Modification of Oligopeptides on Aspartic Acid or Lysine Residues by Solid-Phase Synthesis through On-Resin Side-Chain Conjugation

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1. General Information

1.1 Materials

The reagents and solvents used for building blocks and peptide synthesis were purchased as follows: Fmoc protected amino acids, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC-HCl), diisopropylcarbodiimide (DIC), O-benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), (benzotriazol-1-yl)tris(pyrrrolidinophosphonium hexafluorophosphate (PyBOP), 1-hydroxybenzotriazole (HOBt), N,N-dimethylaminopyridine (DMAP) were purchased from GL Biochem (Shanghai) Ltd. Wang resin, ninhydrin, trisopropylsilane (TIS), tetrakis (triphenylphosphine) palladium [Pd(PPh)4] and phenylsilane (PhSiH3) were purchased from Aladdin (Shanghai). 5-carboxyfluorescein-N-hydroxysuccinimide ester (FAM-OSu), 7-methoxycoumarin-4-acetic acid N-hydroxysuccinimide ester (MCA-OSu), fluorescein isothiocyanate (FITC), biotin N-hydroxysuccinimide ester (biotin-OSu) and glucosamine hydrochloride were purchased from MERYER (Shanghai). Biotin hydrazide, 2-biotinamidoethylamine and dansylhydrazine were purchased from HEOWNS (Tianjing). Polyethylene glycol derivatives were laboratory-made. HPLC-level methanol and acetonitrile and other solvents were purchased from Yuwang group (Shandong).

1.2 Instrumentation

The model of Lyophilizer is LGJ-10 from Beijing SongYuan HuaXing Technology and Development Co., Ltd. The model of multipurpose vibrator is HY-4 from RongHua Instrument (Changzhou). The chemical structures of final products were confirmed by a high-resolution Bruker microTOF Q mass spectrometer. HPLC were performed using P230 II high performance liquid chromatography (Dalian Elite Analytical Instruments Co., Ltd.).

2. Experimental Section

2.1 Synthesis of Fmoc-Tyr(t-Bu)-Resin

The Wang resin (3 mmol, 1.82 g, 1.63 mmol/g) was swelled in DMF (15 mL) for 30 minutes. The solvent was filtered off and the resin was washed with DMF (3×15 mL). Fmoc-Tyr(t-Bu)-OH (12 mmol, 5.51 g), HOBt (12 mmol, 1.62 g), and EDC·HCl (12 mmol, 2.29 g) were dissolved in DMF (15 mL). The reaction mixture was shaken at room temperature for 5 minutes. Then DMAP (1.2 mmol, 146 mg) was added to the suspension. The reaction mixture was shaken for 4 hours. The solvent was filtered off and the resin was washed with DMF (3×15 mL). The resin was suspended in acetic anhydride/pyridine (5:1 v/v, 12 mL) for 12 hours. The solvent was filtered off and the resin was washed with DMF (3×15 mL).

2.2 Synthesis of Arg-Lys-Asp(R)-Val-Tyr

Peptide was synthesized by SPPS using Fmoc/t-Bu strategy on 100 mg of Fmoc-Tyr(t-Bu)-Resin (0.05 mmol, 0.52 mmol/g). The resin was suspended in 20% piperidine in DMF (v/v, 5 mL) for 30 minutes. The solvent was filtered off and the resin was washed with DMF (3×5mL). For the attachment of the second amino acid, Fmoc-Val-OH (0.2 mmol, 68 mg) was anchored to the resin using EDC·HCl (0.2 mmol, 38 mg) and HOBt (0.2 mmol, 27 mg) in DMF (4 mL) for 2 hours. A negative ninhydrin test (colorless) indicated that the reaction was complete. After removal of the Fmoc group in Fmoc-Val-Tyr-Resin, the other Fmoc-protected amino acid derivatives were anchored to the resin in the following sequence: Fmoc-Asp(OAll)-OH (0.2 mmol, 79 mg), Fmoc-Lys(Boc)-OH (0.2 mmol, 94 mg), Fmoc-Arg(Pbf)-OH (0.2 mmol, 130 mg). After attachment of the Fmoc-Arg(Pbf)-OH, the Fmoc group on the N-terminus was not removed.

