported the efficacy of this convergent methodology. Very recently, Martinez *et al.* applied this method for the preparation of a cyclic peptide.[12]

Toward future protein synthesis, we herein established the *O*-acyl isopeptide method-based racemization-free segment condensation method by synthesizing three bioactive peptides (influenza A virus matrix M1 58–66, humanin and orexin-B). The key segments containing C-terminal *O*-acyl Ser/Thr residues were successfully synthesized by use of a lower nucleophilic base cocktail for the removal of the Fmoc group. The middle-sized peptide segments were then coupled to the amino group of the peptide-resin quantitatively under optimized conditions with no epimerization due to the urethane-protected *O*-acyl isopeptide structure. In addition, we successfully performed the segment condensation in a sequential manner, and also in solution phase conditions. Finally, the synthesized isopeptides released their native peptides rapidly (half time: <30 s ~ 10 min) and quantitatively *via* *O*-to-*N* intramolecular acyl migration. These observations would be useful information for chemical protein synthesis based on the segment condensation method.

Results

Synthesis of influenza A virus matrix M1 58–66

Influenza A virus matrix M1 58–66[19] (H–GILGFVFTL–OH, **1**) was synthesized *via* segment condensation with *O*-acyl isopeptide segment **4** (Scheme 1). Segment **4** was constructed by Fmoc-based SPPS on 2-chlorotrityl resin (Scheme 1A). *O*-Acyl isodipeptide unit, Boc–Thr(Fmoc–Phe)–OH[6b] was loaded onto the resin in the presence of diisopropylethylamine (DIPEA) in 1,2-dichloroethane to give **2**. The fully protected isopeptide segment **4** was obtained after couplings of Fmoc-amino acids and base treatments, followed by cleavage from the resin by treating with 20% hexafluoroisopropanol (HFIP) in dichloromethane (DCM). However, base treatments with 20% piperidine in DMF for Fmoc-removal caused severe losses of peptides during SPPS, resulting in low isolated yield (<3%) of **4**. This is probably due to ester decomposition by the base and/or diketopiperazine formation when the Fmoc group of the amino acid next to the isodipeptide unit was removed. Therefore, we adopted 1-methylpyrrolidine (25 v/v%)–hexamethyleneimine (2 v/v%)–HOBt (3 w/v%) in NMP–DMSO (1 : 1) (Reagent A)[20] as a lower nucleophilic base developed by Aimoto *et al.* for an effective preparation of peptide-thioesters using Fmoc-SPPS. HPLC analysis of crude **4**, which was prepared using Reagent A for Fmoc removal, showed that any significant side product was not detected, suggesting that the ester moiety was sufficiently stable under Reagent A treatment (Fig. 3). HPLC purification gave pure **4** with moderate yield (60%, Fig. S1A).

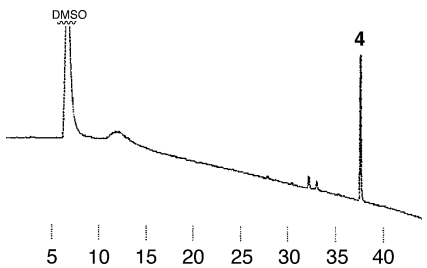
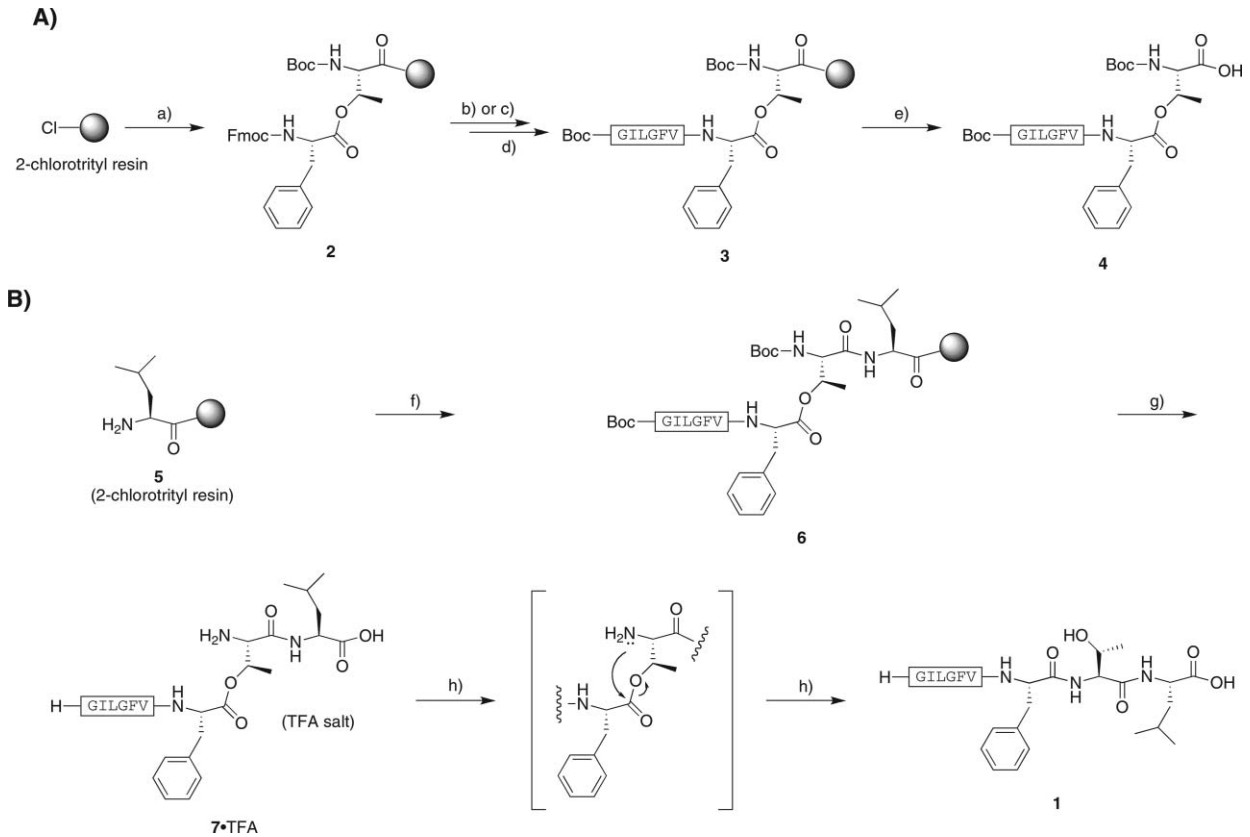


Fig. 3 HPLC profile of crude **4** synthesized using Reagent A. Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS AM302) with binary solvent system: a linear gradient of CH_3CN (0–100% CH_3CN, 40 min) in 0.1% aqueous TFA with a flow rate of 0.9 mL min^{-1} (40 °C), detected at 230 nm.

