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Short Communication

Isopeptide method: development of *S*-acyl isopeptide method for the synthesis of difficult sequence-containing peptides

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Abstract: A novel strategy for a more efficient synthesis of difficult sequence-containing peptides, the *S*-acyl isopeptide method, was developed and successfully applied. A model pentapeptide Ac–Val–Val–Cys–Val–Val–NH_2 was synthesized via its water-soluble *S*-acyl isopeptide using an *S*-acyl isodipeptide unit, Boc–Cys(Fmoc–Val)–OH. An *S*-acyl isopeptide possessing excellent water solubility could be readily and quantitatively converted to the native peptide via an *S*–*N* intramolecular acyl migration reaction at pH 7.4. Thus, the *S*-acyl isopeptide method provides a useful tool in peptide chemistry. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: *S*-acyl isopeptide method; difficult sequence-containing peptide; *S*–*N* intramolecular acyl migration; solid-phase peptide synthesis

The SPPS has been routinely used for chemical synthesis of peptides and proteins. However, synthesis of 'difficult sequence'-containing peptides is still a challenging field in peptide chemistry as these peptides often have poor synthetic yield and low purity in SPPS [1–3]. The difficult sequences are generally hydrophobic and prone to aggregation in solvent during chain elongation and final purification. This aggregation is attributed to inter/intramolecular hydrophobic interactions and hydrogen-bond networks formed among resin-bound peptide chains, resulting in the formation of extended secondary structures such as β-sheets. To solve these problems, pseudo-proline building blocks [1], backbone amide protective groups [2,3] such as Hmb were developed. These special building blocks were designed to disrupt the secondary structure formed by inter/intrachain interactions on the resin.

In 2003, we discovered that replacing the native amide (*N*-acyl moiety) by a β-ester (*O*-acyl moiety) within the peptide backbone at Ser or Thr significantly changed the secondary structure of native peptide [4,5]. Shortly after our disclosure a similar method surfaced in the work of other research groups, including Mutter group [6], Carpino–Bienert–Beyermann group [7]. The target peptide can be easily generated from the formed *O*-acyl isopeptide by an *O*–*N* intramolecular acyl migration reaction [8–26]. These findings led us to the development of a novel strategy, the *O*-acyl isopeptide method, for the preparation of peptides containing difficult sequences (Figure 1). In addition, *O*-acyl isodipeptide units were developed for the routine preparation of *O*-acyl isopeptides in SPPS [11–17], and are now commercially available from Novabiochem (Merck KGaA). The method was successfully applied to the synthesis of Ac–Val–Val–Ser/Thr–Val–Val–NH_2 [4,5,11], Alzheimer's disease-related amyloid β–peptide 1–42 [8,16], and bioactive Influenza A virus-related peptide [15]. Recently, the *O*-acyl isopeptide method was further applied to the click peptide for a chemical biology-oriented research [10], and epimerization free-segment condensation toward an effective preparation of longer peptides and proteins [13]. Our studies consistently indicated that by modifying the peptide backbone to an ester structure at only one position within the whole peptide sequence significantly changes the unfavorable secondary structure of the native peptide, leading to improved coupling, deprotection, and purification efficiency during SPPS. Herein, we applied the isopeptide methodology to the synthesis of Cys-containing peptides, and we developed the 'S-acyl isopeptide method' based on the idea that synthesized hydrophilic *S*-acyl isopeptides can release native peptides via *S*–*N* intramolecular acyl migration reaction [27–29]. The efficacy of *S*-acyl isopeptide method in the synthesis of a model pentapeptide

Abbreviations: DIPCDI, 1,3-diisopropylcarbodiimide; EDC·HCl, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole; NMP, 1-methyl-2-pyrrolidinone; SPPS, solid-phase peptide synthesis.

