Automated Synthesis of Cyclic Disulfide-Bridged Peptides

Summary

Cyclic disulfide-bridged peptides can be prepared rapidly with good purity using microwave-enhanced SPPS. Synthesis of the peptide hormone oxytocin[1] was achieved in under 3 h with 69% purity. Preparation of a peptide agonist of BMP receptor activin-like kinase 3 (Alk3), THR-123[2], was completed in 3 h with 77% purity. CHEC-7[3], a neuroprotective peptide inhibitor of amyloidogenesis, was prepared in under 3 h with 80% purity. Finally, a peptide venom from cone snails containing two disulfide bridges (conotoxin-SI)[4] was synthesized in under 4 h with 67% purity.

Introduction

Cyclic peptides containing disulfides represent a class of compounds with a profound array of biological functions ranging from venoms to integral hormones.[5] The disulfide bond helps stabilize the secondary structure and conformation of peptides, which can contribute favorably to proteolytic stability and target affinity.[6] Because of their promising therapeutic potential, interest in the synthesis of cyclic disulfide-bridged peptides has grown steadily.

Peptides with disulfide bridges can be prepared by SPPS by using orthogonally-protected cysteine amino acids such as Fmoc-(S)-Cys(Mmt)-OH and Fmoc-(S)-Cys(STmp)-OH (**Figure 1**). The Cys(Mmt) group can be selectively deprotected using a dilute solution of trifluoroacetic acid (TFA), whereas the Cys(STmp) group is orthogonally deprotected using dithiothreitol (DTT) as a reducing agent. After deprotection, selective oxidation of the Cys thiol groups to form a disulfide bond can be achieved using N-chlorosuccinimide (NCS) as a mild oxidant.[7] Application of microwave energy to the synthesis of disulfide-bridged peptides allows for more efficient coupling which leads to rapid synthesis times and high purity (CarboMAX™).[8]

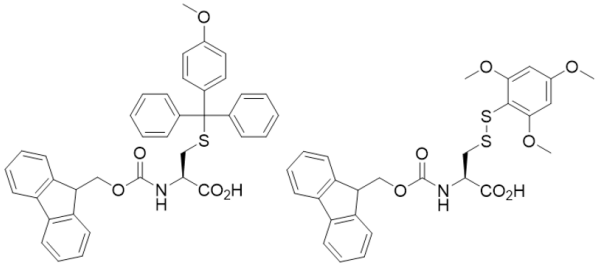

Figure 1: Left: Fmoc-(S)-Cys(Mmt)-OH; Right: Fmoc-(S)-Cys(STmp)-OH

Materials and Methods

Reagents

The following Fmoc amino acids were obtained from CEM Corporation (Matthews, NC) and contain the indicated side chain protecting groups: Ala, Arg(Pbf), Asn(Trt), Asp(OMpe), Gln(Trt), Gly, Ile, Leu, Lys(Boc), Phe, Pro, Ser(tBu), Tyr(tBu), and Val. Rink Amide ProTide™ LL resin and Cl-MPA ProTide™ LL resin were also obtained from CEM Corporation. Fmoc-(S)-Cys(Mmt)-OH and Fmoc-(S)-Cys(STmp)-OH were purchased from EMD Millipore (Burlington, MA). N-chlorosuccinimide (NCS), DL-Dithiothreitol (DTT), 4-methylmorpholine (NMM), N,N'-Diisopropylcarbodiimide (DIC), piperidine, piperazine, trifluoroacetic acid (TFA), 3,6-dioxa-1,8-octanedithiol (DODT), and

triisopropylsilane (TIS) were obtained from Sigma-Aldrich (St. Louis, MO). Dichloromethane (DCM), N,N-Dimethylformamide (DMF), 1-methyl-2-pyrrolidone (NMP), ethanol, anhydrous diethyl ether (Et_2O), acetic acid, HPLC grade water, and acetonitrile were obtained from VWR (West Chester, PA). LC-MS grade water (H_2O) and LC-MS grade acetonitrile (MeCN) were obtained from Fisher Scientific (Waltham, MA).

Peptide Synthesis: Oxytocin, CYIQNCPLG-NH_2

The peptide (**Figure 2**) was synthesized on a 0.10 mmol scale (disulfide formation was performed on a 0.05 mmol scale) using the CEM Liberty Blue™ automated microwave peptide synthesizer on 0.526 g Rink Amide ProTide LL resin (0.19 meq/g substitution). Deprotection was performed with 20% piperidine and 0.1 M Oxyma Pure in DMF. Coupling reactions were performed in 5-fold excess of 0.2 M Fmoc-AA with 1.0 M DIC and 1.0 M Oxyma Pure in DMF (CarboMAX).[8] Fmoc-(S)-Cys(Mmt)-OH was used for C. A solution of 2% TFA in DCM was used for deprotection of Cys(Mmt). Disulfide formation was achieved using a 25 mM solution of NCS in DMF. Cleavage was performed using the CEM Razor™ high-throughput peptide cleavage system with 92.5:2.5:2.5 TFA/H_2O/TIS/DODT. Following cleavage, the peptide was precipitated with Et_2O and lyophilized overnight.

