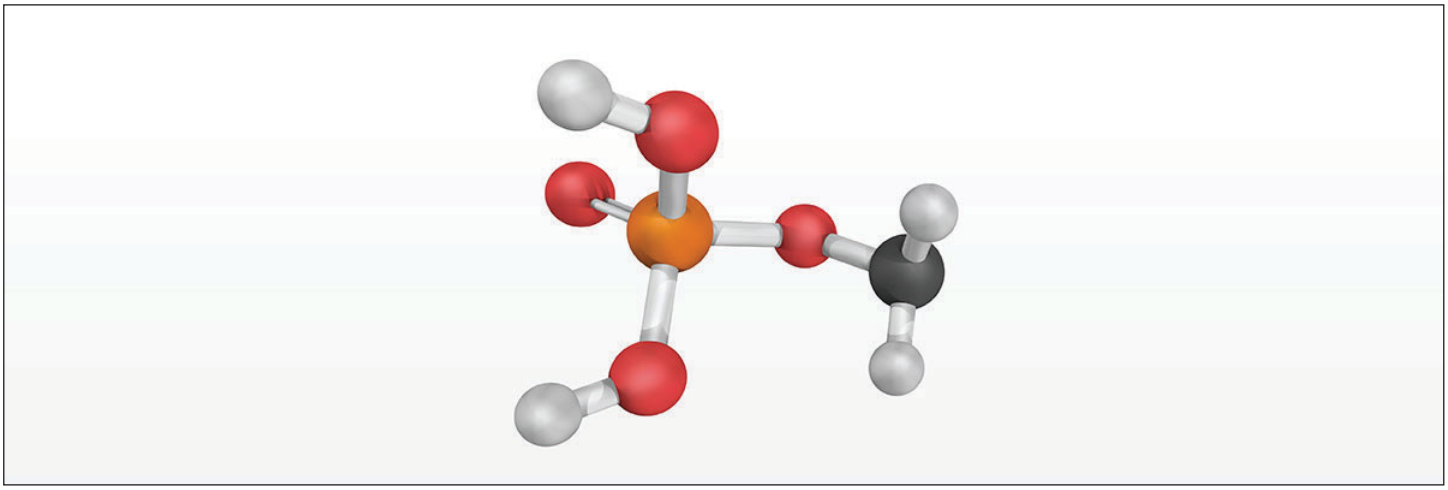
High Efficiency Synthesis of Phosphopeptides



Summary

Highly efficient synthesis of difficult phosphopeptides is demonstrated using the Liberty Blue™ automated microwave peptide synthesizer with CarboMAX™ coupling methodology. The use of microwave energy proved powerful for coupling bulky phosphoamino acid derivatives while the use of CarboMAX coupling methodology stabilized the acid-labile protected phosphate group at high temperature thereby minimizing side reactions. Using this approach the following phosphopeptides were each assembled in less than 2 hours: CTEDQY(pS) LVED-NH_2 (82% purity), CPSPA(pT)DPSLY-NH_2 (73% purity), and CSDGG(pY)MDMSK-NH_2 (62% purity).

Introduction

Phosphorylation is a post-translational modification (PTM) that involves the introduction of a phosphate group onto the threonine, serine, or tyrosine residues of a peptide or protein.[1] Enzymatic phosphorylation regulates the function of many proteins and guides numerous intracellular signal transduction cascades.[2] Due to the integral role of phosphorylation in biology, interest in the synthesis of phosphopeptides has steadily grown in recent years, especially as cell biologists and proteomics researchers seek to better understand and characterize the phosphoproteome.[3] Originally, production of phosphopeptides required synthesis of the entire peptide followed by a post-synthetic phosphorylation step. This post-synthesis approach was often difficult to perform and frequently yielded impure peptides.[4] The introduction of Fmoc-derived, monobenzyl-protected phosphoamino acids such as Fmoc-Thr(PO(OBzl)OH)-OH, Fmoc-Ser(PO(OBzl)OH)-OH, and Fmoc-Tyr(PO(OBzl)OH-OH (**Figure 1**) has significantly improved the synthesis process and enabled successful automation of SPPS for a wide range of phosphopeptides.[5]

