Enhancing Solid-Phase Peptide Synthesis with Green Chemistry Principles

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Introduction

Antimicrobial peptides (AMPs) are evolutionarily conserved peptide sequences often found in plants, animals, and humans. They play a significant role in the host's innate immune response by acting as a first line of defense against invading pathogenic microbes and have a broad spectrum of antimicrobial activity.[1] AMPs target microbial components that facilitate immune response. They are a key focus in the development of novel antibacterial agents due to their rapid pathogen killing ability and alternative mechanism of action, which make it challenging for pathogens to develop antibiotic resistance.

As the demand for peptide-based therapeutics (including AMPs) increases, so does the need for sustainable and environmentally friendly synthesis methodologies.[2] The decapeptide, ACP_{65-74}, is known for its challenging synthesis[3], making it an excellent benchmark for evaluating novel synthesis approaches. In this study, ACP was synthesized using both traditional DMF-based protocols and alternative solvents to explore the application of green chemistry principles in solid-phase peptide synthesis (SPPS) in conjunction with automation platforms. The most effective green synthesis protocol was then applied to two common AMPs, C18[4] and hepcidin[5] for further validation.

Experimental Protocol

Peptides were synthesized on 0.1 mmol scale using a Biotage® Initiator+ Alstra™ peptide synthesizer with 0.5 M DIC, 0.5 M Oxyma and 0.5 M amino acid solutions at 75°C for 5 min in DMF. The AMPs were prepared using 0.6 M HBTU and 0.5 M DIEA for 15 min at 50°C in DMF. Fmoc deprotection was performed using 20% 4-methylpiperidine (pip) for 3 min followed by 10 min at room temperature. Amino acids and coupling reagents were prepared at 0.25 M in 7:3 n-butyl acetate:DMSO solutions for all peptides evaluated.

Peptides were cleaved with a solution of 95% TFA: 2.5% TIPS: 2.5% H_2O at room temperature for at least 3 hours. Hepcidin was cleaved using 92.5% TFA: 2.5% DTT: 2.5% TIPS: 2.5% H_2O for 3 hours to minimize Met oxidation. The cleaved peptides were then ether precipitated and analyzed for purity with an Agilent 1260 Infinity series HPLC equipped with a Restek Raptor™ ARC-18 (2.1 x 50 mm) column.

Results and Discussion

Evaluation of sustainable synthesis solvents with ACP

This study systematically examined post-deprotection wash steps and solvent compositions to identify the most effective and environmentally friendly solution for SPPS using an automation platform while minimizing hardware adjustments.

***Table 1**. Sustainable synthesis evaluation using ACP as a test sequence.*

	Synthesis solvent(s)	Deprotection Base	Post-deprotection washes	Post-deprotection wash solvent	Percent purity
1	DMF	20% piperidine	4x	DMF	88.95
2	**DMF**	**20% piperidine**	**1x**	**DMF**	**76.55**
3	DMF	20% piperidine	1x	DMF + 1% oxyma	76.04
4	DMF	20% piperidine	1x	9:1 EtOAc:DMSO	NP
5	DMF	20% piperidine	1x	7:3 BtOAc:DMSO	4.87
6	9:1 EtOAc:DMSO	20% piperidine	1x	9:1 EtOAc:DMSO	49.39
7	**9:1 EtOAc:DMSO (C) 6:4 EtOAc:DMSO (D)**	**20% piperidine**	**1x**	**9:1 EtOAc:DMSO**	**58.49**
8	9:1 EtOAc:DMSO (C) 6:4 EtOAc:DMSO (D)	20% piperidine	1x	9:1 EtOAc:DMSO + 1% oxyma	NP
9	**7:3 BtOAc:DMSO**	**20% piperidine**	**1x**	**7:3 BtOAc:DMSO**	**72.73**
10	7:3 BtOAc:DMSO	20% piperidine	1x	7:3 BtOAc:DMSO + 1% oxyma	7.46

While reducing post-deprotection washes from four to one slightly decreased crude purity (Table 1), it cut solvent consumption by more than 50% and reduced synthesis time by 29.33% for peptide ACP in DMF. Reducing the use of DMF is important but replacing it with less hazardous binary solvent mixtures is emerging as a more viable and environmentally friendly alternative.