Figure 2: Oxytocin

Peptide Synthesis: THR-123, CYFDDSSNVLCKKYRS-CO_2H

The peptide (**Figure 3**) was synthesized on a 0.10 mmol scale (disulfide formation was performed on a 0.05 mmol scale) using the CEM Liberty Blue automated microwave peptide synthesizer on 0.556 g Cl-MPA ProTide LL resin (0.18 meq/g substitution). Deprotection was performed with 20% piperidine and 0.1 M Oxyma Pure in DMF. Coupling reactions were performed in 5-fold excess of 0.2 M Fmoc-AA with 1.0 M DIC and 1.0 M Oxyma Pure in DMF (CarboMAX).[8] Fmoc-(S)-Cys(Mmt)-OH was used for C. A solution of 2% TFA in DCM was used for deprotection of Cys(Mmt). Disulfide formation was achieved using a 25 mM solution of NCS in DMF. Cleavage was performed using the CEM Razor high-throughput peptide cleavage system with 92.5:2.5:2.5 TFA/H_2O/TIS/DODT. Following cleavage, the peptide was precipitated with Et_2O and lyophilized overnight.

Figure 3: THR-123

Peptide Synthesis: CHEC-7, CHEAAQC-CO_2H

The peptide (**Figure 4**) was synthesized on a 0.10 mmol scale (disulfide formation was performed on a 0.05 mmol scale) using the CEM Liberty Blue automated microwave peptide synthesizer on 0.556 g Cl-MPA ProTide LL resin (0.18 meq/g substitution). Deprotection was performed with 10% piperazine in 1:9 ethanol/ NMP. Coupling reactions were performed in 5-fold excess of 0.2 M Fmoc-AA with 1.0 M DIC and 1.0 M Oxyma Pure in DMF (CarboMAX).[8] Fmoc-(S)-Cys(Mmt)-OH was used for C. A solution of 2% TFA in DCM was used for deprotection of Cys(Mmt). Disulfide formation was achieved using a 25 mM solution of NCS in DMF. Cleavage was performed using the CEM Razor high-throughput peptide cleavage system with 92.5:2.5:2.5 TFA/H_2O/TIS/DODT. Following cleavage, the peptide was precipitated with Et_2O and lyophilized overnight.

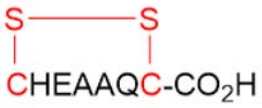

Figure 4: CHEC-7

Peptide Synthesis: Conotoxin-SI, ICCNPACGPKYSC-NH_2

The peptide (**Figure 5**) was synthesized on a 0.10 mmol scale (disulfide formation was performed on a 0.05 mmol scale) using the CEM Liberty Blue automated microwave peptide synthesizer on 0.526 g Rink Amide ProTide LL resin (0.19 meq/g substitution). Deprotection was performed with 20 % piperidine and 0.1 M Oxyma Pure in DMF. Coupling reactions were performed in 5-fold excess of 0.2 M Fmoc-AA with 1.0 M DIC and 1.0 M Oxyma Pure in DMF (CarboMAX).[8] Fmoc-(S)-Cys(Mmt)-OH was used for C, and Fmoc-(S)-Cys(STmp)-OH was used for C. A solution of 5% DTT and 0.1 M NMM in DMF was used to deprotect the Cys(STmp) group. The first disulfide was formed using a 25 mM solution of NCS in DMF. A solution of 2% TFA in DCM was used for the deprotection of Cys(Mmt). The second disulfide bond was formed using the same solution of NCS in DMF. Cleavage was performed using the CEM Razor high-throughput peptide cleavage system with 92.5:2.5:2.5 TFA/H_2O/TIS/DODT. Following cleavage, the peptide was

precipitated with Et_2O and lyophilized overnight.

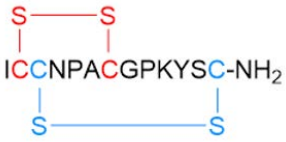

Figure 5: Conotoxin-SI

Peptide Analysis

The peptides were analyzed on a Waters Acquity UPLC system with PDA detector equipped with an Acquity UPLC BEH C8 column (1.7 mm and 2.1 x 100 mm). The UPLC system was connected to a Waters 3100 Single Quad MS for structural determination. Peak analysis was achieved on Waters MassLynx software. Separations were performed with a gradient elution of 0.05% TFA in (i) H_2O and (ii) MeCN.

Results

Microwave-enhanced SPPS of Oxytocin on the Liberty Blue automated microwave peptide synthesizer produced the target peptide in 69% purity (**Figure 6**).