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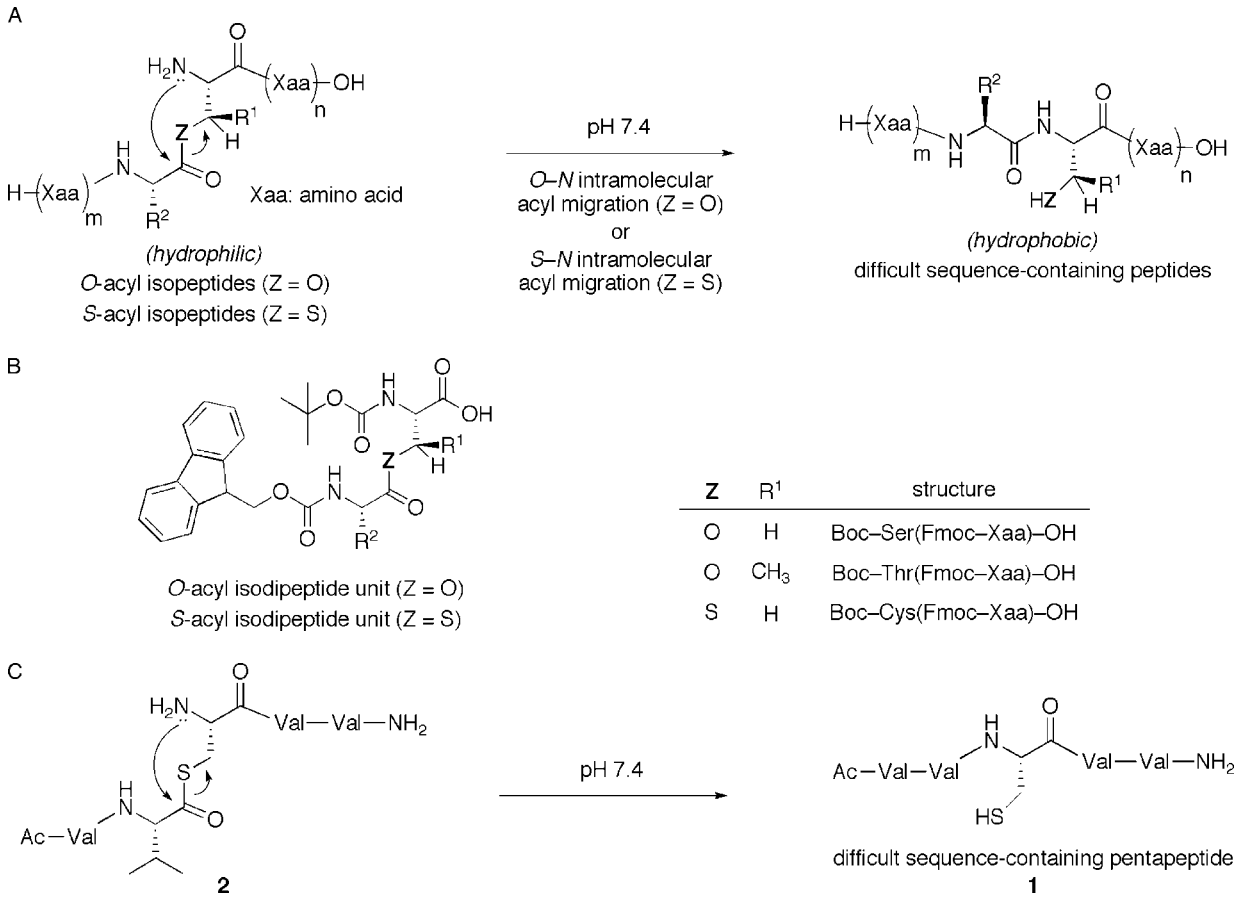

Figure 1 (A) *O*-Acyl isopeptide method and *S*-acyl isopeptide method. (B) *O*-Acyl isodipeptide units and *S*-acyl isodipeptide units. (C) Conversion of *S*-acyl isopeptide **2** to native peptide **1** via *S–N* intramolecular acyl migration reaction.

Ac–Val–Val–Cys–Val–Val–NH_2 (**1**), using a novel *S*-acyl isodipeptide unit Boc–Cys(Fmoc–Val)–OH (**6**), is herein demonstrated.

To prove the concept and demonstrate the effectiveness of the proposed method for the synthesis of difficult sequence-containing peptides, at first, peptide **1** was synthesized by a conventional Fmoc SPPS using the DIPCDI–HOBt method on the Rink amide-AM resin. As shown in Figure 2(A), deletion of Val observed in the crude product after the final deprotection may indicate that the acylation reaction was not completed during SPPS. We reported similar results during the synthesis of difficult sequence-containing pentapeptides possessing Ser or Thr [5,11]. This observation suggested that the hydrophobic nature of the peptide leading to the formation of secondary structures on the resin, prevented Fmoc–Val–OH from accessing the amino group of the growing peptide chain. Moreover, further purification of **1** by preparative-scale HPLC was laborious due to a broad elution profile and the extremely low solubility of the product. The solubility of **1** in H_2O and DMSO were determined as 5.2 μg ml^{-1} and 1.11 mg ml^{-1}, respectively. When the DMSO solution of **1** was used for HPLC purification, the overall yield of **1** was only 4.7%.