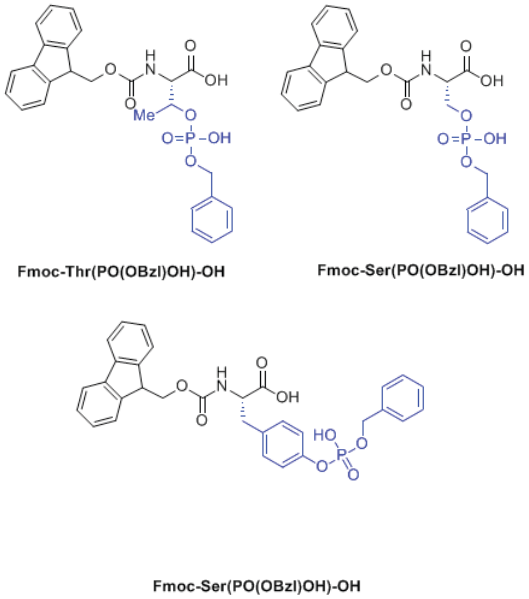


Figure 1: Fmoc-Derived, Monobenzyl-Protected Phosphoamino Acids

Microwave energy has been utilized for the synthesis of phosphopeptides. It has previously been shown that the first deprotection after coupling the phosphoamino acid should be

conducted at room temperature to prevent beta-elimination of the protected phosphate group. It has also been shown that phospho linkages are acid labile and therefore susceptible to removal at high temperature during carbodiimide coupling. CarboMAX methodology which employs the use of 0.4 equivalents of DIEA is powerful for stabilizing acid sensitive linkages including protected phosphate groups. CarboMAX was recently demonstrated to successfully synthesize a phosphopeptide containing 3 protected phosphate groups. Microwave coupling steps have been shown to improve direct and subsequent couplings of phosphorylated residues as well as minimize dephosphorylation and deletion products (CarboMAX).[6]

Materials and Methods

Reagents

All amino acids were obtained from CEM Corporation (Matthews, NC) and contained the following side chain protecting groups: Asn(Trt), Asp(OMpe), Cys(Trt), Gln(Trt), Glu(OtBu), Lys(Boc), Ser(tBu), Thr(tBu), Tyr(tBu). Fmoc monobenzyl-protected phosphoamino acids were also obtained from CEM Corporation (Matthews, NC): *N*-α-Fmoc-O-benzyl-L-phosphoserine (pS), *N*-α-Fmoc-O-benzyl-L-phosphotyrosine (pY), and *N*-α-Fmoc-O-benzyl-L-phosphothreonine (pT). Oxyma Pure and Rink Amide ProTide™ LL resin were obtained from CEM Corporation (Matthews, NC). N,N'-Diisopropylcarbodiimide (DIC) was obtained from CreoSalus (Louisville, KY). Piperidine was obtained from Alfa Aesar (Ward Hill, MA). Trifluoroacetic acid (TFA), 3,6-dioxa-1,8-octanedithiol (DODT), triisopropylsilane (TIS), N,N-Diisopropylethylamine (DIEA) thioanisole, and acetic acid were obtained from Sigma-Aldrich (St. Louis, MO). Dichloromethane (DCM), N,N-dimethylformamide (DMF), and anhydrous diethyl ether (Et_2O) were obtained from VWR (West Chester, PA). LCMS-grade water (H_2O), and LCMS-grade acetonitrile (MeCN) were obtained from Fisher Scientific (Waltham, MA).

Peptide Synthesis

The peptides were prepared at 0.1 mmol scale using the CEM Liberty Blue automated microwave peptide synthesizer on 0.500 g Rink Amide ProTide LL resin (0.20 meq/g substitution). Deprotection was performed with 20% piperidine and 0.1 M Oxyma Pure in DMF. Coupling reactions were performed with a 5-fold excess of Fmoc-AA-OH, 1.0 M DIC in DMF and 1.0 M Oxyma Pure/0.4 M DIEA in DMF (CarboMAX).[6] Cleavage was performed with 85:5:2.5:2.5:5 TFA/thioanisole/TIS/H_2O/DODT. On the CEM Razor™ parallel peptide cleavage system. Following cleavage, the peptide was precipitated in Et_2O and lyophilized overnight.

Method Programming: CTEDQY(pS)LVED-NH_2

Peptide to AA (Non-Tyr) Coupling:
Deprotection (4 mL) was added to the peptide-containing reaction vessel and the solution was microwaved for 1 min at 90 °C. Following deprotection, the peptide was washed with DMF (4 x 4 mL). Then, amino acid (2.5 mL), DIC (1 mL) and Oxyma Pure/DIEA (0.5 mL) were added to the reaction vessel and the solution was microwaved for 4 min at 90 °C. Upon completion, the reaction vessel was drained, preparing the peptide for the next coupling reaction. (**CTEDQ**Y**(pS)LVED**-NH_2)

Peptide to Tyr Coupling:
Deprotection (4 mL) was added to the peptide-containing reaction vessel and the solution was bubbled for 5 min at 23 °C, upon which the reaction vessel was drained and a second portion of deprotection (4 mL) was added. The solution was bubbled for an additional 10 min at 23 °C. Following deprotection, the peptide was washed with DMF (4 x 4 mL). Then, amino acid (2.5 mL), DIC (1 mL) and Oxyma Pure/DIEA (0.5 mL) were added to the reaction vessel and the solution was microwaved for 4 min at 90 °C. Upon completion, the reaction vessel was drained, preparing the peptide for the next coupling reaction. (CTEDQ**Y**(pS)LVED-NH_2)