As shown in Scheme 1B, **4** was coupled to H-Leu-resin (**5**) using the standard 1,3-diisopropylcarbodiimide (DIPCDI)–1-hydroxybenzotriazole (HOBt) method in DMF to give **6**. Crude isopeptide **7** was successfully obtained with high purity (90%, Fig. 4A) after TFA treatment of **6**. In crude **7**, D-*allo*-Thr derivative was not detected (<2.0%), which was confirmed by a comparison with an authentic sample using the analytical HPLC, indicating that epimerization at the activated Thr residue did not occur during segment condensation. In addition, des-Thr derivative (H–GILGFVFL–OH) was not detected in crude **7**, as also confirmed by a comparison with an authentic sample. This



Scheme 1 *Reagents and conditions*: a) Boc–Thr(Fmoc–Phe)–OH, diisopropylethylamine (DIPEA), 1,2-dichloroethane, 2.5 h; b) 20% piperidine in DMF, 20 min; c) 1-methylpyrrolidine (25 v/v%), hexamethyleneimine (2 v/v%), HOBt (3 w/v%), NMP–DMSO (1 : 1) (Reagent A),[20] 20 min; d) Fmoc–AA–OH (2.5 eq) or Boc–Gly–OH (2.5 eq), 1,3-diisopropylcarbodiimide (DIPCDI, 2.5 eq), HOBt (2.5 eq), DMF, 2 h; e) 20% hexafluoroisopropanol (HFIP) in dichloromethane (DCM), 45 min; f) **4** (2.5 eq), DIPCDI (2.5 eq), HOBt (2.5 eq), DMF, 2 h; g) TFA (92.5 v/v%), *m*-cresol (2.5 v/v%), thioanisole (2.5 v/v%), water (2.5 v/v%), 90 min; h) pH 7.4 phosphate buffer, 6 h, room temperature.

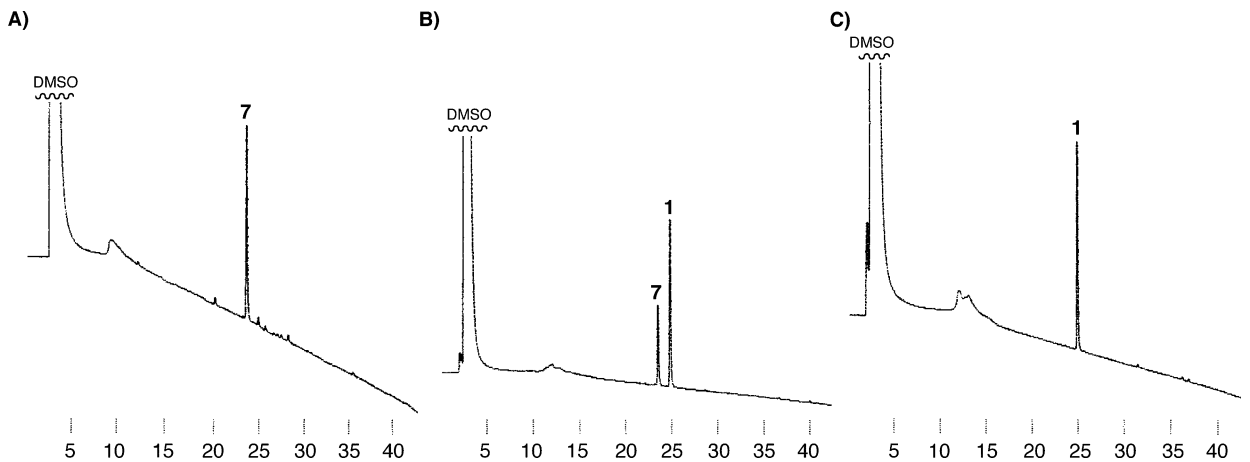
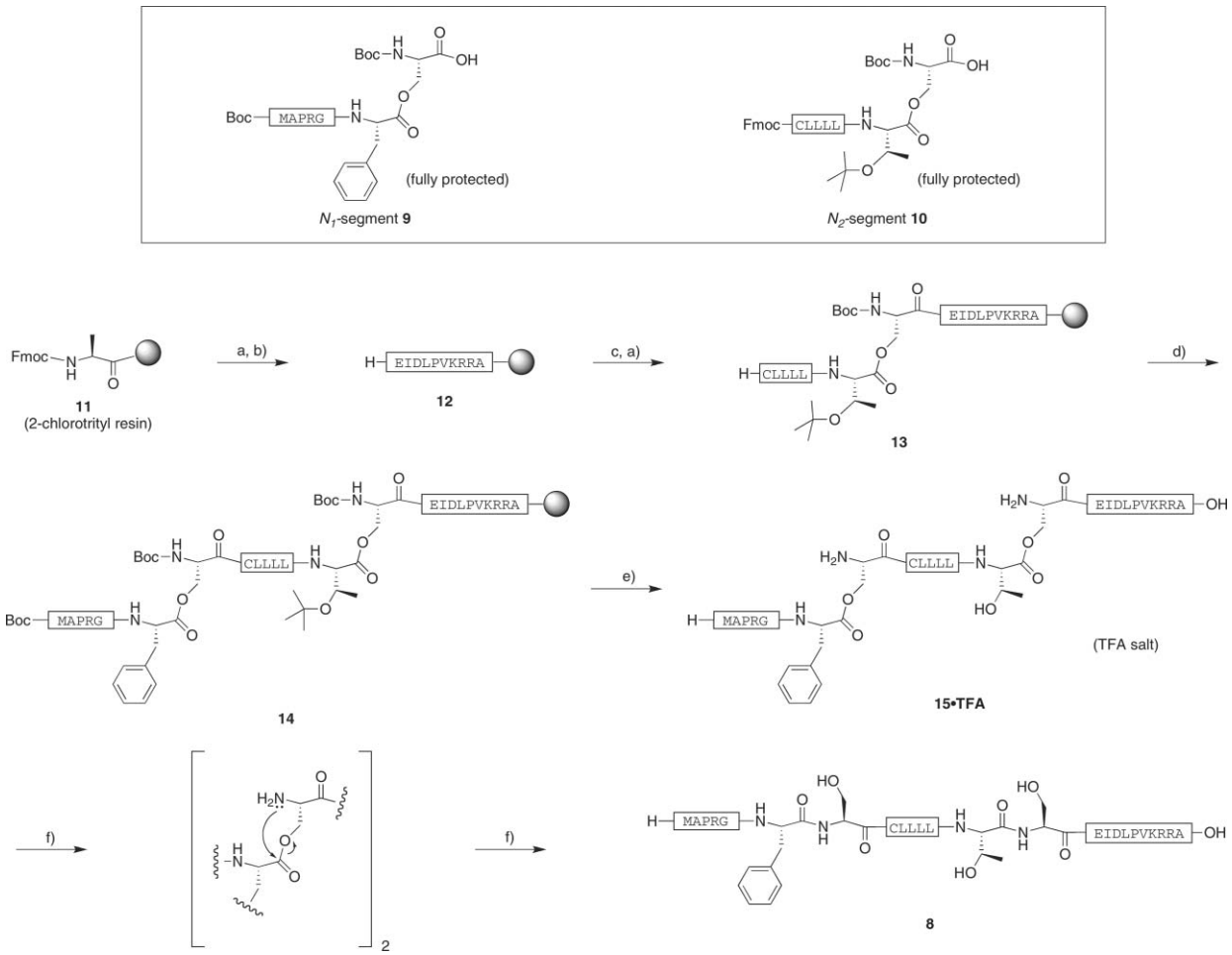


Fig. 4 (A) HPLC profile of crude **7**. (B, C) *O*-to-*N* Intramolecular acyl migration reaction released native peptide **1** from pure **7** (B: time = 30 s, C: time = 30 min). HPLC conditions were similar to those described in Fig. 3.

result indicates that a possible side reaction during condensation (Scheme S1), in which the HOBt ester of **4** reacts intramolecularly to form a mixed anhydride of Boc–GILGFVF–OH and Boc–βMeΔAla–OH (eventually H–GILGFVFL–OH should be given), did not occur. Such a side reaction was previously observed during an HOBt activation step of *O*-acyl isodipeptide.[6c] Finally, isopeptide **7** was purified (Fig. S1B), and converted to the desired peptide **1** *via* an *O*-to-*N* intramolecular acyl migration reaction in pH 7.4 buffered solution (half time: <30 s, Fig. 4B, C).[6b] After final purification, peptide **1** was successfully obtained with a yield of 70% based on resin-loaded-Leu, by the racemization-free segment condensation method (Fig. S1C).