Next, **1** was synthesized by our proposed approach, i.e. the *S*-acyl isopeptide method (Scheme 1). As epimerization during thioesterification was a possible drawback in the solid-phase synthesis, the *S*-acyl isodipeptide unit, Boc–Cys(Fmoc–Val)–OH (**6**, Figure 2(B)) was introduced based on the hypothesis that thioesterification-derived epimerization should be suppressed in solution due to the faster coupling rate compared to that on a solid support. Therefore, *S*-acyl isodipeptide unit **6** was prepared by solution-phase synthesis in three steps from (Boc–Cys–$OH)_2$ that is commercially available from Fluka (Sigma–Aldrich Co.). Thioesterification in solution was free from epimerization, which was confirmed by comparison with an authentic derivative, Boc–Cys(Fmoc–D-Val)–OBzl. Then we synthesized *S*-acyl isopeptide **2** by an Fmoc SPPS using the conventional DIPCDI–HOBt method and isodipeptide unit **6** (Figure 2). 1-Methylpyrrolidine (25 v/v%)-HOBt (4.8 w/v%)-hexamethyleneimine (2 v/v%)-NMP-DMSO (1 : 1) (reagent A [30–33]), was adopted as the Fmoc deprotection reagent to prevent decomposition of the thioester bond during treatment with piperidine/DMF (20%). Finally, the resulting resin-bound peptide was cleaved by TFA (92.5%)-*m*-cresol (2.5%)-thioanisole (2.5%)-water (2.5%), and

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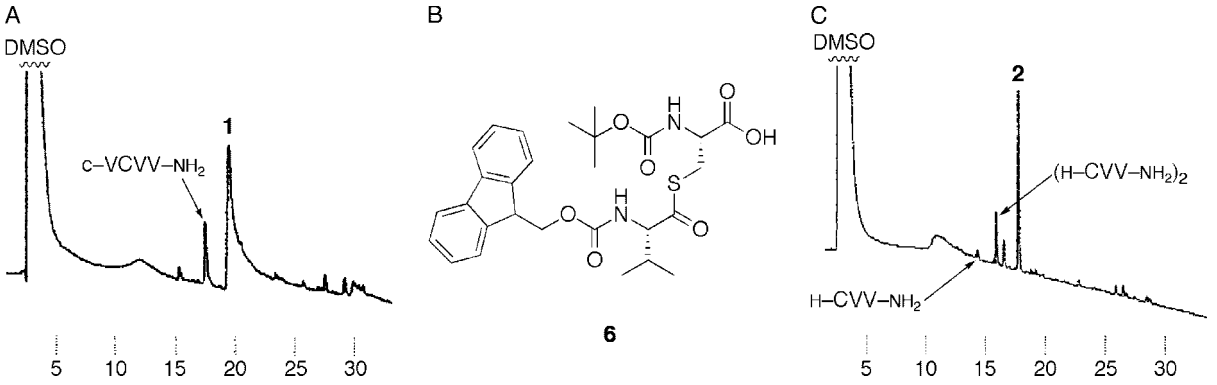
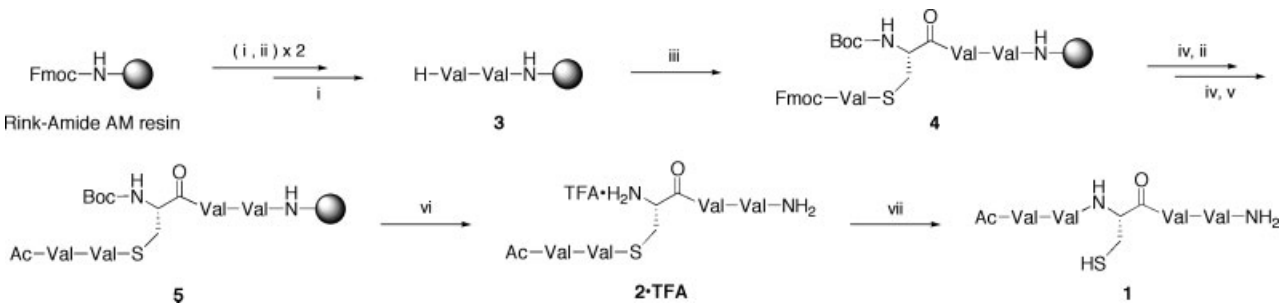


Figure 2 (A) The HPLC profile of crude **1** synthesized in the conventional manner. (B) A novel *S*-acyl isodipeptide unit, Boc–Cys(Fmoc–Val)–OH (**6**). (C) HPLC profile of crude **2** synthesized by *S*-acyl isopeptide method. Analytical HPLC was performed using a C18 reverse-phase column (4.6 × 150 mm^2; 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 at a flow rate of 0.9 ml min^{-1} (40 °C), detected at 230 nm.