Method Programming: CPSPA(pT)DPSLY-NH_2 & CSDGG(pY)MDMSK-NH_2

Peptide to AA Coupling:
Deprotection (4 mL) was added to the peptide-containing reaction vessel and the solution was microwaved for 1 min at 90 °C. Following deprotection, the peptide was washed with DMF (4 x 4 mL). Then, amino acid (2.5 mL), DIC (1 mL) and Oxyma Pure/DIEA (0.5 mL) were added to the reaction vessel and the solution was microwaved for 4 min at 90 °C. Upon completion, the reaction vessel was drained, preparing the peptide for the next coupling reaction.

Results

Microwave-enhanced SPPS of CTEDQY(pS)LVED-NH_2 on the Liberty Blue automated microwave peptide synthesizer produced the target peptide in 82% purity (**Figure 2**). No Tyr deletions were observed. Microwave-enhanced SPPS of CPSPA(pT)DPSLY-NH_2 produced the target peptide in 73% purity

(**Figure 3**). Truncation of amino acids 7–11 was observed in a small quantity. Microwave-enhanced SPPS of CSDGG(pY)MDMSK-NH_2 produced the target peptide in 62% purity (**Figure 4**). Minimal aspartimide formation and methionine oxidation were observed.

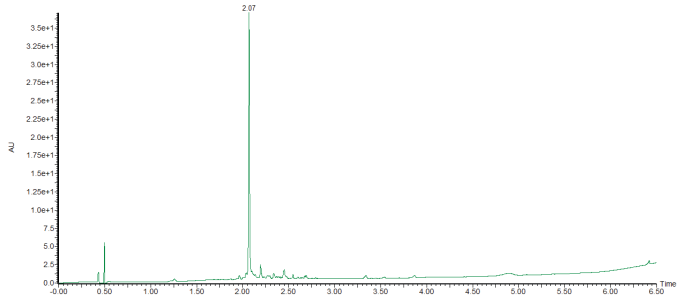


Figure 2: UPLC Chromatogram of CTEDQY(pS)LVED-NH_2

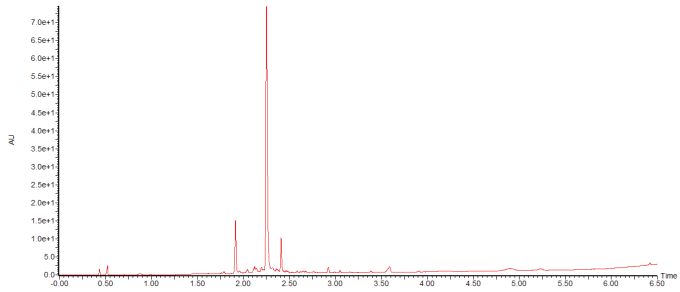


Figure 3: UPLC Chromatogram of CPSPA(pT)DPSLY-NH_2

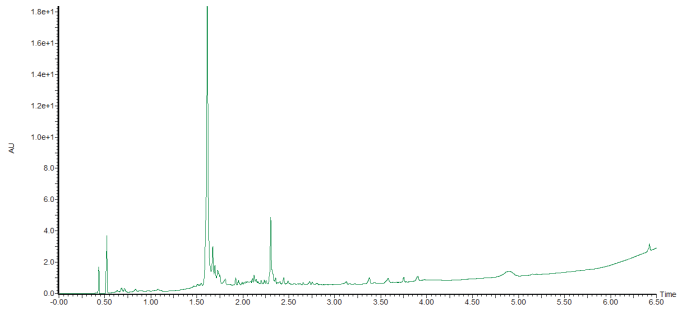


Figure 4: UPLC Chromatogram of CSDGG(pY)MDMSK-NH_2

Conclusion

Microwave-enhanced SPPS on the Liberty Blue automated microwave peptide synthesizer quickly and efficiently produces high quality phosphopeptides, such as CTEDQY(pS)LVED-NH_2, CPSPA(pT)DPSLY-NH_2, and CSDGG(pY)MDMSK-NH_2. Room temperature deprotection following the insertion of phosphoserine minimizes dephosphorylation in the synthesis of CTEDQY(pS)LVED-NH_2. Employment of Fmoc-Asp(OMpe)-OH minimizes the occurrence of aspartimide formation in susceptible sequences, especially CSDGG(pY)MDMSK-NH_2. Microwave-enhanced SPPS can improve direct and subsequent couplings of phosphorylated residues while minimizing undesired side reactions.

References

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