

## Introduction

A major problem in solid phase peptide synthesis is aggregation and poor solvation leading to incomplete deprotection and/or coupling steps that result in low crude purities.<sup>1</sup> This phenomenon is aggravated in the case of long peptides (50-100 amino acid length) where deprotection and coupling efficiencies are dramatically reduced as the peptide chain length increases. The subsequent purification of the crude peptide mixture becomes a complex and time consuming task, and repeated chromatography runs are often required leading to inevitable reduction in yield of the target peptide.<sup>2</sup> These post-synthetic challenges for long peptides are avoidable if the peptide can be synthesized in a high (> 80%) crude purity on the system, in which case a single chromatographic purification step is sufficient to isolate the target peptide in pure form and high yield. We recently reported an optimized process for solid phase peptide synthesis that allows for significant gains in product purity along with only a 4 minute standard cycle time and a 90% reduction in total waste produced.<sup>3</sup> The general applicability of the new High Efficiency Solid Phase Peptide Synthesis (HE-SPPS) method was confirmed on a series of medium sized peptides (10-42 amino acid length). The present work involves application of the HE-SPPS method to the synthesis of well-known and difficult long peptides: SDF-1 $\alpha$  (68-amino acid length), Ubiquitin (76-amino acid length) and AS-48 (75-amino acid length), which have been synthesized on the Liberty Blue™ system in 84-87% crude purity. To the best of our knowledge, previous attempts to synthesize AS-48 have been unsuccessful to date.

## Experimental

### HE-SPPS Materials and Methods

All peptides were synthesized on the CEM Liberty Blue™ Automated Microwave Peptide Synthesizer at 0.10 mmol scale using Fmoc-PAL-PEG-PS resin (0.17 mmol/g substitution). 10% w/v piperazine in NMP/EtOH (9/1) was used as the Fmoc-deblocking reagent. Post-deprotection washing with DMF (4 x 4 mL) was followed by coupling using a 5-fold excess of reagents: Fmoc-AA-OH (0.2 M in DMF, 2.5 mL), DIC (0.5 M in DMF, 1.0 mL) and Oxyma Pure (1.0 M in DMF, 0.5 mL). The peptide resin was cleaved with TFA/TIS/H<sub>2</sub>O/DODT (92.5/2.5/2.5/2.5) using the CEM Accent™ Microwave Cleavage System. The peptide was precipitated in cold ether and the crude material was analyzed without any purification.

### Analysis

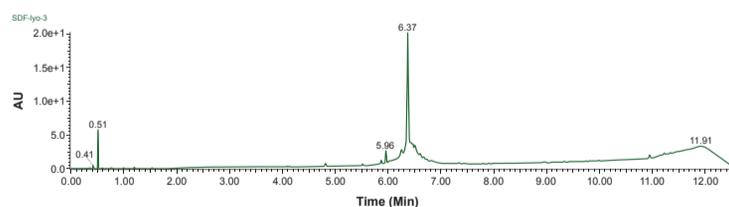
Crude peptides were analyzed on a Waters UPLC ACQUITY H-Class with 3100 Single Quad MS using acetonitrile/water with 0.1% TFA as the solvent system on a C18 Column (1.7  $\mu$ m, 2.1 x 100 mm). (D)-Asp formation due to aspartimide side reaction was analyzed at C.A.T. GmbH & Co., Germany.

## Results and Discussion

(A) SDF-1 $\alpha$ : KPVLSYRCPCRFESHVARANVKHLKILNTPNCALQIVARLKNNNRQVCI DPKLKWIQYLEKALNK (68-mer)

The SDF-1 $\alpha$  peptide was synthesized on the Liberty Blue™ with the standard default methods using DIC/Oxyma coupling and the new deblocking cocktail 10% piperazine in NMP/EtOH. All amino acid cycles used a single 1 min/90 °C deprotection and single 2 min/90 °C coupling for the first 30 amino acid residues and double 2 min/90 °C coupling for the rest of the sequence. His was coupled with a special 2 min/RT-4 min/50 °C method and all Arg residues were coupled with double 2 min/90 °C method. The synthesis of this peptide was completed in 8 hours 15 min with a crude purity of ~84% (Figure 1).

Figure 1. UPLC chromatogram of crude SDF-1 $\alpha$



### SDF-1 $\alpha$ (68-mer)

Crude Purity: ~84%  
Total Synthesis Time: 8 hours 15 min  
Total Wash Solvent Usage (DMF): 1240 mL  
Total Chemical Waste: 1940 mL

(B) Ubiquitin: MQIFVKLTGKITLEVPSDTIENVKAKIQDKGIPDPQQLRFAGKQL EDGRTLSDYNIQKESTLHLVRLRGG (76-mer)

The Ubiquitin peptide sequence consists of four aspartic acid segments DY, DG, DQ and DT that have a tendency to form the aspartimide side product with DG being the most susceptible. The synthesis of this peptide was carried out using Fmoc-Asp(OtBu)-(Dmb)Gly-OH for the DG segment and Fmoc-Asp(OtBu)-OH for all other Asp residues with 1 min/90 °C deprotection using 10% piperazine/0.1 M HOBt. The crude peptide was analyzed using UPLC MS (Figure 2). C.A.T. epimerization analysis revealed a very low epimerization level of 1.61% (D)-Asp. All amino acid cycles used a single 1 min/90 °C deprotection step, a single 2 min/90 °C coupling for the first 30 amino acid residues, and a double 2 min/90 °C coupling for the rest of the sequence. His was coupled with a special 2 min/RT-4min/50 °C microwave method and all Arg residues were coupled with double 2 min/90 °C method.

