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'*O*-Acyl isopeptide method' for the efficient synthesis of difficult sequence-containing peptides: use of '*O*-acyl isodipeptide unit'

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Abstract—A novel '*O*-acyl isodipeptide unit', Boc-Thr(Fmoc-Val)-OH **5** has been successfully used for the efficient synthesis of a difficult sequence-containing pentapeptide based on the '*O*-acyl isopeptide method', in which racemization-inducible esterification could be omitted, suggesting that the use of *O*-acyl isodipeptide units allows the application of this method to fully automated protocols for the synthesis of long peptides or proteins.

The synthesis of 'difficult sequence'-containing peptides is one of the most problematic areas in peptide chemistry. These peptides are often obtained with low yield and purity in solid-phase peptide synthesis (SPPS).[1] These difficult sequences are generally hydrophobic and promote aggregation in solvents during synthesis and purification. This aggregation is attributed to intermolecular hydrophobic interaction and hydrogen bond network among resin-bound peptide chains, resulting in the formation of extended secondary structures such as β-sheets.[1]

In regard to the synthesis of difficult sequence-containing peptides, we have recently disclosed an '*O*-acyl isopeptide method',[2] in which a native amide bond at a hydroxyamino acid residue, for example, Ser was isomerized to the ester bond, followed by an *O*–*N* intramolecular acyl migration reaction (Fig. 1A). The method has been successfully applied to the efficient synthesis of difficult sequence-containing peptides such as Ac-Val-Val-Ser-Val-Val-NH_2 **1** (Fig. 1B)[2b,d,e] and Alzheimer's disease-related amyloid β peptide (Aβ) 1–42.[2c–g]

Our studies indicated that the isomerization of the peptide backbone at only one position of the whole peptide sequence, that is, formation of the ester, significantly changed the unfavorable secondary structure of the difficult sequence-containing peptides, leading to improved coupling and deprotection efficacy during SPPS. Mutter et al.[3a,c] and Carpino et al.[3b] have also confirmed the efficacy of the '*O*-acyl isopeptide method'. Herein, a novel '*O*-acyl isodipeptide unit', Boc-Thr(Fmoc-Val)-OH **5** was successfully used to efficiently synthesize a difficult sequence-containing pentapeptide (Ac-Val-Val-Thr-Val-Val-NH_2 **3**).

In the synthesis of peptide **3** by standard SPPS using Fmoc-amino acids,[4] an undesired peptide, Fmoc-Val-Val-Thr-Val-Val-NH_2 was obtained at a similar rate to peptide **3** after the final deprotection (Fig. 2A). This indicated that the Fmoc group of the pentapeptide-resin was not deprotected during SPPS, similar to what we have previously reported for the synthesis of **1**.[2b,d,e] This suggests that the highly hydrophobic nature of Fmoc-peptide-resin prevented the base from accessing the Fmoc group, thus forming insoluble micro-aggregates on the resin. Further purification of **3** in preparative scale HPLC was laborious due to the extremely low solubility of the product (the solubility of **3** in H_2O, MeOH, and DMSO being 0.008 ± 0.003, 0.059 ± 0.004 and 1.89 ± 0.14 mg mL^{-1}, respectively). When the

