Ultrafast Peptide Synthesis at Elevated Temperature

Summary

The Liberty PRIME™ 2.0 microwave peptide synthesizer is the most advanced system available for peptide synthesis. It is based on the use of a new one-pot coupling and deprotection methodology for solid phase peptide synthesis that drastically reduces cycle time and waste usage to unprecedented levels. The system provides a complete cycle time of only 2 min 10 sec (for all 20 standard Fmoc amino acids) with only 8 mL of chemical waste produced, half the amount of CEM's highly efficient Liberty Blue 2.0 peptide synthesizer. The Liberty PRIME 2.0 HT24 is an ideal system for high-throughput peptide synthesis of both standard and complex peptides, with the ability to automatically synthesize up to 24 peptides in a day.

Introduction

Traditional solid phase peptide synthesis involves the use of iterative and separate deprotection and coupling steps with washing in-between. This is based on the assumption that undesirable amino acid insertions can occur without complete draining and washing between each step. In 2013 our group demonstrated that washing after the coupling step can be eliminated without effect on peptide purity.[1]

The Liberty PRIME takes this further by using a new one-pot coupling and deprotection process as shown in **Figure 1.**[2] This technique involves addition of the deprotection reagent (base) directly to the undrained post-coupling mixture. The ability to do this is based on the insight that faster reaction kinetics in the solution phase promote rapid hydrolysis or self-condensation of the active ester, thereby avoiding potential side reactions at the resin bound amino functionality. The Fmoc removal then proceeds uninterrupted at elevated temperature. An optimized use of reagents results in an essentially neutral reaction mixture towards the end of deprotection step. This new procedure offers several advantages such as (a) approximately 90% reduction in solvent requirement for the deprotection step, (b) 75% reduction in solvent requirement for post-deprotection washings, (c) faster deprotection step, since the microwave ramp time is not needed, and (d) shorter cycle time due to absence of post-coupling drain step.

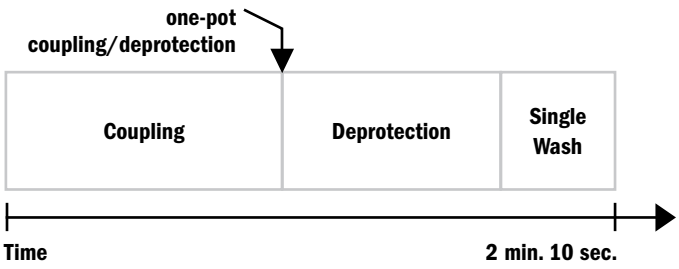

Figure 1. One-pot coupling/deprotection process used on Liberty PRIME 2.0.

Utilization of the one-pot coupling/deprotection methodology requires the ability to consistently add precise small volumes of concentrated base. To achieve this, the Liberty PRIME 2.0 incorporates a dedicated pumping module with the ability to rapidly add the deprotection reagent precisely at the end of the coupling step in volumes as low as 0.25 mL. The pre-calibrated pump module does not require on-going calibration thereby avoiding drifting delivery amounts.

Additionally, the Main Wash and the activator (Oxyma Pure) are also delivered through similar individual pumps within the module for improved performance.

Figure 2. Integrated pump module utilized on the Liberty PRIME 2.0.

The Liberty PRIME 2.0 also utilizes the CarboMAX™ coupling process that improves existing carbodiimide chemistry at elevated temperature by using a higher ratio of carbodiimide relative to the amino acid (2 equivalents).[3] This coupling chemistry has demonstrated the ability to provide a more rapid formation of the O-acylisourea intermediate thus leading to faster coupling rates while correspondingly reducing epimerization.4 Highly efficient 1 minute coupling times on the Liberty PRIME 2.0 utilizing CarboMAX chemistry at 105 °C provides exceptional acylation rates with minimal epimerization rates for all 20 standard Fmoc amino acids (including cysteine and histidine).

A selection of well-known difficult peptides were synthesized to demonstrate the performance of the Liberty PRIME automated microwave peptide synthesizer. **Note:** All peptides were synthesized on a 1st generation Liberty PRIME system.

Materials and Methods

Reagents

All Fmoc amino acids were obtained from CEM Corporation (Matthews, NC) and contained the following side chain protecting groups: Arg(Pbf), Asn(Trt), Asp(OMpe), Cys(Trt), Gln(Trt), Glu(OtBu), His(Boc), Lys(Boc), Ser(tBu), Thr(tBu), Trp(Boc) and Tyr(tBu). Oxyma Pure and Rink Amide ProTide® LL resin were also obtained from CEM Corporation. Rink Amide MBHA LL and Fmoc-Gly-wang LL resins were obtained from MilliporeSigma (Burlington, MA). N,N-Diisopropylcarbodiimide (DIC), Pyrrolidine, Trifluoroacetic acid (TFA), 3,6-Dioxa-1,8-octanedithiol (DODT), Triisopropylsilane (TIS) 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). HPLC-grade water (H_2O) and HPLC-grade acetonitrile (MeCN) were obtained from Fisher Scientific (Waltham, MA).