Scheme 2 *Reagents and conditions*: a) Reagent A, 20 min; b) Fmoc–AA–OH (2.5 eq), DIPCDI (2.5 eq), HOBt (2.5 eq), DMF, 2 h; c) **10** (2.5 eq), DIPCDI (2.5 eq), HOAt (2.5 eq), NMP, 2 h; d) **9** (2.6 eq), HATU (2.5 eq), HOAt (2.6 eq), 2,4,6-collidine (2.5 eq), 1,8-bis(dimethylamino)naphthalene (2.5 eq), DCM–NMP (1 : 4), 4 h; e) TFA (94%), 1,2-ethanedithiol (EDT, 2.5%), water (2.5%), triisopropylsilane (TIS, 1%), 90 min; f) pH 7.4 phosphate buffer, 37 °C, overnight.

Synthesis of humanin

Next, we synthesized humanin (H–MAPRGFSCLLLLT-SEIDLPVKRRA–OH, **8**)[21] to examine the advantage of the method in the synthesis of a longer peptide. Humanin, a neuroprotective factor in Alzheimer's disease,[21a,b] is reported as a "difficult sequence"-containing peptide because of highly hydrophobic $L^9L^{10}L^{11}L^{12}$ sequence.[21c] In our hands, the total isolated yield of **8** was only 6% using the conventional stepwise Fmoc-SPPS because of the poor quality of crude **8** (Fig. S2). Thus, **8** was synthesized *via* sequential segment condensation using protected *O*-acyl isopeptide **9** and **10** (Scheme 2). Both N_1-segment **9** and N_2-segment **10** could be efficiently prepared using Reagent A as an Fmoc deprotection reagent without byproduct derived from the base-mediated ester decomposition (Fig. 5). Both segments were purified by RP-HPLC (Fig. S3, 24% yield for **9** and 40% yield for **10**). Using the DIPCDI–HOAt method in NMP, N_2-segment **10** was coupled to peptide-resin **12**, which was synthesized by standard Fmoc SPPS on a 2-chlorotrityl resin. To monitor for the completeness of the reaction, peptide-resin **13** was in part cleaved by a TFA cocktail, and the obtained peptide was analyzed by HPLC (Fig. 6A). In the HPLC chart, deprotected/cleaved **13** was obtained as a major product without deprotected/cleaved **12**, indicating that the segment condensation reaction of **10** onto peptide-resin **12** was proceeded quantitatively. However, the following condensation reaction of N_1-segment **9** with **13** was not successfully achieved by the DIPCDI–based method, as verified by an analysis of the deprotected/cleaved compound after the coupling reaction, probably because of the low reactivity of **13**. Hence, we adopted HATU–HOAt–2,4,6-collidine–1,8-bis(dimethylamino)naphthalene in DCM–NMP (1 : 4) for the coupling. The resulting peptide-resin **14** was subsequently treated with a TFA cocktail to give **15**. As shown in Fig. 6B, deprotected/cleaved **13** was not detected in crude **15**, suggesting that segment condensation of **9** under the conditions proceeded quantitatively. Also, none of D-Ser[7], des-Ser[7], D-Ser[14] or des-Ser[14] derivative was identified (<2.0%, verified by comparison with authentic samples using the analytical HPLC) in crude **15**, indicating that neither epimerization nor intramolecular side reaction with formation of mixed anhydrides occurred during the coupling reactions of **9** and **10**, respectively. Pure **15** (Fig. S3C) was then dissolved in pH 7.4 phosphate buffer to afford target

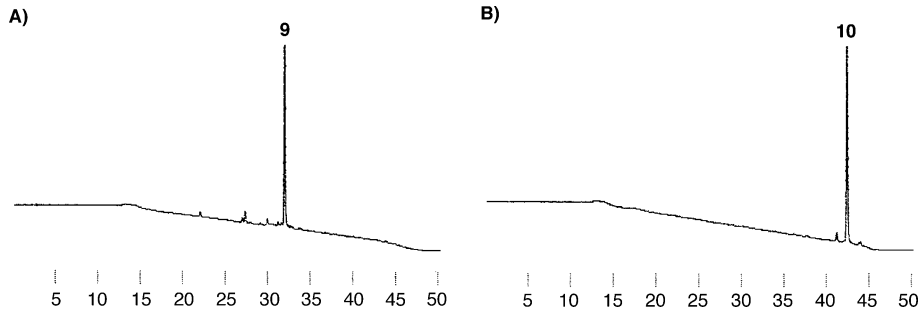


Fig. 5 HPLC profiles of crude isopeptide segments (A) **9** and (B) **10** of humanin synthesis. HPLC conditions were similar to those described in Fig. 3.