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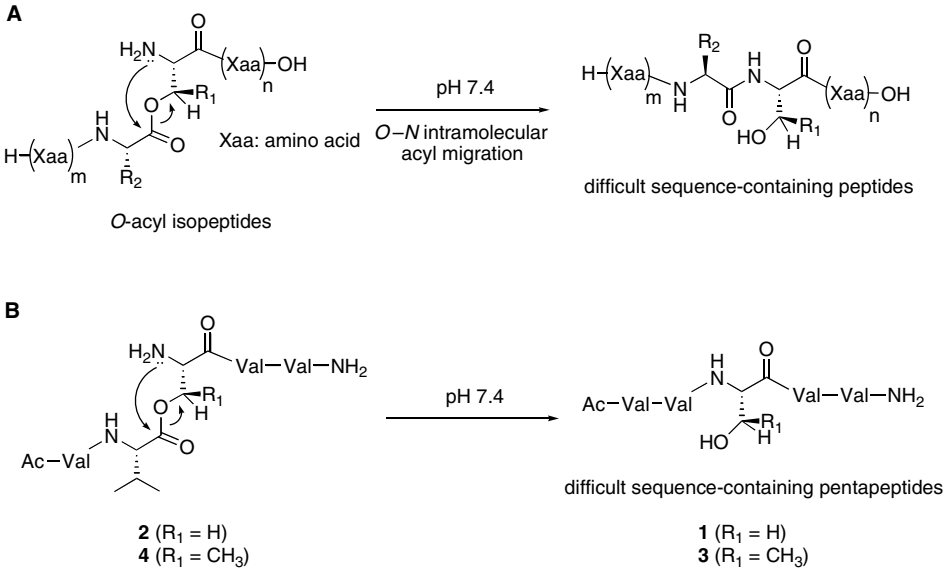


Figure 1. (A) '*O*-Acyl isopeptide method': the synthetic strategy for difficult sequence-containing peptides via the *O–N* intramolecular acyl migration reaction of *O*-acyl isopeptides, (B) application of the *O*-acyl isopeptide method for the synthesis of pentapeptides **1** and **3**.

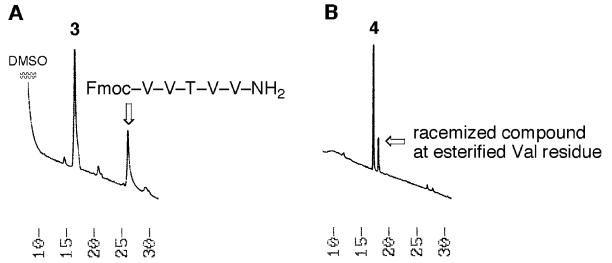


Figure 2. HPLC profiles of crude (A) peptide **3** (synthesized by the standard SPPS) and (B) its *O*-acyl isopeptide **4**. Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS AM302) with a binary solvent system: a linear gradient of CH_3CN (0–100% CH_3CN, 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1} (40 °C), detected at 230 nm.

DMSO solution of **3** was used for HPLC purification, the overall yield of **3** was only 1.4%.[4]

For the case of *O*-acyl isopeptide method,[5] Boc-Thr-OH was coupled to the H-Val-Val-NH-resin, and subsequent acylation with Fmoc-Val-OH to the β-hydroxyl group of Thr was performed using the DIPCDI–DMAP method in CH_2Cl_2 to obtain ester. After coupling with another Val residue, *N*-acetylation and TFA treatment, *O*-acyl isopeptide **4**·TFA was obtained without forming Fmoc-containing by-product (Fig. 2B). Hence, the protected peptide resin was efficiently synthesized with no interference from the difficult sequences. The results support our hypothesis that the modification of **3** to the ester structure **4** changed the secondary structure of peptide to that more favorable for Fmoc-deprotection. Moreover, the solubility of **4**·TFA in H_2O or MeOH was 46.2 ± 22.7 (5775-fold) or 266.5 ± 65.4 (4517-fold) mg mL^{-1}, respectively, higher than that of *N*-acyl peptide **3**, because of the ionized amino group in the isopeptide. Accordingly, a solution of **4**·TFA in MeOH could easily be applied to preparative HPLC, and **4**·TFA was purified using the 0.1% aqueous TFA–CH_3CN system as the eluant to obtain pure **4** with an isolated yield of 28.0%.