Scheme 1 Reagents and conditions: (i) 20% piperidine/DMF, 20 min; (ii) Fmoc–Val–OH (2.5 eq.), DIPCDI (2.5 eq.), HOBt (2.5 eq), DMF, 2 h; (iii) **6** (2.5 eq.), DIPCDI (2.5 eq.), HOBt (2.5 eq.), CH_2Cl_2, 2 h; (iv) reagent A [30–33], 3 min × 1, 5 min × 1; (v) Ac_2O (1.5 eq.), TEA (1.0 eq.), DMF, 2 h; (vi) TFA : thioanisole : *m*-cresol : H_2O (92.5 : 2.5 : 2.5 : 2.5), 90 min; (vii) phosphate buffer, pH 7.4, 25 °C.

S-acyl isopeptide **2**·TFA was obtained as shown in Figure 2(C). Usually, addition of a highly malodorous reagent, 1,2-ethanedithiol (EDT), is required in the TFA cocktail to deprotect the trityl group from thiol group of Cys. In the *S*-acyl isopeptide method, the thiol group of Cys was protected as a thioester with the next Val, thus the use of EDT was omitted. Formation of the des-Val byproduct was not observed. This result demonstrated that the modification of the native amide backbone to the thioester moiety changed the secondary structure of peptide in a way that is more favorable for SPPS. HPLC analysis and MS of the crude product identified the presence of H–Cys–Val–Val–NH_2 (this tripeptide was highly prone to dimerization.), whereas Ac–Val–Val–OH was not observed, suggesting that partial decomposition of the thioester structure had occurred during the on-resin assembly of the peptide. These results imply that base-mediated hydrolysis of thioester and/or diketopiperazine formation took place during SPPS. Thus similar to the previous study [30–33], the cleavage of thioester bonds during SPPS was not completely suppressed. Additionally, the byproduct resulting from epimerization, H–Cys(Ac–Val–D-Val)–Val–Val–NH_2, was observed in 1% yield, while H–D-Cys(Ac–Val–Val)–Val–Val–NH_2 was not detected, which was confirmed by comparison with an authentic sample of each compound. Epimerization of thioesterified Val residue may occur during subsequent base treatment through the SPPS as it was previously reported by Aimoto group [32]. The crude *S*-acyl isopeptide **2** was dissolved in TFA, applied to preparative-scale HPLC, and eluted using 0.1% aqueous TFA–CH_3CN. Since **2** was promptly solubilized and was eluted as a narrow single peak, we could easily purify the crude product by preparative-scale HPLC to obtain pure **2** as a TFA salt with a total isolated yield of 10%. As a consequence of the presence of an ionized amino group within the structure of isopeptide, the water solubility of **2**·TFA was determined as 8.5 mg ml^{-1}, which was 1400-fold higher than that of native peptide **1**. Then pure **2** was quantitatively converted to native peptide **1** in phosphate buffer (pH 7.4) via *S*–*N* intramolecular acyl migration reaction (Figure 3). This migration was very fast and was completed in less than 1 min at room temperature. Consequently, the overall yield of **1**, synthesized by the *S*-acyl isopeptide method, was 10%, which was twofold higher than that of the conventional method.

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The *S*-acyl isopeptide **2**·TFA was stable for at least one day in DMSO or MeOH solution at room temperature, which was estimated by HPLC analysis. In contrast, in phosphate buffer (pH 7.4) at room temperature, it spontaneously released the native peptide **1**. This *S*–*N* intramolecular acyl migration was slower in water with a $t_{1/2}$ of approximately 33 h. Moreover, *S*-acyl isopeptide **2**·TFA as an amorphous powder after lyophilization, was stable for at least 3 months at room temperature. These results suggested that an *S*-acyl isopeptide could be stored without decomposition, and afforded the native peptide immediately in a buffer when needed.