Peptide Synthesis

All peptides were synthesized on a 1st generation Liberty PRIME system (CEM Corp.) at 0.10 mmol scale using CarboMAX coupling with onepot coupling and deprotection. Coupling with Fmoc-AA-OH/DIC/Oxyma (5/10/5) in DMF was performed for 60 sec at 105 °C. The deprotection step was initiated by adding 0.5 mL of 25% pyrrolidine/DMF directly to the undrained post-coupling solution and the reaction was continued for an additional 40 sec at 100 °C before draining. This was followed by a single 4 mL wash. Cleavage was performed for 30 min at 40 °C using the CEM Razor high-throughput peptide cleavage system with TFA/H_2O/TIS/DODT. Following cleavage, peptides were precipitated in cold Et_2O and lyophilized overnight.

Peptide Analysis

Crude peptides without any purification 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) H2O and (ii) MeCN.

Results

The one-pot coupling/deprotection process on the Liberty PRIME produced high-purity peptides with approximately 50% reduction in cycle time and a 66% reduction in total chemical waste, compared to the standard methods utilized on CEM's Liberty Blue automated microwave peptide synthesizer. A standard cycle at 0.10 mmol scale on the Liberty PRIME uses a single wash of 4 mL between the deprotection and subsequent coupling, and produces only 8 mL of chemical waste with a total cycle time of 2 min 10 seconds. **Table 1** (page 3) shows the experimental data in terms of crude purity, total synthesis time and total chemical waste for a set of eight peptides synthesized on the Liberty PRIME.

Table 1. Peptide Synthesis on the Liberty PRIME.

Peptide	Sequence	Crude Purity	Total Synthesis Time	Total Chemical Waste
$^{65\text{-}74}$ACP	VQAAIDYING-NH_2	94%	25 min	92 mL
ABC-20 mer	VYWTSPFMKLIHEQCNRADG-NH_2	83%	48 min	172 mL
JR-10 mer	WFTTLISTIM-NH_2	70%	25 min	92 mL
Exenatide	HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS-NH_2	57%	1 h 36 min	273 mL
$^{7\text{-}37}$GLP1	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG	47%	1 h 14 min	217 mL
PnIA(A10L)	GCCSLPPCALNNPDYC-NH_2	77%	43 min	112 mL
Circulin A	GIPCGESCVWIPCISAALGCSCKNKVCYRN	79%	1 h 10 min	252 mL
Parigidin-br-1	GGSVPCGESCVFIPCITSLAGCSCKNKVCYYD	74%	1 h 14 min	264 mL

Resins used: Rink amide Protide LL (0.18 mmol/g) for 65-74ACP, ABC-20 mer, JR-10 mer, Exenatide, and PnIA(A10L); Fmoc-Gly-wang LL (0.32 mmol/g) for 7-37GLP1; Rink Amide MBHA LL (0.31 mmol/g) for Circulin A and Parigidin-br-1.

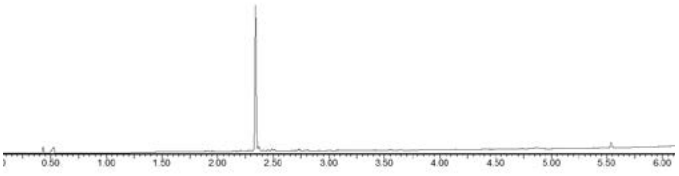

Figure 2. UPLC-MS Analysis of crude 65-74ACP.

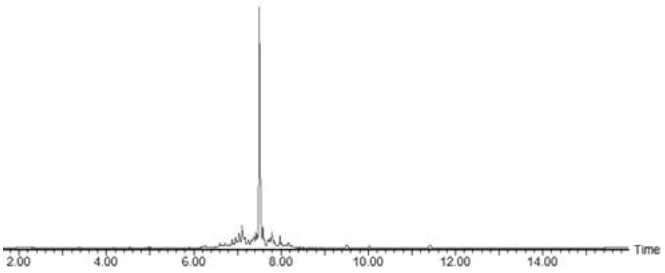

Figure 5. UPLC-MS Analysis of crude Exenatide.

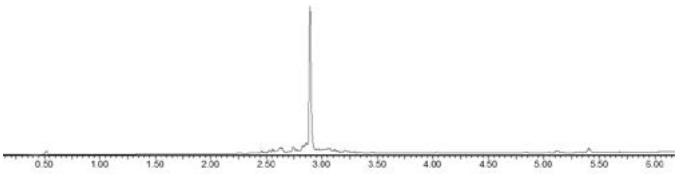

Figure 3. UPLC-MS Analysis of crude ABC-20 mer.

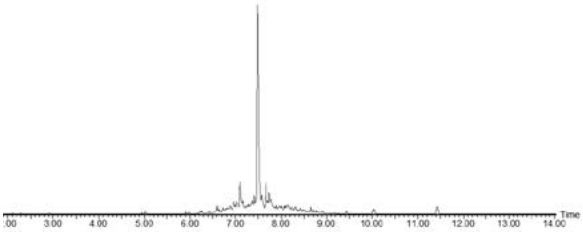

Figure 6. UPLC-MS Analysis of crude 7-37GLP1.