However, a large amount of racemization (21%) of the esterified Val residue occurred in the DIPCDI–DMAP method (Fig. 2B), which was confirmed by an independent synthesis of H-Thr(Ac-Val-D-Val)-Val-Val-NH_2. This extent of racemization is remarkably higher than that of the esterification between Val and Ser in **2** (0.8%),[2b,d,e] which is probably due to steric hindrance at the secondary hydroxyl group in Thr as compared to Ser. We also observed a slightly higher amount of racemization (33%) when 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT) (3.0 equiv)–*N*-methylimidazole (NMI) (12 equiv) in CH_2Cl_2[6] was used for the esterification, which agrees with a literature report.[6c] We considered that the large extent of racemization should be a serious disadvantage in the synthesis of Thr-containing peptides using the *O*-acyl isopeptide method.

To avoid this problem, we decided to adapt an *O*-acyl isodipeptide unit, Boc-Thr(Fmoc-Val)-OH **5** (Fig. 3A),

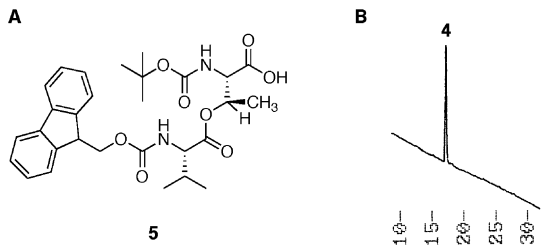
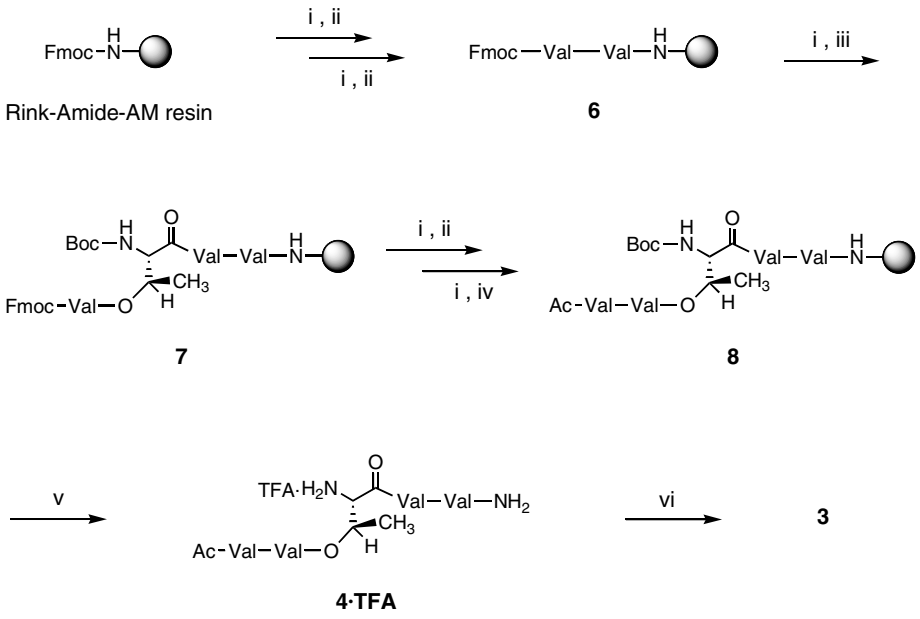


Figure 3. (A) *O*-Acyl isodipeptide unit **5** and (B) crude isopeptide **4** (synthesized using **5**). Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS AM302) with binary solvent system: a linear gradient of CH_3CN (0–100% CH_3CN, 40 min) in 0.1% aqueous TFA 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 equiv), DIPCDI (1,3-diisopropylcarbodiimide, 2.5 equiv), HOBt (2.5 equiv), DMF, 2 h; (iii) **5** (2.5 equiv), DIPCDI (2.5 equiv), HOBt (2.5 equiv), DMF, 2 h; (iv) Ac_2O (1.5 equiv), TEA (1.0 equiv), DMF, 2 h; (v) TFA–*m*-cresol–thioanisole–H_2O (92.5:2.5:2.5:2.5), 90 min; (vi) phosphate buffered saline (PBS), pH 7.4, 25 °C.