In summary, we developed the *S*-acyl isopeptide method for the synthesis of difficult sequence-containing peptides and this method was successfully applied to the synthesis of model pentapeptide **1**. The results indicated that not only Ser or Thr but also Cys could be used for effective synthesis of difficult sequence-containing peptides via isopeptide method. Additionally, *S*-acyl isopeptide **2**·TFA was stable in solutions such as DMSO and MeOH, as well as an amorphous powder. Especially in contrast to the native peptide **1** in the solid state, *S*-acyl isopeptide was resistant to spontaneous disulfide bond formation, suggesting that an *S*-acyl isopeptide could also be utilized as a stable precursor of Cys-containing peptides. These results indicated that the extension of the isopeptide strategy to the *S*-acyl isopeptide method would provide a useful tool in the peptide chemistry. Further studies on the applications of *S*-acyl isopeptide method for efficient synthesis of difficult sequence-containing peptides are now in progress, including combination with a silyl carbamate–acid fluoride system [33,34] developed for thioester-containing peptide synthesis.

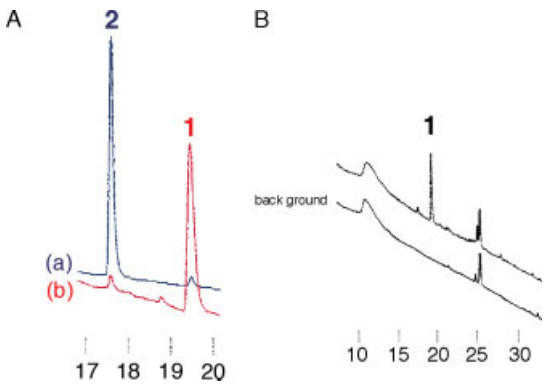

Figure 3 (A) *S*–*N* Intramolecular acyl migration reaction from isopeptide **2** to native peptide **1**. The migration reaction was monitored by HPLC at (a) 0 s (blue line) and (b) 30 s (red line). (B) HPLC profile of **1** synthesized by the *S*-acyl isopeptide method. Analytical HPLC was performed using the same conditions as described in Figure 2.

EXPERIMENTAL

Ac–Val–Val–Cys–Val–Val–NH_2 (1, by the conventional method)

Peptide **1** was synthesized on Rink amide-AM resin (200 mg, 0.142 mmol) according to the general Fmoc-based solid-phase procedure in Ref. 11. The peptide was cleaved from the resin using TFA (94%)–EDT (2.5%)–H_2O (2.5%)–triisopropylsilane (1%) for 90 min, and purified by RP-HPLC with a 0.1% aqueous TFA–CH_3CN system. Yield: 3.7 mg (4.7%); ESI-MS: calculated for $(M + Na)^+$: 581.32, found: 581.15; HPLC analysis at 230 nm: purity was 91%.

Boc–Cys(Fmoc–Val)–OH (6)

N,*N*′-(di-*t*-Butoxycarbonyl)-L-cystine di-benzyl ester ((Boc–Cys–OBzl)$_2$) was synthesized based on the method described in Ref. 35,36. BnBr (1.62 ml, 14 mmol) was added to a stirring solution of *N*,*N*′-(di-*t*-butoxycarbonyl)-L-cystine (Boc–Cys–OH)$_2$ (600 mg, 1.4 mmol) in DMF (8 ml) containing $NaHCO_3$ (458 mg, 5.4 mmol) at 0 °C, and the mixture was stirred overnight at room temperature under Ar, diluted with AcOEt, and washed successively with water 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 : 2) to yield (Boc–Cys–OBzl)$_2$. Yield: 841 mg (99%); HPLC analysis at 230 nm: purity was higher than 94%. Spectroscopic data were identical to the reported in Ref. 35.

After that, PPh_3 (160 mg, 0.61 mmol) was added to a stirring solution of (Boc–Cys–OBzl)$_2$ (377 mg, 0.61 mmol) in $CHCl_3$ (5 ml) containing H_2O (64.2 µl, 3.6 mmol) and the mixture was stirred for 2 h at room temperature. Then, *N*-(9H-fluoren-9-ylmethoxycarbonyl)-L-valine (Fmoc–Val–OH, 1032 mg, 3.0 mmol), EDC·HCl (581 mg, 3.0 mmol), and HOBt (411 mg, 3.0 mmol) were added, and the mixture was stirred additionally for 6 h, diluted with AcOEt, and washed successively with water, 1 N 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 (Et_2O : hexane = 1 : 2.5) to yield Boc–Cys(Fmoc–Val)–OBzl. Epimerization during above reaction was not detected, which was confirmed by comparison with independently synthesized D-Val derivative. Yield: 633 mg (82%); ESI-MS: calculated for $(M + Na)^+$: 655.25, found: 654.95; HPLC analysis at 230 nm: purity was higher than 94%; NMR (CD_3OD, 300 MHz): δ 7.91–7.88 (m, 1H), 7.77–7.74 (m, 1H), 7.44–7.33 (m, 11H), 5.13 (s, 2H), 4.38–4.22 (m, 3H), 4.14–4.12 (m, 1H), 4.02–3.97 (m, 1H), 3.04–2.91 (m, 2H), 2.15–2.08 (m, 1H), 1.36 (s, 9H), 0.91–0.85 (m, 6H).