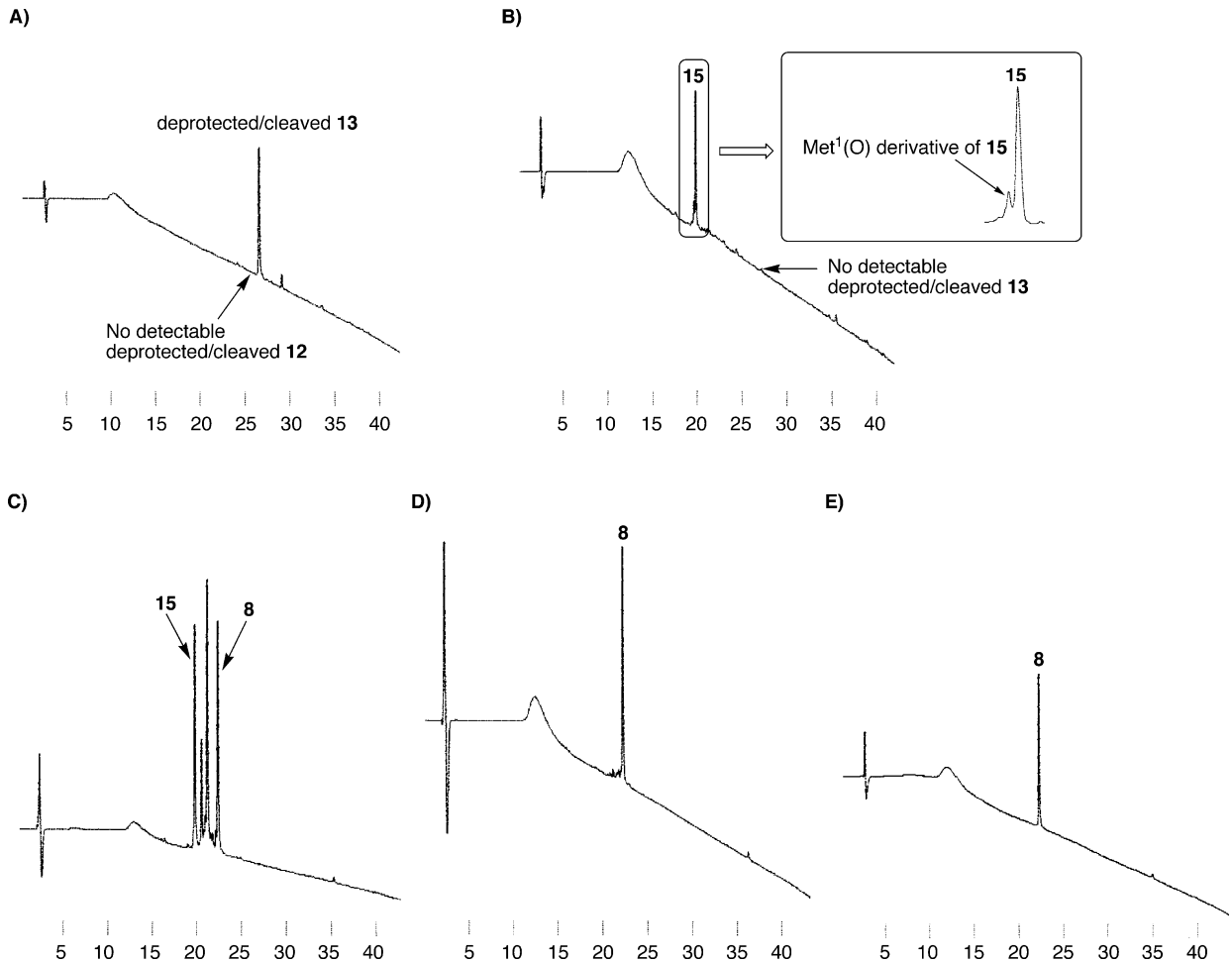


Fig. 6 Humanin (**8**) was synthesized by the racemization-free segment condensation method on resin. (A) To monitor the completeness of the reaction, peptide-resin **13** was in part cleaved by TFA cocktail, and the crude peptide was analyzed by HPLC; (B) crude **15**; (C, D) pure **15** was dissolved in pH 7.4 phosphate buffer at 37 °C to afford **8** (C: time = 10 min, D: time = overnight); (E) **8** after purification. HPLC conditions were similar to those described in Fig. 3.

peptide **8** *via* an *O*-to-*N* intramolecular acyl migration reaction (half time: *ca.* 10 min at 37 °C, Fig. 6C, D), followed by a final HPLC purification to give pure **8** (Fig. 6E) with a total yield of 41% based on the resin-bound-Ala residue. These results suggest that middle-sized peptides could be efficiently synthesized by use of the *O*-acyl isopeptide method-based segment condensation reaction.

Synthesis of orexin-B

Solution phase segment condensation might be more suitable as compared to solid phase for the synthesis of longer peptides or proteins, due to the use of less aggregative (thus more reactive) peptides (without resin). In fact, segment condensation in the solution phase has successfully been

applied to chemical protein synthesis.[1i,22] Thus, we studied the application of the *O*-acyl isopeptide method to solution phase segment condensation by synthesizing orexin-B (H–RSGPPGLQGRLQRLQRLLQASGNHAAGILTM–NH_2, **16**), a 28-mer neuropeptide associated with sleeping and feeding.[23] N_1-Segment **17** was synthesized with a yield of 34%. *O*-Acyl isopeptide N_2-segment **18** was efficiently prepared in a similar manner to **4** with a yield of 42% (Fig. 7). As shown in Scheme 3, N_2-segment **18** was coupled to peptide-amide **19**, which was prepared using Sieber amide resin with a yield of 37% (Fig. 8A), using EDC·HCl–HOAt–2,4,6-collidine in DMF–$CHCl_3$ (1 : 1) to afford crude **20** (Fig. 8B). In crude **20**, starting materials **18** and **19** were almost completely consumed, indicating that segment coupling was quantitatively completed. In addition, neither D-Ser nor des-Ser derivatives were detected (<2.0%), which were confirmed by comparison with authentic samples using the analytical HPLC. After deprotection of the Fmoc group (Fig. 8C) and purification of **21** by RP-HPLC (Fig. S4C), a second segment condensation reaction efficiently gave **22** by the EDC·HCl–HOAt method in DMF–$CHCl_3$ (1 : 1) (Fig. 8D). Fully deprotected isopeptide **23** was then obtained through a final deprotection with TFA (Fig. 8E), and isopeptide **23** was purified by RP-HPLC (Fig. 8F). Pure **23** was treated with dimethyl sulfide (DMS)–NH_4I[24c,d] in TFA to reduce methionine sulfoxide[24] to methionine, affording isopeptide **24** (Fig. 8G). Finally, crude **24** was dissolved in pH 7.4 phosphate buffer to trigger *O*-to-*N* intramolecular acyl migration reaction (half time: <30 s) to afford orexin-B **16** (Fig. 8H, I). Purification by RP-HPLC gave pure **16** with a reasonable total yield (44%, based on isopeptide

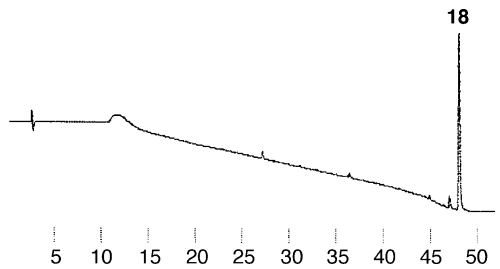
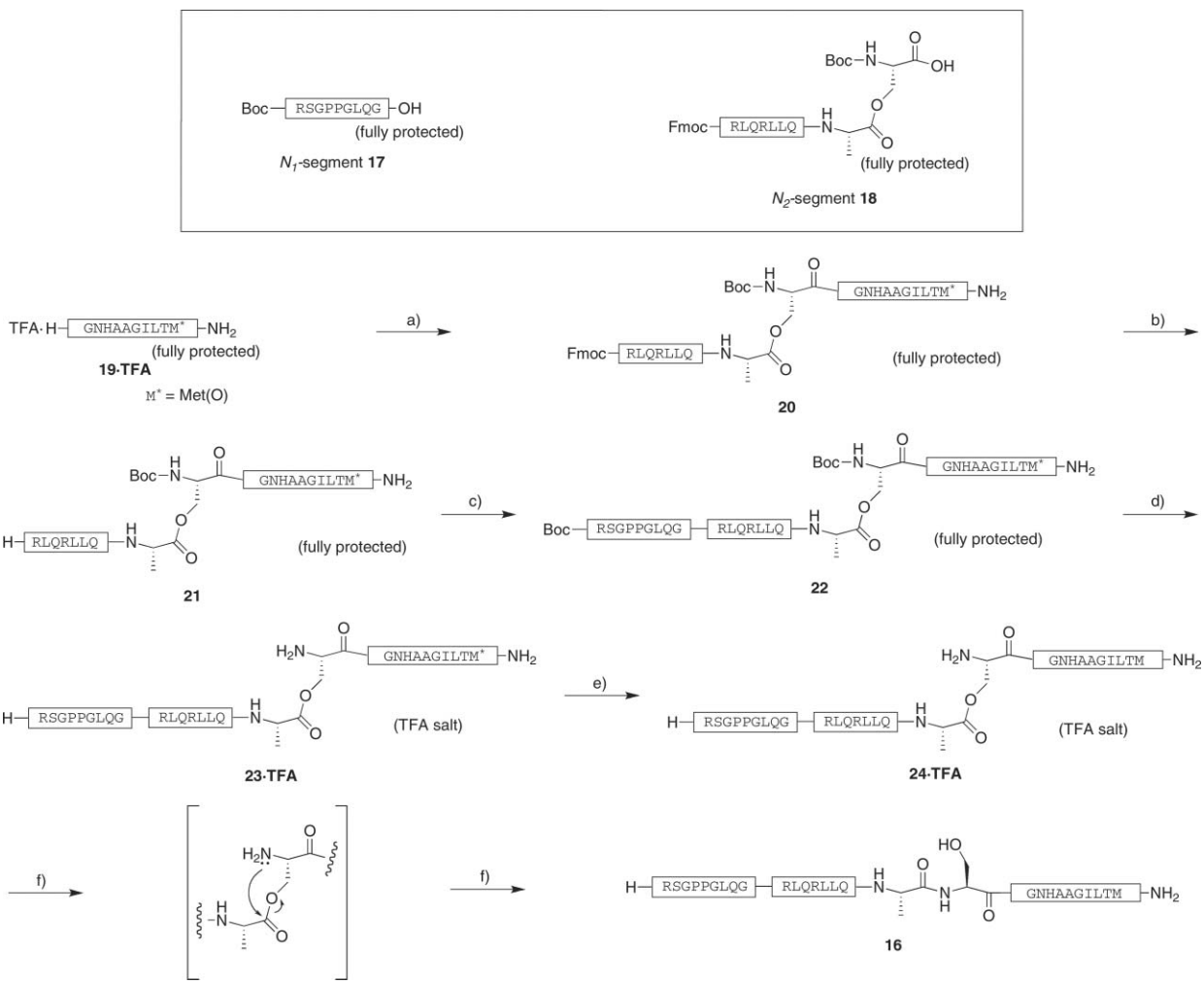