which was synthesized by solution phase,[7] for the synthesis of **3** based on the *O*-acyl isopeptide method (Scheme 1).[8] The use of isodipeptide **5** could omit the racemization-inducing esterification reaction. The *O*-acyl isodipeptide **5**, which readily solubilized in DMF, was coupled to the H-Val-Val-NH-resin using the standard DIPCDI–HOBt method (2 h) to obtain **7**. The completeness of the coupling was verified by the Keiser test. After coupling with another Val residue followed by *N*-acetylation and TFA–*m*-cresol–thioanisole–H_2O (92.5:2.5:2.5:2.5) treatment, *O*-acyl isopeptide **4**·TFA was obtained. As shown in Figure 3B, HPLC analysis of crude **4** (synthesized using *O*-acyl isodipeptide unit **5**) exhibited a high purity of the desired product **4** with no by-product derived from the difficult sequence or racemization. The use of isodipeptide **5** did not lead to any additional side reaction. Moreover, since H-Thr-Val-Val-NH_2 was not formed as a by-product, we concluded that (1) the ester bond between Val and Thr was stable in both piperidine and TFA treatments and (2) diketopiperazine was not formed when the last Fmoc group was removed. Consequently, we could obtain pure **4** without further purification, with an isolated yield of 44.5%.

Compound **4**·TFA was stable at 4 °C for at least 2 years. On the other hand, when **4**·TFA was dissolved and stirred in phosphate buffered saline (PBS, pH 7.4) at rt, quantitative *O–N* intramolecular acyl migration to the corresponding parent peptide **3** was observed with no side reaction (Fig. 4A).[9] Isopeptide **4** exhibited more than 5-fold faster migration with a half-life of 23 min than that observed in **2** containing Ser (half-life = 2 h).[2b,d,e] The faster migration in **4** may be attributed to a unique interlocking effect of the β-methyl group in Thr, which has conformational restrictions, such as

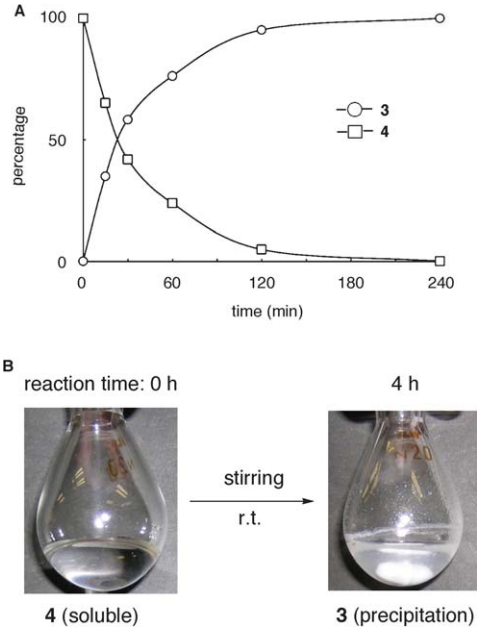


Figure 4. (A) A graph and (B) photographs in conversion of *O*-acyl isopeptide **4** to **3** via the *O–N* intramolecular acyl migration in phosphate buffered saline (pH 7.4, 25 °C).

a *gem*-effect by geminal methyl substitution.[10] Finally, as depicted in Figure 4B, **3** was formed as a white precipitate from **4**. The resulting precipitate was centrifuged and washed with water and methanol to afford highly pure **3**. The overall yield of **3** in *O*-acyl isopeptide method was 42.7%.[8]

In conclusion, '*O*-acyl isopeptide method' with a novel *O*-acyl isodipeptide unit, Boc-Thr(Fmoc-Val)-OH **5**, in which the racemization-inducing esterification reaction

could be omitted, has been successfully applied to the efficient synthesis of a difficult sequence-containing pentapeptide by improving the nature of difficult sequence during SPPS. This suggests that the use of *O*-acyl isodipeptide units allows the application of '*O*-acyl isopeptide method' to fully automated protocols for the synthesis of long peptides or proteins.

