Supporting Information
For
Improved Fmoc SPPS of oxytocin with high bioactivity

Pengcheng Sun, Wenli Tang, Yu Huang, Bi-Huang Hu*
College of Oceanography, Hainan University, Haikou, Hainan Province, 570228, China.
E-mail: bhhu@hainu.edu.cn; Tel: +86 0898 66289567
* corresponding author.

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1. General Information:

Materials and methods

Rink Amide-AM Resin (loading: 0.55 mmol/g, 0.37 mmol/g, 0.87 mmol/g, 100-200 mesh) was purchased from Tianjin Nankai Hecheng Science & Technology Co., Ltd., Tianjin City, China. Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Cys(Acm)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Ile-OH, Fmoc-Tyr(tBu)-OH, Boc-Cys(Acm)-OH, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyleniumhexafluorophosphate (HBTU), N,N-diisopropylethylamine (DIEA), triisopropylsilane (TIS) were purchased from Suzhou Highfine Biotech. Co. Ltd, Jiangsu Province, China. The authentic Oxytocin R.S. (European pharmacopoeia reference standard, code: 00700000, Batch: 5.0, Id: 00M420) was purchased from EDQM, Council of Europe. Silica gel for RH-HPLC (C18, 10 µm, 100 Å, Fuji, Japan), N,N-Dimethylformamide (DMF), acetonitrile (ACN), iso-Propyl alcohol (IPA), trifluoroacetic acid (TFA) were from Shaanxi Xintong chemical industry co. Ltd., Xian City, Shaanxi Province. Piperazine, Iodine, NaH2PO4, Ninhydrin were from Tianjin Damao chemical reagents co. Ltd.

Analytical HPLC: Agilent 1260 Infinity, UV detection at 220 nm, column (kromasil C18, 5µm, 100Å, 4.6×250 nm). Oxytocin and the analyzed impurities were eluted using gradient elution with a binary solvent system at 25°C and a flow rate of 1.0 ml/min: eluent A (0.1 M NaH2PO4/H2O), eluent B (ACN-H2O=1:1); gradient (0-30 min): 70% A-40% A. Preparative RP-HPLC: Agilent Prepstar SD-1, UV detection at 220 nm, column (Fujii C18, 10 µm, 100 Å, 50×250 mm), flow rate at 50.0 ml/min; eluent A (0.1 M NaH2PO4/H2O), eluent B (ACN-H2O=1:1); gradient (0–60 min): 60%A-50%A.

2. Synthetic Procedures:

Oxytocin synthesis: Oxytocin was synthesized by SPPS using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on 5 g of Rink Amide-AM Resin (loading: 0.55 mmol/g). The SPPS reactions were carried out in a 50 ml reaction vessel. After each step in the formation of the peptide, solvents and soluble reagents were removed under vacuum. The Fmoc groups were removed by treatment with 5% piperazine/DMF or 20 min. At this point, a positive ninhydrin test (dark blue) indicated the presence of free amino groups.

For the attachment of the first amino acid, Fmoc-Gly-OH (2 eq.,) was anchored to the resin using HBTU/DIEA in a 1.9 eq/2.4 eq. ratio, respectively in DMF for 2 hours. A negative ninhydrin test indicated that the reaction was complete. After removal of the Fmoc group in Fmoc-Gly-Resin, the following L-protected amino acid derivatives were used in the following sequence: Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Cys(Acm)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Ile-OH, Fmoc-Tyr(tBu)-OH, and Boc-Cys(Acm)-OH.