Fig. 7 HPLC profile of crude isopeptide segment **18**. HPLC conditions were similar to those described in Fig. 3.



Scheme 3 *Reagents and conditions*: a) **18** (0.8 eq), EDC·HCl (1.2 eq), HOAt (2.4 eq), 2,4,6-collidine (1.0 eq), DMF-$CHCl_3$ (1 : 1), 4 h; b) 20% piperidine in DMF, 5 min; c) **17** (2.4 eq), EDC·HCl (4.2 eq), HOAt (3.6 eq), 2,4,6-collidine (3.0 eq), DMF–$CHCl_3$ (1 : 1), 18 h; d) TFA (95%), TIS (2.5%), water (2.5%), 90 min; e) dimethyl sulfide (DMS, 20 eq), NH_4I (20 eq), TFA–water (20 : 1); f) pH 7.4 phosphate buffer, 3 h, room temperature.

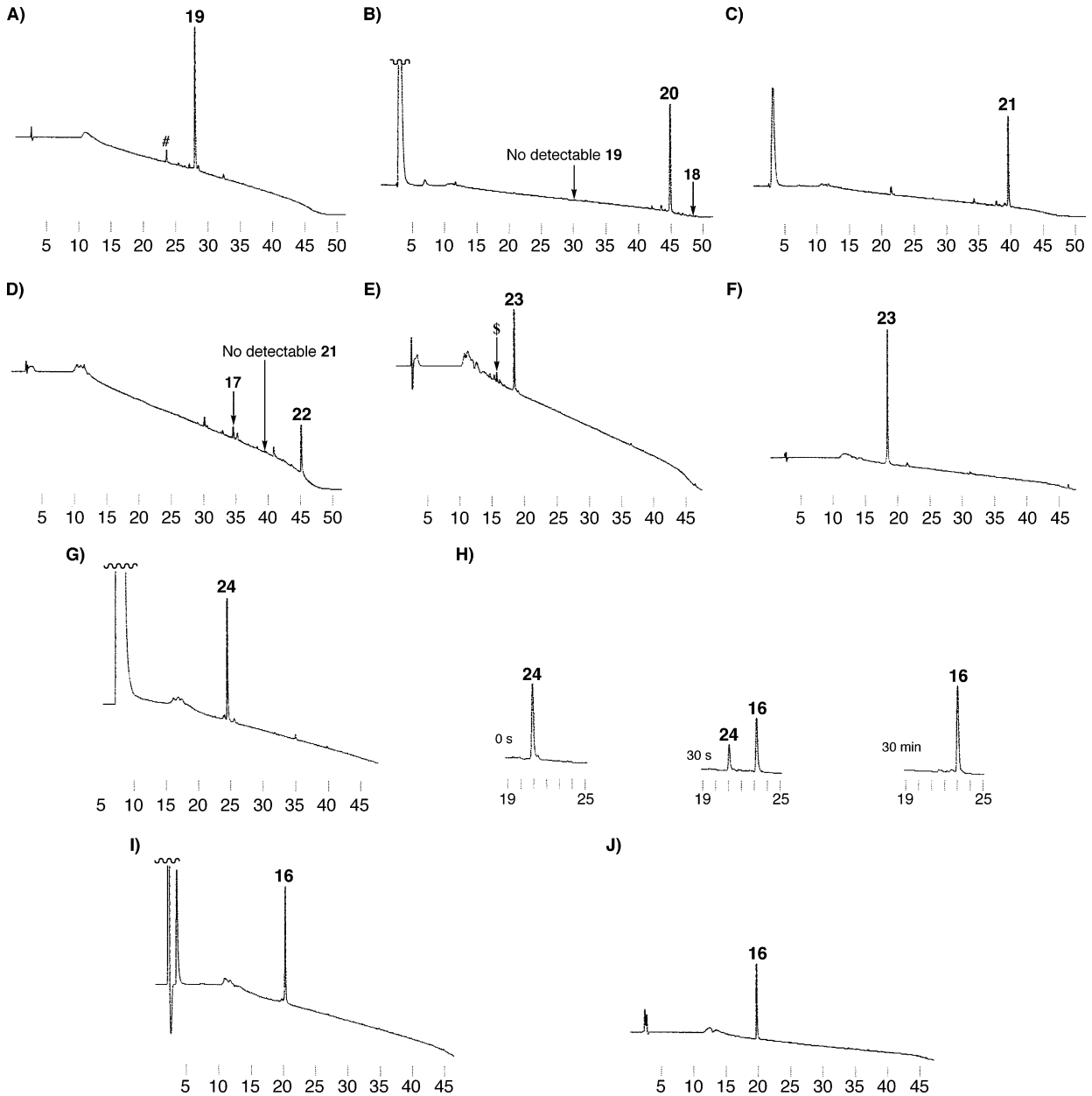


Fig. 8 Orexin-B (**16**) was synthesized by the segment condensation in solution phase. The condensation reaction was monitored by HPLC; (A) pure **19**, (B) 1st condensation (crude **20**), (C) Fmoc deprotection by 20% piperidine in DMF (crude **21**), (D) 2nd condensation (crude **22**), (E, F) final deprotection with TFA cocktail followed by purification using RP-HPLC (E: crude **23**, F: pure **23**), (G) following reduction of Met(O) gave crude isopeptide **24**. (H, I, J) *via* *O*-to-*N* intramolecular acyl migration (H, half time: <30 s), native peptide **16** was obtained (I: crude **16**, J: pure **16**). HPLC conditions are in a same manner described in Fig. 3, except for (H) in which binary solvent system: a linear gradient of CH_3CN (15–55% CH_3CN, 40 min) in 0.1% aqueous TFA was used. # = des-Trt-**19**, $ = deprotected **17**.

segment **18**, Fig. 8J). The successful synthesis of **16** indicates that the convergent synthesis using an isopeptide segment in solution phase could be advantageous to the synthesis of longer peptides or proteins.