After that, Pd/C (300 mg) was added to the stirring solution of Boc–Cys(Fmoc–Val)–OBzl (100 mg, 0.158 mmol) in EtOH (5 ml) in the presence of formic acid (33 µl, 0.79 mmol), and the reaction mixture was vigorously stirred for 1 h at 40 °C. Pd/C was filtered off through celite, then the next portions of Pd/C (300 mg) and formic acid (33 µl, 0.79 mmol) were added. The reaction mixture was vigorously stirred for 1 h at 40 °C again, then Pd/C was filtered off through Celite. The solvent was removed *in vacuo* and the crude compound dissolved in EtOH, filtered using 0.46-µm filter unit, and immediately injected into preparative-scale HPLC with a 0.1% aqueous

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TFA–CH_3CN system. The desired fractions were collected and immediately lyophilized to afford *S*-acyl isodipeptide unit **6** as a white amorphous powder. Yield: 18.4 mg (22%); ESI-MS: calculated for $(M + Na)^+$: 565.20, found: 564.95; HPLC analysis at 230 nm: purity was higher than 94%; NMR (CD_3OD, 300 MHz): δ 7.79 (d, $J = 3.7$ Hz, 2H), 7.73–7.68 (m, 2H), 7.41–7.29 (m, 2H), 4.50–4.46 (m, 1H), 4.38–4.32 (m, 1H), 4.30–4.22 (m, 2H), 4.17–4.12 (m, 1H), 3.45–3.41 (m, 1H), 3.23–3.12 (m, 1H), 2.26–2.20 (m, 1H), 1.40 (s, 9H), 0.97–0.86 (m, 6H).

Ac–Val–Val–Cys–Val–Val–NH_2 (1, by the *S*-acyl isopeptide method)

After the preparation of the H–Val–Val–NH-resin (Rink amide-AM resin, 100 mg, 0.068 mmol) in the same manner described in the synthesis of **1** using the conventional method, Boc–Cys(Fmoc–Val)–OH (**6**, 92.2 mg, 0.17 mmol) was coupled in the presence of DIPCDI (27.0 µl, 0.17 mmol) and HOBt (26.0 mg, 0.17 mmol) for 2 h in dry DCM (1.5 ml). Fmoc–Val–OH (57.7 mg, 0.17 mmol) was coupled in the presence of DIPCDI (27.0 µl, 0.17 mmol) and HOBt (26.0 mg, 0.17 mmol) for 2 h in DMF (1.5 ml), successive *N*-acetylation was carried out using Ac_2O (9.6 µl, 0.10 mmol) and DIPEA (11.8 µl, 0.068 mmol) after removal of each Fmoc group by reagent A [30–33] for 3 min × 1 and 5 min × 1. The resulting protected peptide resin was treated with TFA (2.3 ml)–*m*-cresol (61.1 µl)–thioanisole (61.1 µl)–H_2O (61.1 µl) for 90 min, concentrated *in vacuo*, washed with diethyl ether, and centrifuged to give the crude *S*-acyl isopeptide **2**·TFA (24.9 mg). This crude peptide (7.5 mg) was dissolved in TFA, and immediately injected into preparative-scale HPLC with a 0.1% aqueous TFA–CH_3CN system. The desired fractions were collected and immediately lyophilized to afford pure **2**·TFA as a white amorphous powder. Yield: 1.3 mg (10%); ESI-MS: calculated for $(M + Na)^+$: 581.32, found: 581.15; HPLC analysis at 230 nm: purity was higher than 94%.

Purified *S*-acyl isopeptide **2**·TFA (0.149 mg) was then dissolved in phosphate buffer (100 mM, pH 7.4, 1.0 ml) and stirred for 1 min at room temperature. The resultant precipitate was centrifuged and washed with water and MeOH followed by lyophilization to give **1** as a white powder. Yield: 0.124 mg (99%); ESI-MS: calculated for $(M + Na)^+$: 581.32, found: 581.15; HPLC analysis at 230 nm: purity was higher than 94%; 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 conventional method.

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