Chain extension was carried out by coupling the Fmoc amino acids with Fmoc amino acid-HBTU/DIEA (2: 1.9 : 2.4) in DMF for 1.5 h. After synthesis of the fully protected peptide on the resin, the cyclization reaction (removal of S-Acm and disulfide formation) was carried out on resin with iodine (3.5 g) in DMF (35 ml) for 2.5 h. The iodine solution was removed, and the peptide resin was washed with DMF (30ml×4) and IPA (30 ml×4), and dried in vacuo. Finally, the peptide-resin was treated with a mixture of 20 ml TFA/triisopropylsilane/H2O (95:2.5:2.5) for 30 min each time (20 ml×6 times). The oxytocin/TFA solution was collected and evaporated, and ethyl acetate (150 ml) was added. After storage at -20°C overnight, the crude oxytocin was collected by filtration, washed with ethyl acetate and dried in vacuo, yielding 2.4 g of crude oxytocin (yield: 85%). The purity was determined by HPLC (tR= 13.6min, 92% purity, Fig. S1).

Crude oxytocin purification: The crude oxytocin (0.57 g) was dissolved in water (50 ml) and passed
through a 0.45 µm filter. The filtrate was loaded and subjected to preparative RP-HPLC with collection of the fractions containing oxytocin with a purity > 99%. After the organic solvent was removed, the resulting concentrated solution was desalted with a preparative RP-HPLC column: eluent A (0.1% acetic acid), eluent B (ACN); gradient of 90%A-10%A in 40 min. After oxytocin was purified and desalted, lyophilization afforded 1.2 g of oxytocin (99.3%, Fig. S2). ESI-MS (C₄₃H₆₆N₁₂O₁²S₂, Fig. S3): m/z calculated: 1006.44; found: 1007.50 [M+H]+. Oxytocin bioactivity: 588 IU/mg, measured with the method in the “European pharmacopeia 9.0, 2017, 3250-3251.” On the basis of EP 9.0, 1mg of oxytocin peptide is equivalent to 600 IU of biological activity, we determined the biological activity by HPLC with external standard method; oxytocin R.S. (European pharmacopoeia reference standard) and our synthetic oxytocin were separately injected into HPLC system, according to their peak area, we can calculate the content of synthetic oxytocin (C₄₃H₆₆N₁₂O₁²S₂) from the declared content of C₄₃H₆₆N₁₂O₁²S₂ in oxytocin R.S..

Synthesis of oxytocin with methods 1 and 2:
Method 1: Reactions via SPPS were carried out in a 10 ml reaction tube with 1 g of Rink Amide-AM Resin (loading: 0.55 mmol/g). After removal of the Fmoc groups on the resin by treatment with 8 ml of piperazine/DMF (5%), Fmoc-Gly-OH (2 eq.) was anchored to the resin with HBTU (1.9 eq.)/DIEA (2.4 eq.) in DMF for 2 hours. A negative ninhydrin test indicated the coupling reaction was completed. After removal of the Fmoc group in the C-terminal amino acid, chain extensions were carried out with 2 eq. of the same amino acid derivatives as described previously for the synthesis of oxytocin, except that the N-terminal amino acid was coupled using Fmoc-Cys(Acm)-OH. Each coupling step used HBTU and DIEA in a 1.9 eq./2.4 eq. ratio, respectively in 4 ml DMF for 1.5 h. Once the fully protected peptide was formed, the terminal Fmoc group was removed. The followed procedures are the same as in the synthesis of oxytocin.

Method 2: Identical to method 1 except that the N-terminal amino acid was coupled using (Boc-Cys(Trt)-OH). During the cyclization reaction, the terminal amino acid retained Boc protection.

Synthesis of possible oxytocin-related impurities a-f:
Impurity a: Synthesized by SPPS similar to the synthesis of oxytocin on 1 g of Rink Amide-AM Resin (loading: 0.55 mmol/g) in a 10 ml reaction tube. Once the fully protected peptide had been synthesized, it was subject to global deprotection and cleavage from the resin by treatment with a mixture of TFA-trisopropylsilane-H₂O (95:2.5:2.5) for 30 min time, (3 ml×6). The peptide was isolated by precipitation with cold diethyl ether, centrifuged, dissolved in water, and sequentially lyophilized. It was then characterized by ESI-MS: m/z calc. for H-Cys(Acm)-Tyr-Ile-Gln-Asn-Cys(Acm)-Pro-Leu-Gly-NH₂ (C₄₉H₇₈N₁₄O₁₄S₂,) 1150.53; found 1151.35 [M+H]+ (Fig. S4).