After synthesis of the fully protected peptide on the resin, the resin was reacted with Pd(PPh3)4 (0.2 mmol, 231 mg) and PhSiH3 (0.5 mmol, 56 mg) in DCM (4 mL) to remove the OAll group. The reaction mixture was shaken at room temperature for 12 hours. The solvent was filtered off and the resin was washed with DCM (3×5 mL) and DMF (3×5 mL).
The side-chain modification was performed using different modifiers under corresponding conditions (see Scheme 1 in the paper). For TA₁: MPEG₆-OH (0.2 mmol, 59 mg), HOBt (0.2 mmol, 27 mg), DIC (0.2 mmol, 25 mg), DMAP (0.1 mmol, 12 mg) were dissolved in DMF (4 mL). For TA₂-TH₆: R-H (0.2 mmol), HOBt (0.2 mmol, 27 mg), HBTU (0.2 mmol, 75 mg) and DIPEA (0.2 mmol, 26 mg) were dissolved in DMF (4 mL). For all reactions, the reaction mixture was shaken at room temperature for 24 hours. The solvent was filtered off and the resin was washed with DCM (3×5 mL) and DMF (3×5 mL). R-H included MPEG₆-NH₂ (0.2 mmol, 57 mg), glucosamine hydrochloride (0.2mmol, 43 mg), 2-biotinamidoethylamine (0.2mmol, 65 mg), biotin hydrazide (0.2mmol, 52 mg) and dansylhydrazine (0.2mmol, 53 mg).

The resin was suspended in 20% piperidine in DMF (v/v, 5 mL) for 30 minutes. The solvent was filtered off and the resin was washed with DMF (3×5 mL). The resin was shrunk with MeOH (2×5 mL). The peptide was cleaved from the resin with 50% TFA in DCM (v/v, 5 mL) for 2 hours. The reaction mixture was filtered and the filtrate was concentrated under vacuum. The residue was dissolved in water, then freeze-dried to give the solid.

2.3 Synthesis of Arg-Lys(R′)-Asp-Val-Tyr

Peptide was synthesized by SPPS using Fmoc/t-Bu strategy on 100 mg of Fmoc-Tyr(t-Bu)-Resin (0.05 mol, 0.52 mmol/g). The resin was suspended in 20% piperidine in DMF (v/v, 5 mL) for 30 minutes to remove the Fmoc group. The solvent was filtered off and the resin was washed with DMF (3×5 mL). For the attachment of the second amino acid, Fmoc-Val-OH (0.2 mmol, 68 mg) was anchored to the resin using EDC·HCl (0.2 mmol, 38 mg) and HOBt (0.2 mmol, 27 mg) in DMF (4 mL) for 2 hours, when a negative ninhydrin test was detected. After removal of the Fmoc group, attachment of Fmoc-Asp(0Bu-t)-OH (0.2 mmol, 82 mg) to the resin and removal of the Fmoc group were performed using the same reaction conditions as Fmoc-Val-OH. Then, Fmoc-Lys(Mtt)-OH (0.2 mmol, 125 mg) was anchored to the resin using HOBT (0.2 mmol, 27 mg), HBTU (0.2 mmol, 75 mg) and DIPEA (0.2 mmol, 26 mg) in DMF (4 mL) for 2 hours, when a negative ninhydrin test was detected. After removal of the Fmoc group, attachment of Fmoc-Arg(Pbf)-OH (0.2 mmol, 130 mg) to the resin was performed using the same reaction conditions as Fmoc-Lys(Mtt)-OH.

The resin was treated with 5% TFA and 5% TIS in DCM (v/v, 5 mL, 2×5 minutes) to remove the Mtt group. A positive ninhydrin test (dark blue) indicated the reaction was complete. Then the resin was washed with DMF (3×5 mL), DCM (3×5 mL) and DMF (3×5 mL).

The side-chain modification was performed using different modifiers under corresponding conditions (see Scheme 1 in the paper). For TL₁-TL₂: R′-OH (0.2 mmol), HOBt (0.2 mmol, 27 mg), HBTU (0.2 mmol, 75 mg) and DIPEA (0.2 mmol, 26 mg) were dissolved in DMF (4 mL). The reaction mixture was shaken at room temperature for 4 hours. The solvent was filtered off and the resin was washed with DCM (3×5 mL) and DMF (3×5 mL). R′-OH included MPEG₆-CH₂-COOH (0.2 mmol, 71mg) and MPEG₆-(CH₃)₂-COOH (0.2mmol, 82 mg). For TL₃-TH₆: R′-OSu (0.2 mmol) or FITC (0.2 mmol, 78 mg) was dissolved in DMF (4 mL), then DIPEA (0.2 mmol, 26 mg) was added. The reaction mixture was shaken at room temperature for 8 hours. The reaction using FITC was shielded from light. Then, the solvent was filtered off and the resin was washed with DCM (3×5 mL) and DMF (3×5 mL). R′-OSu included biotin-OSu (0.2 mmol, 68 mg), MCA-OSu (0.2 mmol, 66 mg) and FAM-OSu (0.2 mmol, 95 mg).