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4. The peptide **3** was synthesized on Rink amide aminomethyl (AM) resin (200 mg, 0.126 mmol) according to the general Fmoc-based solid-phase procedure. After the resin was washed with DMF (1.5 mL, ×5), Fmoc-Val-OH (107 mg, 0.315 mmol), and Fmoc-Thr(^{t}Bu)-OH (111 mg, 0.315 mmol) were coupled in the presence of 1,3-diisopropylcarbodiimide (DIPCDI, 49.2 μL, 0.315 mmol) and 1-hydroxybenzotriazole (HOBt, 48.2 mg, 0.315 mmol) in DMF (1.5 mL) for 2 h according to the sequence. The Fmoc-group was removed by 20% piperidine/DMF (20 min). *N*-Acetylation was carried out with Ac_2O (14.3 μL, 0.151 mmol) in the presence of TEA (17.5 μL, 0.126 mmol) for 2 h. The peptide was cleaved from the resin using TFA (4.7 mL) in the presence of thioanisole (126 μL), *m*-cresol (126 μL), and distilled water (126 μL) for 90 min at rt, concentrated in vacuo, washed with Et_2O, centrifuged, suspended with water, and lyophilized to give the crude peptide (45.9 mg). This crude peptide (20 mg) was saturated in DMSO, filtered using a 0.46 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 the desired peptide **3** as a white amorphous powder. Yield: 0.4 mg (1.4%); HRMS (FAB): calcd for $C_{26}H_{49}N_6O_7$ $(M+H)^+$: 557.3663, found: 557.3657; HPLC analysis at 230 nm: purity was higher than 95%.
5. After the preparation of the H-Val-Val-NH-resin (Rink amide AM resin, 200 mg, 0.126 mmol) in the same manner as described in the synthesis of **3** using the conventional method, Boc-Thr-OH (82.9 mg, 0.378 mmol) was coupled in the presence of DIPCDI (59.2 μL, 0.378 mmol) and HOBt (57.8 mg, 0.378 mmol) in DMF (1.5 mL). Subsequent coupling with Fmoc-Val-OH (128 mg, 0.378 mmol) to the β-hydroxyl group of Thr was performed using the DIPCDI (59.2 μL, 0.378 mmol)–DMAP (3.1 mg, 0.0252 mmol) method in CH_2Cl_2 (1.5 mL) for 16 h (×2), followed by coupling with another Val residue, *N*-acetylation using Ac_2O (17.8 μL, 0.15 mmol)–TEA (17.6 μL, 0.126 mmol), TFA (4.7 mL)–thioanisole (128 μL)–*m*-cresol (128 μL)–distilled water (128 μL) treatment for 90 min at rt, concentration in vacuo, Et_2O wash, centrifugation, suspension in water, and lyophilization to give the crude *O*-acyl isopeptide **4**·TFA (51.5 mg). This crude peptide (20 mg) was dissolved in MeOH, filtered using a 0.46 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, affording the desired *O*-acyl isopeptide **4**·TFA as a white amorphous powder (9.2 mg, 28.0%). HRMS (FAB): calcd for $C_{26}H_{49}N_6O_7$ $(M+H)^+$: 557.3663, found: 557.3666; HPLC analysis at 230 nm: purity was higher than 95%.
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7. EDC·HCl (104 mg, 0.539 mmol) was added to a stirring solution of *N*-(*t*-butoxycarbonyl)-L-threonine benzyl ester (Boc-Thr-OBzl)[11] (139 mg, 0.449 mmol), *N*-(9*H*-fluoren-9-ylmethoxycarbonyl)-L-valine (Fmoc-Val-OH, 183 mg, 0.539 mmol), and DMAP (5.5 mg, 0.045 mmol) in dry $CHCl_3$ (10 mL) at 0 °C. The mixture was slowly warmed to rt over 2 h, stirred additionally for 5 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 $MgSO_4$ and the solvent was removed in vacuo. The resulting oil was purified by silica gel column chromatography (AcOEt–hexane 1:4) to yield Boc-Thr(Fmoc-Val)-OBzl (266 mg, 0.422 mmol, 94%). Epimerization during the above reaction was not detected, which was confirmed by comparison with independently synthesized D-valine derivative. After that, Pd/C was added (12 mg) to the stirring solution of the Boc-Thr(Fmoc-Val)-OBzl (236 mg, 0.374 mmol) in AcOEt (10 mL), and the reaction mixture was vigorously stirred for 3 h (it is worthy to notice that the higher amount of