Discussion

We have recently disclosed a novel segment condensation method based on the *O*-acyl isopeptide method with synthesizing a model pentapeptide. In this method, the use of an *N*-segment possessing a C-terminal Ser/Thr residue allows an effective segment condensation without any epimerization, thanks to the C-terminal *O*-acyl isopeptide structure with a urethane-protected Ser/Thr residue. To confirm the applicability of this racemization-free segment condensation to the synthesis of proteins, we synthesized herein three bioactive peptides (influenza A virus matrix M1 58–66, humanin and orexin-B) with optimization of reaction conditions. Conventional Fmoc SPPS including piperidine treatment for Fmoc removal gave no desired *O*-acyl isopeptide segment for the synthesis of influenza

A virus matrix M1 58–66, however, we found that the use of Reagent A (1-methylpyrrolidine–hexamethyleneimine–HOBt–NMP–DMSO) instead of 20% piperidine for Fmoc removal enabled an effective construction of the *O*-acyl isopeptide segments while avoiding a base-mediated ester cleavage. The segment condensation reactions of middle-sized segments onto peptide-resin were then completed under the conventional DIPCDI–HOAt method and the stronger HATU–HOAt–2,4,6-collidine–1,8-bis(dimethylamino)naphthalene method without side reactions. Additionally, toward future chemical protein synthesis, sequential segment coupling and solution phase condensation were also successfully achieved. In each segment condensation reaction, a side reaction such as epimerization and des-Ser/Thr formation was not observed. Finally, the synthesized isopeptides released their native peptides rapidly (half time: <30 s ~ 10 min) and quantitatively *via* *O*-to-*N* intramolecular acyl migration reaction in pH 7.4 phosphate buffer.

These results suggested that the racemization-free segment condensation reaction of middle-sized peptide segments becomes possible at not only the C-terminal Gly/Pro but also Ser/Thr residues of the *N*-segment. Using these segment condensation reactions in solution as well as on resin would enable a future chemical protein synthesis. In addition, final deprotected peptides and proteins were effectively purified by HPLC, because a simple isomerization to an *O*-acyl isopeptide remarkably and temporarily changed the physicochemical properties of the native peptide. Especially, higher water solubility of *O*-acyl isopeptide would enable easy purification of hydrophobic peptides. Finally, an *O*-to-*N* intramolecular acyl migration reaction triggered the native amide bond formation quantitatively under neutral conditions. These observations would be useful information for future chemical protein synthesis based on the segment condensation method.

Conclusion

We have described optimized conditions for the preparation of isopeptide segments and the segment condensation reaction with synthesizing three bioactive peptides. The use of Reagent A was suitable for the construction of the isopeptide segments. Sequential segment coupling reactions both on resin and in solution were successfully achieved without side reactions. Additionally, the synthesized isopeptides released their native peptides rapidly *via* *O*-to-*N* intramolecular acyl migration reaction. Considering these features, the *O*-acyl isopeptide method-based segment condensation methodology would contribute to the field of chemical synthesis of long peptides and proteins.

Experimental

General procedures

Fmoc-amino acid side-chain protections were selected as follows: *t*Bu (Asp, Glu, Ser, Thr), Boc (Lys), Pmc (Arg), Trt (Cys, Asn, Gln, His). All protected amino acids and resins were purchased from Calbiochem-Novabiochem Japan Ltd. (Tokyo, Japan) and Watanabe Chemical Ind., Ltd. (Hiroshima, Japan). Other chemicals were 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. 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 in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1} (temperature: 40 °C), detected at 230 nm. Preparative HPLC was carried out on a C18 reverse phase column (20 × 250 mm; YMC Pack ODS SH343–5) with a binary solvent system: a linear gradient of CH_3CN in 0.1% aqueous TFA at a flow rate of 5.0 mL min^{-1} (temperature: 40 °C), detected at 230 nm. Solvents used for HPLC were of HPLC grade. MALDI-TOF-MS spectra were recorded on Voyager DE-STR 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.

Solid phase peptide synthesis

In general, the peptide chains were assembled on a 2-chlorotrityl resin by sequential coupling of activated N^{α}-Fmoc-amino acid (2.5 eq) in DMF (1–2 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 (1.5 mL, × 5) and the completeness of each coupling was verified by the Kaiser test.[25] N^{α}-Fmoc deprotection was carried out by treatment with Reagent A[19] (2 mL, 1 min × 1 and 20 min × 1), followed by washing with NMP (1.5 mL, × 5), DMF (1.5 mL, × 10) and $CHCl_3$ (1.5 mL, × 5). After complete elongation of the peptide chains, the peptide resins were washed with $CHCl_3$ (1.5 mL, × 3) and MeOH (1.5 mL, × 3), and then dried for at least 2 h *in vacuo.*

(A) **Cleavage of protected peptide segments.** The protected peptides were cleaved from the resin with 20% HFIP in DCM for 45 min, concentrated *in vacuo*, dissolved in MeOH or DMSO, filtered using a 0.45 mm 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, affording the desired isopeptide segment. For the synthesis of protected peptide amide segment, Sieber amide resin was used, and 1% TFA in DCM treatment gave a crude protected peptide amide.

(B) **Cleavage of native peptides.** 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 at 0 °C followed by centrifugation at 4,000 rpm for 5 min (× 3). In the synthesis of humanin, a cocktail of TFA : 1,2-ethanedithiol (EDT) : distilled water : triisopropylsilane (TIS) (94 : 2.5 : 2.5 : 1) was used, instead of TFA–thioanisole–*m*-cresol–water. The resultant peptides were dissolved in 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 °C, and lyophilized at least 12 h.

Boc–Thr^{65}(Boc–Gly^{58}–Ile–Leu–Gly–Phe–Val–Phe^{64})–OH (4). The 2-chlorotrityl chloride resin (125 mg, 0.188 mmol) and Boc–Thr(Fmoc–Phe)–OH[6b] (73.6 mg, 0.125 mmol) were taken to the manual solid-phase reactor under an argon atmosphere and stirred for 2.5 h in the presence of DIPEA (13.1 μl, 0.125 mmol) in 1,2-dichloroethane (1.5 ml). After washing with DMF (1.5 ml, × 5), capping was performed with MeOH (125 μl) in the presence of

DIPEA (32.7 μl, 0.188 mmol) in DMF for 10 min. After washing with DMF (× 5), DMF–water (1 : 1, × 5), $CHCl_3$ (× 2) and MeOH (× 2) followed by drying *in vacuo*, the loading ratio was determined (0.071 mmol) photometrically from the amount of Fmoc chromophore liberated upon treatment with 50% piperidine in DMF for 30 min at 37 °C. The following Fmoc-protected amino acids (0.18 mmol) and Boc–Gly–OH (0.18 mmol) were manually coupled in the presence of DIPCDI (27.9 μl, 0.18 mmol) and HOBt (27.6 mg, 0.18 mmol) in DMF (1.5 ml) for 2 h after removal of each Fmoc group by Reagent A (1.5 mL) for 20 min (peptide-resin: 179 mg). The protected peptide was then cleaved from the resin (150 mg) with HFIP (1.0 ml)–DCM (4.0 ml) for 45 min, concentrated *in vacuo*, dissolved in MeOH (2.0 ml), filtered using a 0.45 μm 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, affording the desired isopeptide segment **4** as a white amorphous powder (37.5 mg, 35.5 μmol, 60%). HRMS (FAB): calcd for $C_{53}H_{80}N_8O_{14}Na$ $(M + Na)^+$: 1075.5692, found: 1075.5699; HPLC analysis at 230 nm: purity was higher than 95%.