The resin was suspended in 20% piperidine in DMF (v/v, 5 mL) for 30 minutes. The solvent was filtered off and the resin was washed with DMF (3×5 mL). The resin was shrunk with MeOH (2×5 mL). The peptide was cleaved from the resin with 50% TFA in DCM (v/v, 5 mL) for 2 hours. The reaction mixture was filtered and the filtrate was concentrated under vacuum. The residue was dissolved in water, then freeze-dried to give the solid.

2.4 Synthesis of TL₁ and TL₂ using Fmoc-Lys(MPEG₆(CH₂)₆CO)-OH or Fmoc-Lys(MPEG₆(CH₃)₂CO)-OH

Fmoc-Lys(MPEG₆(CH₂)₆CO): To a solution of MPEG₆(CH₂)₆COOH (5 mmol, 1.8 g) or MPEG₆(CH₃)₂COOH (5 mol, 2.1 g) in DCM (10 mL), HOSu (6 mmol, 0.7 g) and EDC·HCl (6 mmol, 1.2 g) was added. The reaction mixture was stirred at room temperature for 4 hours. Then, the solution was washed with 5% HCl solution (5 mL),
water (5 mL), 5% NaHCO₃ solution (5 mL) and water (5 mL). The organic layer was dried over anhydrous Na₂SO₄ and filtered, then the filtrate was concentrated under vacuum to afford active ester. To a solution of Fmoc-Lys-OH·HCl (5 mol, 2.0 g) in carbonate buffer (pH 9, 20 mL), the active ester was added. The reaction mixture was stirred at room temperature for 1 hours. Then, the aqueous solution was extracted with DCM (3×5 mL). The combined organic layer was dried over anhydrous Na₂SO₄ and filtered, then the filtrate was concentrated under vacuum to afford Fmoc-Lys(MPEG₆(CH₂)₂CO)-OH (2.4 g, yield 69%) or Fmoc-Lys(MPEG₆(CH₂)₅CO)-OH (2.8 g, yield 74%).

**TL₁ and TL₂**: Peptide was synthesized by SPPS using Fmoc/t-Bu strategy on 100 mg of Fmoc-Tyr(t-Bu)-Resin (0.05 mmol, 0.52 mmol/g). The resin was suspended in 20% piperidine in DMF (v/v, 5 mL) for 30 minutes. The solvent was filtered off and the resin was washed with DMF (3×5 mL). For the attachment of the second amino acid, Fmoc-Val-OH (0.2 mmol, 68 mg) was anchored to the resin using EDC·HCl (0.2 mmol, 38 mg) and HOBt (0.2 mmol, 27 mg) in DMF (4 mL) for 2 hours. A negative ninhydrin test (colorless) indicated that the reaction was complete. After removal of the Fmoc group, the other Fmoc-protected amino acid derivatives were anchored to the resin in the following sequence: Fmoc-Asp(Obu-t)-OH (0.2 mmol, 79 mg), Fmoc-Lys(MPEG₆(CH₂)₂CO)-OH (0.2 mmol, 141 mg) or Fmoc-Lys(MPEG₆(CH₂)₅CO)-OH (0.2 mmol, 152 mg), Fmoc-Arg(Pbf)-OH (0.2 mmol, 130 mg). After attachment of Fmoc-Arg(Pbf)-OH, the resin was suspended in 20% piperidine in DMF (v/v, 5 mL) for 30 minutes. The solvent was filtered off and the resin was washed with DMF (3×5 mL). The resin was shrunk with MeOH (2×5 mL). The peptide was cleaved from the resin with 50% TFA in DCM (v/v, 5 mL) for 2 hours. The reaction mixture was filtered and the filtrate was concentrated under vacuum. The residue was dissolved in water, then freeze-dried to give the solid (TL₁: 42 mg, purity 46%; TL₂: 39 mg, purity 62%). The HPLC yield of TL₁ and TL₂ was 37% and 43% respectively.