Impurity b: Synthesized with the same procedure as impurity a, except that Fmoc-Cys(Trt)-OH was used instead of Fmoc-Cys(Trt)-OH and the N-terminal amino acid of the truncated peptide chain was Fmoc-Tyr(tBu)-OH. ESI-MS: m/z calc. for H-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH₂ (C₄₀H₆₂N₁₁O₁₁S₁) 905.07; found 906.31 [M+H]+ (Fig. S5).

Impurity c: Synthesized in the same manner as impurity a, except that S-Trt protecting groups in the cysteine residues were used instead of S-Acm. ESI-MS: m/z calc. for H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH₂ (C₄₃H₆₆N₁₂O₁₂S₂) 1008.44; found 1009.30 [M+H]+ (Fig. S6).

Impurity d: Synthesized in the same manner as impurity a, except that Fmoc-Tyr(tBu)-OH was using for the coupling of the last residue. ESI-MS: m/z calc. for H-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH₂ (C₄₃H₆₆N₁₂O₁₂S₂) 976.48; found 977.33 [M+H]+ (Fig. S7).

Impurity e: Synthesized in the same manner as impurity a, except that the N-terminal amino acid building block was Boc-Cys(Trt)-OH instead of Boc-Cys(Trt)-OH. ESI-MS: m/z calc. for H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH₂ (C₄₆H₇₂N₁₃O₁₃S₂) 1079.29; found 1080.32 [M+H]+ (Fig. S8).
Impurity f: Synthesized in the same manner as impurity a, except that Fmoc-Cys(Trt)-OH was used instead of Fmoc-Cys(Acm)-OH. ESI-MS: \( m/z \) calcd. for H-Cys(Acm)-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH\(_2\) (C\(_{46}\)H\(_{72}\)N\(_{13}\)O\(_{13}\)S\(_2\)) = 1079.29; found: 1080.33 [M+H]\(^+\) (Fig. S9).

4. HPLC chromatograms and mass spectrometry data:

Fig. S1. HPLC chromatogram of crude oxytocin

Fig. S2. HPLC chromatogram of purified oxytocin
Fig. S3. ESI-MS spectrum of oxytocin
m/z calculated: 1006.44, found: 1007.50 [M+H]^+

H-Cys(Acm)-Tyr-Ile-Gln-Asn-Cys(Acm)-Pro-Leu-Gly-NH₂

Fig. S4. ESI-MS spectrum of impurity a
m/z calcd. for (C₄₉H₇₈N₁₄O₁₄S₂) 1150.53; found 1151.35 [M+H]^+
Fig. S5. ESI-MS spectrum of impurity b
m/z calcd. for \( \left( C_{40}H_{62}N_{11}O_{11}S_{1} \right) \) 905.07; found 906.31 [M+H]^+
Fig. S6. ESI-MS spectrum of impurity c
m/z calcd. for (C_{43}H_{66}N_{12}O_{12}S_{2}) 1008.44; found 1009.30 [M+H]^+
Fig. S7. ESI-MS spectrum of impurity d
m/z calcd. for \((C_{43}H_{68}N_{12}O_{12}S_{1})\) 976.48; found 977.33 [M+H]^+
Fig. S8. ESI-MS spectrum of impurity e

m/z calcd. for (C₄₆H₇₂N₁₃O₁₃S₂) 1079.29; found 1080.32 [M+H]^+
Fig. S9. ESI-MS spectrum of impurity f

m/z calcd. for (C_{46}H_{72}N_{13}O_{13}S_{2}): 1079.29; found: 1080.33 [M+H]^+