65-*O*-Acyl-*iso*influenza A virus matrix M1 58–66 (7). Fmoc–Leu–resin (2-chlorotrityl resin, 34.4 mg, 0.013 mmol) was prepared in a similar manner as described above. After Fmoc deprotection, the protected isopeptide segment (**4**, 34.8 mg, 0.033 mmol) was condensed in the presence of DIPCDI (5.2 μL, 0.034 mmol) and HOBt (5.1 mg, 0.033 mmol) in DMF (400 μL) for 2 h (peptide-resin **6**: 43.4 mg). Then, the peptide was cleaved from the resin using TFA (0.8 mL) in the presence of thioanisole (21.7 μL), *m*-cresol (21.7 μL) and distilled water (21.7 μL) for 90 min at room temperature, concentrated *in vacuo*, washed with diethyl ether, centrifuged, suspended in water, and lyophilized to give the crude peptide **7** as a white amorphous powder (14.1 mg). Crude **7** (5.0 mg) was dissolved in DMSO (0.7 mL), filtered using a 0.45 μm filter unit, applied to preparative HPLC, and eluted using 0.1% aqueous TFA–CH_3CN. The peak fractions were collected and immediately lyophilized to afford desired peptide **7**·TFA as a white amorphous powder (4.6 mg, 3.9 μmol, 85%). MALDI-MS (TOF): M_{calc}: 966.2; $M + H_{found}$: 967.4; HPLC analysis at 230 nm: purity was higher than 95%.

Influenza A virus matrix M1 58–66 (1)[6b]. A solution of pure isopeptide **7**·TFA (2.0 mg, 1.7 μmol) in DMSO (40 μL) was added to pH 7.4 phosphate buffer (100 mM, 1.96 mL), and the solution was stirred for 6 h at room temperature. After lyophilization, crude **1** was washed by water (1 mL, × 2), dissolved in DMSO (1.2 mL), filtered using a 0.45 μm filter unit, applied to preparative HPLC, and eluted using 0.1% aqueous TFA–CH_3CN. The peak fractions were collected and immediately lyophilized to afford desired peptide **1**·TFA as a white amorphous powder (1.5 mg, 1.4 μmol, 82%). MALDI-MS (TOF): M_{calc}: 966.2; $M + H_{found}$: 967.7; 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 synthesized **1** was identical to that of an authentic sample that was previously synthesized in Ref. 6b.

Humanin (8) synthesized by conventional stepwise manner. Fmoc–Ala–resin (**11**, 2-chlorotrityl resin, 100 mg, 0.027 mmol) was prepared in a similar manner as described above. Sequential Fmoc-protected amino acids (0.068 mmol) were manually coupled in the presence of DIPCDI (10.5 μL, 0.068 mmol) and HOBt (10.4 mg, 0.068 mmol) for 2 h in DMF (1.0 mL) after removing each Fmoc group with Reagent A (1 mL) for 20 min. The resulting protected peptide-resin (120.6 mg) was treated with TFA (1.85 mL)–EDT (50.0 μL)–TIS (50.0 μL)–water (50.0 μL) for 90 min, concentrated *in vacuo*, washed with diethyl ether, centrifuged, dissolved in water, and lyophilized to give crude peptide **8** (21.9 mg). Crude **8** (3.0 mg) was dissolved in DMSO, filtered using a 0.45 μm filter unit, applied to preparative HPLC, and eluted using 0.1% aqueous TFA-CH_3CN. The peak fractions were collected and immediately lyophilized to afford desired peptide **8** as a white amorphous powder. Yield: 0.7 mg, 0.21 μmol, 6% based on the resin-bound-Ala; MALDI-MS (TOF): M_{calc}: 2687.2; $M + H_{found}$: 2688.2; 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 synthesized **8** was identical to that of commercially available humanin (PEPTIDE INSTITUTE, INC., Osaka, Japan).

Boc–Ser[7](Boc–Met[1]–Ala–Pro–Arg(Pmc)–Gly–Phe[6])–OH (9). **9** was synthesized (0.128 mmol scale) in a similar manner to that described for **4** using Boc–Ser(Fmoc–Phe)–OH[6b] (97.7 mg, 0.170 mmol). Yield: 38.4 mg, 0.031 mmol, 24%; HRMS (FAB): calcd. for $C_{57}H_{86}N_{10}O_{16}S_2Na$ $(M + Na)^+$: 1253.5562, found: 1253.5566; HPLC analysis at 230 nm: purity was higher than 95%.

Boc–Ser[14](Fmoc–Cys[8](Trt)–Leu–Leu–Leu–Leu–Thr[13](*t*Bu))–OH (10). **10** was synthesized (0.121 mmol scale) in a similar manner to that described for **4** using Boc–Ser(Fmoc–Thr(*t*Bu))–OH[6b] (99.4 mg, 0.170 mmol). Yield: 66.6 mg, 0.048 mmol, 40%; HRMS (FAB): calcd. for $C_{77}H_{103}N_7O_{14}SNa$ $(M + Na)^+$: 1404.7181, found: 1404.7170; HPLC analysis at 230 nm: purity was higher than 95%.

7,14-Di-*O*-Acyl-*iso*humanin (15) synthesized by the segment condensation method. After H–humanin(15–24)–resin (**12**, 2-chlorotrityl resin, 35.0 mg, 0.010 mmol) was constructed, N_2-segment **10** (33.2 mg, 0.024 mmol) was coupled in the presence of DIPCDI (3.7 μL, 0.024 mmol) and HOAt (3.3 mg, 0.024 mmol) in NMP (0.5 mL) for 2 h. After removing the Fmoc group using Reagent A (1.0 mL) for 20 min, N_1-segment **9** (32.0 mg, 0.026 mmol) was coupled in the presence of HATU (9.5 mg, 0.025 mmol), HOAt (3.5 mg, 0.026 mmol), 2,4,6-collidine (3.3 μL, 0.025 mmol) and 1,8-bis(dimethylamino)naphthalene (5.4 mg, 0.025 mmol) (preactivated in DCM (0.2 mL) for 30 sec) in DCM–NMP (1 : 4, 1 mL) for 4 h. Resulting peptide-resin **14** (42.0 mg) was treated with TFA (0.94 mL)–EDT (25 μL)–TIS (10 μL)–water (25 μL) for 90 min, concentrated *in vacuo*, washed with diethyl ether, centrifuged, dissolved in water, and lyophilized to give crude isopeptide **15** (17.6 mg). MALDI-MS (TOF): M_{calc}: 2687.2; $M + H_{found}$: 2688.2; HPLC analysis at 230 nm: purity was 83% (on the other hand, Met[1](O) derivative (8%) was detected). Crude **15** (4.0 mg) was dissolved in MeOH–DMSO (95 : 5, 500 μL), filtered using a 0.45 μm filter unit, applied to preparative HPLC, and eluted using 0.1% aqueous TFA–CH_3CN. The peak fractions were collected and immediately lyophilized to afford **15**·TFA as a white amorphous powder (3.6 mg, 1.0 μmol, 44% based on resin-bound-Ala). MALDI-MS (TOF): M_{calc}: 2687.2; $M + H_{found}$: 2688.1; HPLC analysis at 230 nm: purity was higher than 95%.