Pd/C caused by-products formation). 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 and then the final product was washed out by methanol to give pure **5** (186 mg, 0.346 mmol, 92%). HRMS (FAB): calcd for $C_{29}H_{36}N_2O_8Na$ $(M+Na)^+$: 563.2369, found: 563.2373; HPLC analysis at 230 nm: purity was higher than 95%; NMR (CD_3OD, 400 MHz): δ 7.79 (d, $^3J(H,H) = 7.3$ Hz, 2H, CH), 7.75–7.66 (m, 2H, CH), 7.38 (t, $^3J(H,H) = 7.5$ Hz, 2H, CH), 7.33–7.29 (m, 2H, CH), 5.44–5.41 (m, 1H, CH), 4.38 (d, $^3J(H,H) = 7.0$ Hz, 2H, CH_2), 4.25–4.22 (m, 2H, CH), 4.05–4.01 (m, 1H, CH), 2.11–2.02 (m, 1H, CH), 1.44 (s, 9H, CH_3), 1.25 (d, $^3J(H,H) = 6.4$ Hz, 3H, CH_3), 0.91, 0.89 (2d, $^3J(H,H) = 7.7$, 7.0 Hz, 6H, CH_3).

8. After the preparation of the H-Val-Val-NH-resin (Rink amide AM resin, 100 mg, 0.071 mmol) in the same manner described in the synthesis of **3** using the conventional method, Boc-Thr(Fmoc-Val)-OH **5** (100 mg, 0.18 mmol) was coupled in the presence of DIPCDI (29.0 μL, 0.18 mmol) and HOBt (28.4 mg, 0.18 mmol) in DMF (1.5 mL). Subsequent coupling with Fmoc-Val-OH (62.8 mg, 0.18 mmol), *N*-acetylation using Ac_2O (10.5 μL, 0.11 mmol)–TEA (10.4 μL, 0.071 mmol), TFA (2.47 mL)–thioanisole (66.7 μL)–*m*-cresol (66.7 μL)–distilled water (66.7 μL) treatment for 90 min at rt, concentration in vacuo, Et_2O wash, centrifugation, suspension in water, and lyophilization gave the pure *O*-acyl isopeptide **4**·TFA as a white amorphous powder (21.2 mg, 44.5%). HRMS (FAB): calcd for $C_{26}H_{49}N_6O_7$ $(M+H)^+$: 557.3663, found: 557.3666; HPLC analysis at 230 nm: purity was higher than 95%. Purified *O*-acyl isopeptide **4**·TFA (3.0 mg) was then dissolved in phosphate-buffered saline (PBS, pH 7.4, 3 mL) at rt and stirred overnight at rt. The resultant precipitate was centrifuged and washed with water and MeOH followed by drying in vacuo to give **3** as a white powder. Yield: 2.4 mg (96%); HRMS (FAB): calcd for $C_{26}H_{49}N_6O_7$ $(M+H)^+$: 557.3663, found: 557.3667; 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 product was identical to that of **3**, which was synthesized independently by the conventional method.[4]
9. To PBS (495 μL, pH 7.4) were added DMSO (4 μL) and solution of **4** (1 μL, 10 mM in DMSO), and the mixture was stirred at rt. At the desired time points, DMSO (500 μL) was added to the samples and the mixture was directly analyzed by RP-HPLC. HPLC was performed using a C18 (4.6 × 150 mm; YMC Pack ODS AM302) reverse-phase column with a binary solvent system: linear gradient of CH_3CN (0–100%, 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1}, detected at UV 230 nm.
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