Incorporation of pseudoprolines and Dmb-dipeptides in the sequence minimizes peptide aggregation leading to improved synthesis results.<sup>4</sup> Using pseudoprolines (IT and LS), Dmb- dipeptides (DG and AG) and the newly available Asp derivative Fmoc-Asp(OPhP)-OH<sup>5</sup> in the Ubiquitin sequence with 1 min/90 °C deprotection and 2 min/90 °C coupling was carried out. (Figure 3).

Figure 2. UPLC chromatogram of crude Ubiquitin using Fmoc-Asp(OtBu)-OH and Dmb dipeptide (DG) with 1 min/90 °C deprotection

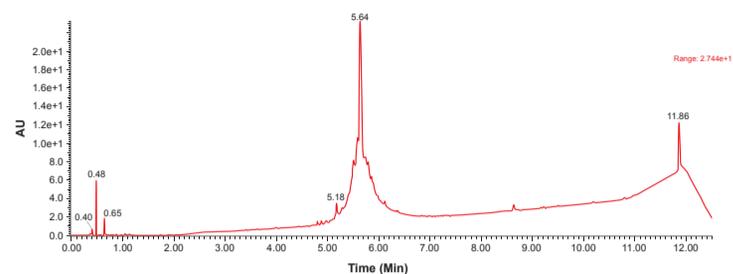
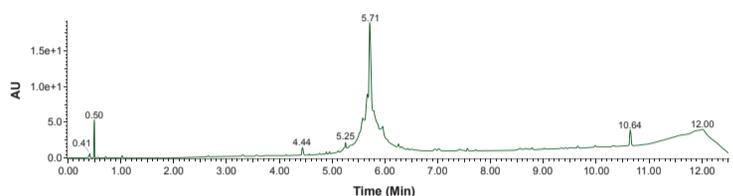


Figure 3. UPLC chromatogram of crude Ubiquitin using Fmoc-Asp(OPhP)-OH for DY segment, pseudoprolines (IT and LS) and Dmb dipeptides (DG and AG) with 1 min/90 °C deprotection



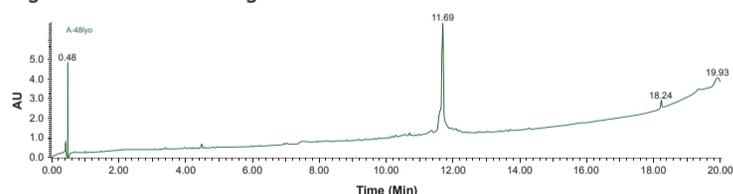
### Ubiquitin (76-mer)

Total Synthesis Time: 9 hours 8 min  
Total Wash Solvent Usage (DMF): 1366 mL  
Total Chemical Waste: 2146 mL

(C) AS-48: VVEAGGWTTIVSILTAVGSGLSLLAAAGRESIKAYLKKKIKKGRAVIA WMAKEFGIPAAVAGTVLNHVKKK (75-mer)

The AS-48 peptide was also synthesized using the DIC/Oxyma coupling method and 10% piperazine in NMP/EtOH for Fmoc-deprotection. All amino acid cycles used a double 1 min/90 °C deprotection, double 2 min/90 °C coupling for the first 30 residues and triple 2 min/90 °C coupling for the rest of the sequence. His was coupled with a special 2 min/RT-4min/50 °C microwave method and all Arg residues were coupled with triple 2 min/90 °C method. The synthesis of this peptide was completed on the Liberty Blue™ system in 15 hours 27 min with a crude purity of 87% (Figure 4).

Figure 4. UPLC chromatogram of crude AS-48



### AS-48 (75-mer)

Crude Purity: 87%  
Total Synthesis Time: 15 hours 27 min  
Total Wash Solvent Usage (DMF): 1366 mL  
Total Chemical Waste: 2450 mL

## Conclusions

The combination of microwave based HE-SPPS method and the ultrafast automation of Liberty Blue™ resulted in the synthesis of long peptides with excellent crude purities in shortest possible synthesis time, and a 90% reduction in solvent usage and chemical waste. The first successful synthesis of the AS-48 (75-mer) peptide accomplished on the Liberty Blue™ is an important milestone in the field of Fmoc-SPPS.

## References

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- The authors gratefully acknowledge EMD Novabiochem for providing a test sample of Fmoc-Asp(OPhP)-OH.