Humanin (8) synthesized by the segment condensation method. Pure **15** (2.0 mg, 0.57 μmol) was dissolved in pH 7.4 phosphate buffer (100 mM, 2 mL), stirred overnight at room temperature and lyophilized to give crude **8**. This crude peptide was dissolved in water, filtered using 0.45 μm filter unit, and immediately injected into preparative HPLC with a 0.1% aqueous TFA–CH_3CN system. The desired fractions were collected and immediately lyophilized to afford desired peptide **8**·TFA as a white amorphous powder (1.8 mg, 0.53 μmol, 93%). MALDI-MS (TOF): M_{calc}: 2687.2; M + H_{found}: 2688.2; HPLC analysis at 230 nm: purity was 95%; the retention time on HPLC (0–100% CH_3CN for 40 min, 230 nm) of synthesized **8** was identical to that of commercially available humanin (PEPTIDE INSTITUTE, INC., Osaka, Japan).

Boc–Arg[1](Pmc)–Ser(*t*Bu)–Gly–Pro–Pro–Gly–Leu–Gln(Trt)–Gly[9]–OH (17). **17** was synthesized (0.089 mmol scale) in a similar manner to that described for peptide **4**. Yield: 45.7 mg, 0.030 mmol, 34%; MALDI-MS (TOF): M_{calc}: 1532.4; M + Na_{found}: 1555.8; HPLC analysis at 230 nm: purity was higher than 95%.

Boc–Ser[18](Fmoc–Arg[10](Pmc)–Leu–Gln(Trt)–Arg(Pmc)–Leu–Leu–Gln(Trt)–Ala[17])–OH (18). **18** was synthesized (0.015 mmol scale) in a similar manner to that described for **4** using Boc–Ser(Fmoc–Phe)–OH[6b] (97.7 mg, 0.170 mmol). Yield: 15.0 mg, 6.2 μmol, 42%; HPLC analysis at 230 nm: purity was higher than 95%. Fully protected isopeptide **18** could not be analyzed by MALDI-MS (TOF) due to its low ionization property. Once the N-terminal Fmoc group was removed, the obtained product could be analyzed using MALDI-MS (TOF): M_{calc}: 2201.7; M + H_{found}: 2203.0. Similarly, after TFA treatment, the obtained product could be analyzed by MALDI-MS (TOF): M_{calc}: 1306.5; M + H_{found}: 1307.5.

H–Gly[19]–Asn(Trt)–His(Trt)–Ala–Ala–Gly–Ile–Leu–Thr(*t*Bu)–Met[28](O)–NH_2 (19). **19** was synthesized using Sieber amide resin (113 mg, 0.070 mmol) in a similar manner to that described for peptide **4**, cleaved with 1% TFA in DCM for 10 min (× 5). Crude **19** was concentrated *in vacuo*, and purified by RP-HPLC. The peak fractions were collected and immediately lyophilized to afford desired peptide **19**·TFA as a white amorphous powder. Yield: 42.9 mg, 0.026 mmol, 37%; MALDI-MS (TOF): M_{calc}: 1539.3; M + Na_{found}: 1562.9; HPLC analysis at 230 nm: purity was higher than 95%.

Partially protected 18-*O*-acyl-Met[28](O)-*iso*orexin-B(10–28) (21). A solution of **19**·TFA (8.2 mg, 5.0 μmol) and 2,4,6-collidine (0.7 μL, 5.2 μmol) in DMF (50 μL) was added to a solution of **18** (10.0 mg, 4.1 μmol), EDC·HCl (1.2 mg, 6.3 μmol) and HOAt (1.7 mg, 12 μmol) in $CHCl_3$ (150 μL). The mixture was stirred for 4 h at room temperature. After the solvent was removed *in vacuo*, the residue was triturated in 1 mL of CH_3CN–water (1 : 1) in an ice bath. After centrifugation, the supernatant was removed to afford crude **20** as a white precipitate. Then, the Fmoc group of crude **20** was removed using 20% piperidine in DMF for 5 min at room temperature. The solvent was removed *in vacuo* and the resulting peptide was dissolved in DMSO and purified by RP-HPLC with a 0.1% aqueous TFA–CH_3CN system. The peak fractions were collected and immediately lyophilized to afford desired protected isopeptide **21**·TFA as a white amorphous powder. Yield: 10.3 mg, 2.7 μmol, 66%; MALDI-MS (TOF): M_{calc}: 3723.6; M + Na_{found}: 3746.0; HPLC analysis at 230 nm: purity was higher than 95%.

18-*O*-Acyl-Met[28](O)-*iso*orexin-B(1–28) (23). To a solution of pure **17** (1.9 mg, 1.2 μmol), EDC·HCl (0.4 mg, 2.1 μmol) and HOAt (0.5 mg, 3.7 μmol) in $CHCl_3$ (100 μL) was added a solution of **21**·TFA (4.0 mg, 1.0 μmol) and 2,4,6-collidine (0.2 μL, 1.5 μmol) in DMF (50 μL). After the solution was stirred for 4 h at room temperature, another potions of pure **17** (1.9 mg, 1.2 μmol), EDC·HCl (0.4 mg, 2.1 μmol) and 2,4,6-collidine (0.2 μL, 1.5 μmol) were added to the mixture, and stirred for more 14 h at room temperature. The crude mixture was concentrated *in vacuo*, and triturated in 1 mL of CH_3CN–water (1 : 1) in an ice bath. After centrifugation, the supernatant was removed. The resulting peptide was suspended in CH_3CN (2 mL) and lyophilized to afford crude fully protected isopeptide **22** (5.7 mg) as a white powder. Obtained **22** (5.7 mg) was treated with TFA (0.95 mL)–TIS (25 μL)–water (25 μL) (92.5 : 2.5 : 2.5) for 90 min at room temperature, concentrated *in vacuo*, washed with diethyl ether, centrifuged, suspended with water, and lyophilized to give crude **23**·TFA (3.5 mg). Crude **23** (3.0 mg) was dissolved in 0.9 mL of MeOH–DMSO (3 : 1) and immediately purified by preparative RP-HPLC with a 0.1% aqueous TFA–CH_3CN system. The desired fractions were collected and immediately lyophilized to afford desired **23**·TFA as a white amorphous powder. Yield: 2.0 mg, 0.56 μmol, 70%; MALDI-MS (TOF): M_{calc}: 2915.3; M + H_{found}: 2916.4; HPLC analysis at 230 nm: purity was higher than 95%.

Orexin-B (16). To a solution of pure **23** (2.0 mg, 0.56 μmol) in TFA–water (2 mL–100 μL) was added NH_4I (1.6 mg, 11 μmol) and dimethyl sulfide (0.8 μL, 11 μmol), and the solution was allowed to stand for 30 min at 0 °C. After concentration *in vacuo*, the crude peptide was dissolved in 1 mL of water and washed by 1 mL of CCl_4. Then, the aqueous layer was lyophilized to give a crude isopeptide mixture. Finally, the obtained crude mixture was dissolved in 1 mL of phosphate buffer (100 mM, pH 7.4) and stirred for 3 h at room temperature. After lyophilization, the crude peptide was dissolved in 1 mL DMSO–water (1 : 1), filtered using a 0.46 μm filter unit, applied to preparative HPLC, and eluted using a 0.1% aqueous TFA–CH_3CN. The desired fractions were collected and immediately lyophilized to afford desired peptide **16** as a white amorphous powder. Yield: 1.8 mg, 0.54 μmol, 96%; MALDI-MS (TOF): M_{calc}: 2899.3; M + H_{found}: 2900.5; 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 synthesized **16** was identical to that of authentic sample from commercially available orexin-B (PEPTIDE INSTITUTE, INC., Osaka, Japan).

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