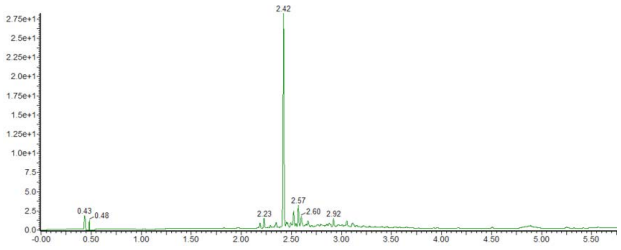

Figure 6: UPLC Chromatogram of Oxytocin

Microwave-enhanced SPPS of THR-123 on the Liberty Blue automated microwave peptide synthesizer produced the target peptide in 77% purity (**Figure 7**).

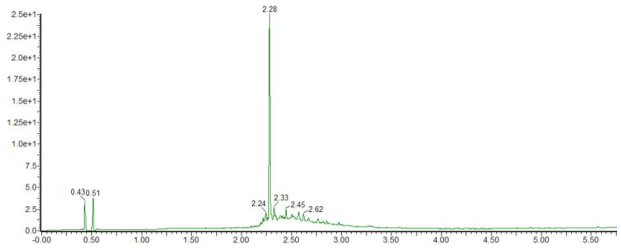

Figure 7: UPLC Chromatogram of THR-123

Microwave-enhanced SPPS of CHEC-7 on the Liberty Blue automated microwave peptide synthesizer produced the target peptide in 80% purity (**Figure 8**). Note: peaks at 5.72 min (see **Figure 9**) and 5.85 (see **Figure 10**) min both have the target peptide mass and are not the result of epimerization.

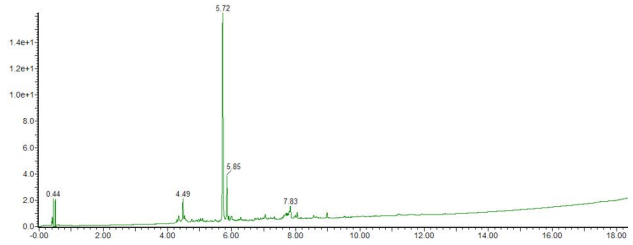

Figure 8: UPLC Chromatogram of CHEC-7

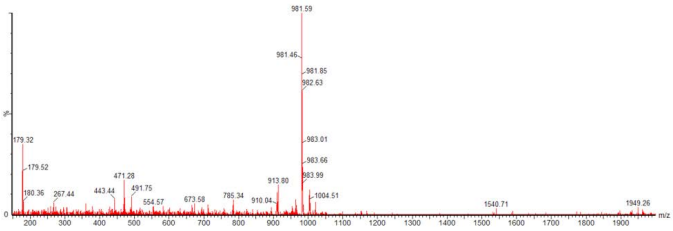

Figure 9: Mass spectrum of peak with retention time of 5.72 min

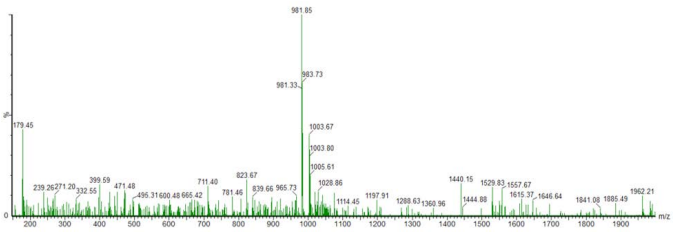

Figure 10: Mass spectrum of peak with retention time of 5.85 min

Microwave-enhanced SPPS of Conotoxin-SI on the Liberty Blue automated microwave peptide synthesizer produced the target peptide in 67% purity (**Figure 11**).

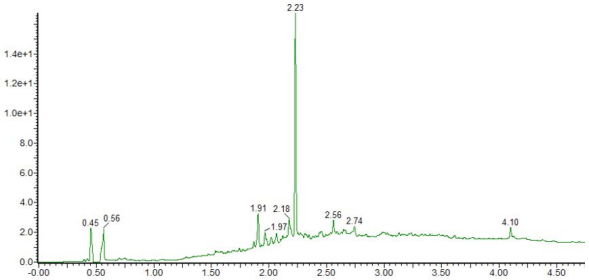

Figure 11: UPLC Chromatogram of Conotoxin-SI

Conclusion

Disulfide-bridged cyclic peptides can be synthesized rapidly and efficiently using automated microwave-enhanced SPPS. Conventional room temperature synthesis of oxytocin requires over 13 h to generate the disulfide-bridged peptide.[7] Using microwave-enhanced SPPS, the peptide was synthesized in under 3 h with 69% purity. Cyclic, disulfide-bridged peptides with C-terminal acids,THR-123 and CHEC-7, were quickly synthesized in high purity (77% and 80% yield respectively) in under 3 h. Conventional room temperature synthesis of conotoxin-SI, which contains two disulfide bridges, requires 20 h.[7] On the other hand, microwave-enhanced SPPS affords the peptide in under 4 h with a purity of 67%.

References

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(8) CEM Application Note (AP0124) - *"CarboMAX - Enhanced Peptide Coupling at Elevated Temperature."*

(9) CEM Technical Note (P/N: 600837) - *"Cl-MPA ProTide and Cl-TCP(Cl) ProTide Resin Loading and Protected Cleavage Procedures."*

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