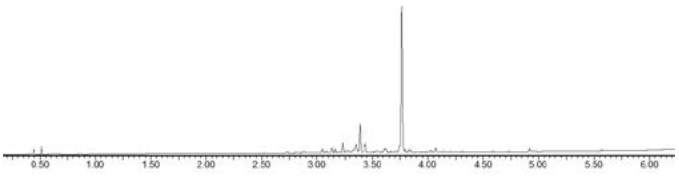

Figure 4. UPLC-MS Analysis of crude JR-10 mer.

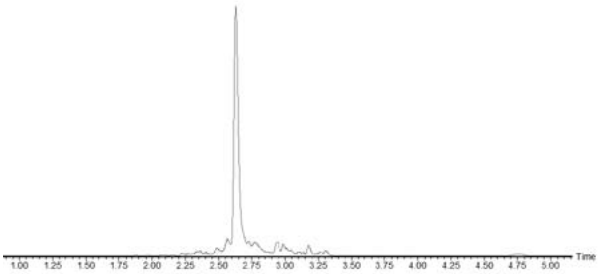

Figure 7. UPLC-MS Analysis of crude PNIA(A10L).

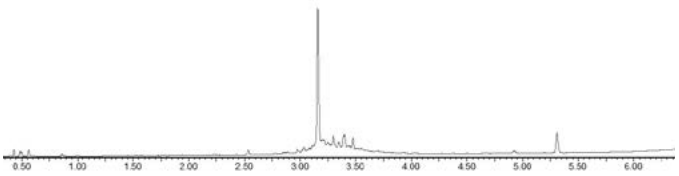

Figure 8. UPLC-MS Analysis of crude Circulin A.

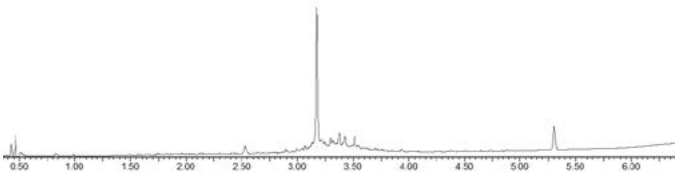

Figure 9. UPLC-MS Analysis of crude Parigidin-br-1.

The potential for epimerization was then investigated on the elevated temperature coupling methods used on the Liberty PRIME. In particular, cysteine and histidine are known to be sensitive to epimerization during coupling. The epimerization level was therefore investigated through a well-known standard method involving hydrolysis, subsequent derivatization, and gas chromatography analysis (C.A.T. GmbH). As shown in **Table 2**, epimerization levels observed with HBTU/DIEA activation at room temperature were found to be higher than those from 90 °C standard or CarboMAX couplings as well as from 105 °C CarboMAX coupling on the Liberty PRIME. Use of Fmoc-His(Boc)-OH instead of Fmoc-His(Trt)-OH allowed coupling temperatures of 90 °C or 105 °C without any increase in epimerization levels. These results further demonstrate that standard HE-SPPS or CarboMAX coupling methods are particularly well-suited for peptide synthesis at elevated temperature.

Table 2. Epimerization levels of cysteine and histidine in ABC 20mer with CarboMAX coupling.

%-D Epimer	Conventional (RT Coupling) HBTU/DIEA	Liberty Blue (90 °C CarboMAX) DIC/Oxyma	Liberty PRIME (105 °C CarboMAX) DIC/Oxyma
D-His	1.79[1,a]	1.12[b]	1.05[b]
D-Cys[c]	1.38[1]	0.64	0.68

[a]Fmoc-His(Trt)-OH; [b]Fmoc-His(Boc)-OH; [c]Fmoc-Cys(Trt)-OH.

Conclusion

The one-pot coupling/deprotection process used on the Liberty PRIME automated microwave peptide synthesizer allowed for a broad range of peptides to be synthesized extremely efficiently. A standard cycle with the improved CarboMAX coupling chemistry was utilized for all 20 amino acids (including cysteine and histidine) that greatly simplifies the need for sequence based optimization. The Liberty PRIME 2.0 with its standard 2-minute cycle time is ideal for high-throughput peptide synthesis of both standard and complex peptides, with an unparalleled reduction in chemical waste.

References

[1] J. Collins, K. Porter, S. Singh and G. Vanier, “High-Efficiency Solid Phase Peptide Synthesis (HE-SPPS),” Org. Lett., vol. 16, pp. 940-943, 2014.

[2] Patent Pending: US20170226152; WO2017070512.

[3] Patent Pending: US15686719; EP17188963.7; US20160176918; EP3037430; JP2016138090; CN105713066; AU2017204172.

[4] CEM Corporation. CarboMAX - Enhanced Peptide Coupling at Elevated Temperature, 2018. CEM Corporation Website; Application Notes. https://cem.com/en/carbomax-enhanced-peptide-coupling-at-elevated-temperatures (accessed April 25, 2022).

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