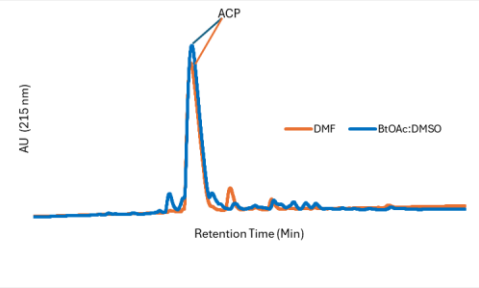

***Figure 1.** HPLC overlay of ACP comparing synthesis using default conditions in DMF (orange) and reduced washing in 7:3 BtOAc:DMSO (blue).*

To assess the viability of greener, binary solvent mixtures in automated synthesis, both 9:1 EtOAc:DMSO and 7:3 BtOAc:DMSO were evaluated. Among the various synthesis conditions tested (Table 1), the method using 7:3 BtOAc:DMSO and 20% pip achieved the highest crude purity (72.73%) among the sustainable alternatives. While there is certainly room for improvement, these preliminary results suggest that 7:3 BtOAc:DMSO can in fact serve as a viable alternative to DMF in automated SPPS, Figure 1. This method was subsequently applied to the synthesis of AMPs, C18 and hepcidin, to further validate its effectiveness across a broader range of peptide sequences.

Evaluation of sustainable synthesis methods for AMPs

The synthesis of AMPs presents additional challenges due to differences in hydrophobicity, aggregation tendencies, and steric hindrance within their sequences - factors commonly encountered in peptide synthesis. C18 (LWKIGKKIWRVLWNWR) is an AMP belonging to the cecropin family, with minimum inhibitory concentration (MIC) of 4 µg/mL against methicillin resistant *S. aureus* (MRSA), making it a promising therapeutic candidate for severe MRSA infections.[5]

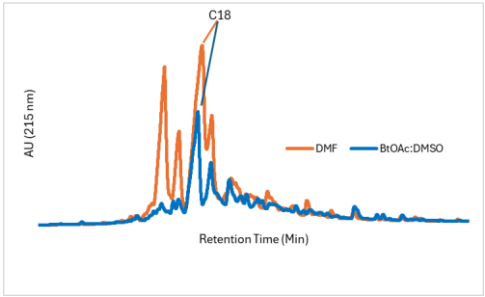

***Figure 2.** HPLC overlay of peptide C18 comparing synthesis using default conditions in DMF (orange) and reduced washing in 7:3 BtOAc:DMSO (blue).*

Default synthesis conditions of C18 yielded crude purity of 29.97%. When synthesized using sustainable solvents and a single post-deprotection wash, crude purity decreased further to 21.76%, Figure 2. The lower crude purity of C18 is due to its high hydrophobic content, which likely promotes intramolecular aggregation on the resin, hindering efficient coupling. While the difference in percent purity is not significant with the more sustainable protocol, the sample content changes dramatically. Impurities present during synthesis with DMF are essentially eliminated when using the binary mix, suggesting that the alternative solvent system better solubilizes the elongating peptide, simplifying future optimization requirements.

We also evaluated hepcidin, a 25 amino acid peptide, that plays a critical role in iron homeostasis and provides insight into hematologic disorders such as anemia of chronic kidney disease.[6] To evaluate synthesis efficiency, hepcidin was synthesized with Acm-protected cysteine residues which protects against oxidation and undesired disulfide bond formation. Default synthesis conditions of hepcidin however, yielded crude purity 56.72%.

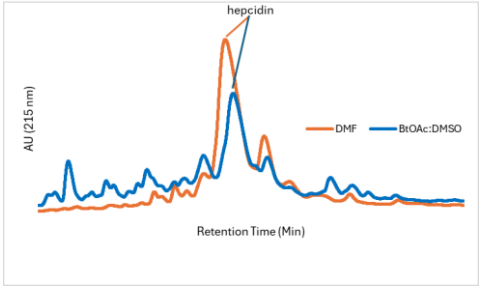

***Figure 3.** HPLC overlay of hepcidin comparing synthesis using default conditions in DMF (orange) and reduced washing in 7:3 BtOAc:DMSO (blue).*

When synthesized using the more sustainable protocol, the crude purity further decreased further to 42.24%, Figure 3. The emergence of new impurities with minimized washes may be attributed to insufficient removal of reaction by-products and increased side reactions, leading to undesired modifications. Interestingly, the chromatographic profile of hepcidin does not change as dramatically as C18, reinforcing the sequence dependent nature of SPPS success or failure. The reduction in purity aligns with the expected impact of fewer post-deprotection washes, rather than a fundamental limitation of the solvent system. This underscores the importance of proper solvent selection and wash steps to maintain synthesis efficiency.

Conclusion

The transition to greener SPPS is critical for reducing environmental impact while maintaining synthetic efficiency. This study demonstrates that 7:3 BtOAc:DMSO with 20% pip is a promising alternative to DMF, achieving only a 23.72% average purity reduction while reducing solvent consumption by more than two-fold. AMPs present unique synthetic challenges due to hydrophobicity and steric hindrance. We present a viable starting point for future optimization. The data highlights the feasibility of automated, sustainable peptide synthesis. With minor hardware adjustments, it is anticipated that the crude purities can be further improved when using greener solvent systems.

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