2.5 Analysis and identification of thymopentin derivatives

The desired modified peptides were analyzed by analytical RP-HPLC (SinoChrom ODS-BP column (4.6 mm×250 mm, 5 μm); 0.1% TFA in H₂O and 0.1% TFA in CH₃CN (5%-95%, 60 minutes); 1 mL·min⁻¹; 220 nm) and HR-ESI-MS analysis was employed to confirm the identity of the peptides. The HPLC yields were calculated according to the HPLC purities and the mass of the crude peptides. Finally, the crude product was purified by preparative RP-HPLC (SinoChrom ODS-BP column (20 mm×250 mm, 5 μm); 0.1% TFA in H₂O and 0.1% TFA in CH₃CN (5%-95%, 60 minutes); 16 mL·min⁻¹; 220 nm). The analytical data of thymopentin derivatives were shown in Table S1.
Table S1. RP-HPLC and HR-ESI-MS analytical data of thymopentin derivatives.

<table>
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<tr>
<th>Compound</th>
<th>Crude product (mg)</th>
<th>Purity of crude product (%)</th>
<th>HPLC yield (%)</th>
<th>Pure product (mg)</th>
<th>Isolated yield (%)</th>
<th>Purity of pure product (%)</th>
<th>Retention time (min)</th>
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^a [M+H]^+

^b [M+H+Na]^2+
3. HPLC chromatograms and mass spectrometry data

**Figure S1.** HPLC chromatogram of TA1.

**Figure S2.** The HR-ESI-MS of TA1.

HR-ESI-MS: m/z [M+2H]²⁺ calcld for C₄₃H₇₅N₉O₁₅: 479.7770; found: 479.7806.
Figure S3. HPLC chromatogram of TA₂.

Figure S4. The HR-ESI-MS of TA₂.

HR-ESI-MS: m/z [M+H]^+ calcd for C_{43}H_{76}N_{10}O_{14}: 957.5615; found: 957.5636.
**Figure S5.** HPLC chromatogram of TA3.

**Figure S6.** The HR-ESI-MS of TA3.

HR-ESI-MS: m/z [M+2H]^2⁺ calc for C₃₆H₆₀N₁₀O₁₃: 421.2243; found: 421.2239.
Figure S7. HPLC chromatogram of TA4.

Figure S8. The HR-ESI-MS of TA4.

HR-ESI-MS: m/z [M+H]^+ calcd for C_{42}H_{69}N_{13}O_{10}S: 948.5084; found: 948.5087.
Figure S9. HPLC chromatogram of TAs.

Figure S10. The HR-ESI-MS of TAs.

HR-ESI-MS: m/z [M+2H]^{2+} calcd for C₄₀H₆₅N₁₃O₁₀S: 460.7428; found: 460.7495.
Figure S11. HPLC chromatogram of TA₆.

Figure S12. The HR-ESI-MS of TA₆.

Figure S13. HPLC chromatogram of TL1.

Figure S14. The HR-ESI-MS of TL1.

HR-ESI-MS: m/z [M+2H]$^{2+}$ calcld for C$_{45}$H$_{77}$N$_9$O$_{17}$: 508.7791; found: 508.7812.
Figure S15. HPLC chromatogram of TL2.

Figure S16. The HR-ESI-MS of TL2.
HR-ESI-MS: m/z [M+H+Na]^{2+} calcd for C_{49}H_{85}N_{9}O_{17}: 547.8014; found: 547.7995.
Figure S17. HPLC chromatogram of TL3.

Figure S18. The HR-ESI-MS of TL3.

HR-ESI-MS: m/z [M+2H]^2+ calcd for C_{40}H_{63}N_{11}O_{11}S: 453.7287; found: 453.7282.
Figure S19. HPLC chromatogram of TL₄.

Figure S20. The HR-ESI-MS of TL₄.

HR-ESI-MS: m/z [M+2H]²⁺ calcd for C₅₂H₅₇N₉O₁₃: 448.7111; found: 448.7115.
Figure S21. HPLC chromatogram of TL5.

Figure S22. The HR-ESI-MS of TL5.

HR-ESI-MS: m/z [M+2H]^2+ calcd for C_{51}H_{59}N_{9}O_{15}: 519.7143; found: 519.7118.
Figure S23. HPLC chromatogram of TL₆.

Figure S24. The HR-ESI-MS of TL₆.

HR-ESI-MS: m/z [M+2H]²⁺ caleed for C₅₁H₆₀N₁₀O₁₄S: 535.208; found: 535.2041.