Improved ecological efficiency

Green manufacturing of Bivalirudin with PEC

Due to solvent waste and energy consumption, the manufacturing of pharmaceutical ingredients has a significant environmental impact. Therefore, the industry demands new and innovative techniques for greener drug production. This study highlights DMF-free solid-phase peptide synthesis (SPPS) and its compatibility with the low-solvent consuming Peptide Easy Clean (PEC) purification technology to manufacture Bivalirudin in a green approach. We also highlight a novel TFA (Trifluoroacetic acid) catch method to eliminate the post-SPPS precipitation step, which consumes a large amount of hazardous ether.

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1. Introduction

Improving the ecological impact during the large-scale manufacture of new drugs and generics can help resolve urgent environmental and health challenges. While we observe significant progress toward the greener manufacturing of small molecule drugs, producing larger biomolecules like polypeptides remains an environmentally inefficient process. Despite some notable advances in greening peptide synthesis, the ecological footprint of subsequent ether precipitation and purification by high-pressure liquid chromatography (HPLC) remains significant due to the enormous energy and solvent consumption.

Inspired by the list of 12 principles for green chemistry[1] and a series of publications from Bachem and Novo Nordisk about using green binary solvent mixtures in SPPS[2-3], we aim to use the PEC technology for developing sustainable processes for peptide manufacturing.

Using the peptide drug Bivalirudin as an example, this case study highlights two levers toward greener processes: Avoiding hazardous solvents and reducing solvent waste. In particular, we employed non-hazardous solvents for SPPS (no DMF), precipitation- and ether-free transfer to the purification step using the novel TFA catch method, and a 75% reduction of solvent waste during orthogonal PEC purification.

2. Method

Green SPPS synthesis. Bivalirudin (H-fPRP GGGGN GDFEE IPEEYL-OH) was synthesized on a 0.5 mmol scale using an automated synthesizer (PurePep™ Chorus from Gyros Protein Technologies). H-Leu-2-CTR resin (1.02 g, loading = 0.49 mmol/g) was pre-swollen in DMSO/EtOAc 4:6. The resin was treated with the amino acid (4 eq.), Oxyma (4 eq.), and Diisopropylcarbodiimide (DIC, 5 eq.) stock solutions for 60 min. Arginine coupling was performed twice. After coupling, capping was performed. Fmoc removal was carried out (2x 5 min). Between all steps except before capping, washing was performed. At the end of the final cycle, the resin was washed with Isopropanol (10 mL) and dried under a vacuum. The binary solvent mixtures used are listed in Table 1.

Table 1: Solubility of reagents in binary solvent

Step	Reagent (Conc.)	Solvent
Stock solutions	Amino acids (0.5M) DIC (1M) Oxyma (1M)	DMSO/EtOAc 4:6 Pure EtOAc DMSO/EtOAc 4:6
Coupling	Amino Acid (4 eq. 0.25M) DIC (5 eq. 0.31M) Oxyma (4 eq. 0.25M)	Resulting mix: DMSO/EtOAc 3:7
Fmoc removal	Piperidine (20% (v/v))	DMSO/EtOAc 6:4
Capping	Ac_2O (37% (v/v)) Pyridine (32% (v/v))	DMSO/EtOAc 1:9
Washings	-	DMSO/EtOAc 2:8

The coupling of PEC-Linker RC+ was performed on the full-length peptide in the last step in DMSO/EtOAc 1:9.

TFA catch and PEC purification. 2 mL TFA cocktail (TFA/H_2O/TIS: 95:2.5:2.5) were used for 20 µmol of the PEC-Linker-coupled peptide on resin. After 2h shaking and subsequent filtration, the peptide-TFA solution was cooled to 0 °C followed by addition of 0.24 mL pyridine and 0.76 mL acetonitrile. This cocktail mixture was directly immobilized on 0.650 g activated polymethacrylate beads (60 µM, ~92 µmol/g). The immobilization was performed for 300 min, followed by washing away impurities and adding 1 mL of 2 wt% cysteine to block unreacted aldehydes (15 min). The reduction was carried out by shaking the beads with 100 mg of DTT in 0.5 mL of 5 w% aq. $NaHCO_3$ for 15 min. Excess of DTT and DTT(ox) was removed through washings. The PEC-Linker cleavage was initiated by adding 0.8 mL of 95% TFA. After 45 min, 0.8 mL 95% TFA was added, and the filtrate was collected in 50 mL tubes. Each sample was washed/eluted twice with 2 mL 95% aq. TFA. Chilled diethyl ether was used to precipitate the peptide before dissolution and lyophilization (10.1 mg final yield)

Analysis. UPLC-UV chromatograms and mass spectra were recorded with an analytical Acquity H-Class UPLC-ESI-MS system from Waters on a C-18 column (1.7 µm, 2.1 x 500 mm). In the mobile phase, mixtures of water (A) and MeCN (B) with 0.1% TFA were used.

3. Results and Discussion

Safer solvents enable a better synthesis

This study investigated DMSO/EtOAc mixtures as a green alternative to DMF. We have considered two guidelines to choose the right proportions of DMSO/EtOAc.

1. Solubility of the reagents at ideal concentrations

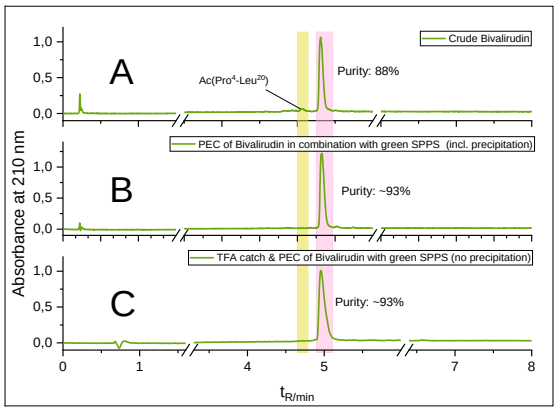
Derived from our optimized SPPS conditions, we work with 0.5M amino acid, 1M DIC, and 1M Oxyma stock solutions. Solubility experiments revealed that 40% DMSO in EtOAc is required to solubilize all necessary amino acids and Oxyma to the desired concentration. Pure EtOAc is adequate to prepare 1M DIC (Table 1).

2. Varying polarity improves reaction rates

Adjusted binary mixtures allowed us to perform the final coupling reaction in a lower-polarity solvent, DMSO/EtOAc (3:7), and Fmoc removal in a higher-polarity solvent, DMSO/EtOAc (6:4). This flexibility enables ideal reaction conditions for each step (i.e., polar solvent stabilizes E1cb intermediate during Fmoc removal).

We performed the capping step in DMSO/EtOAc (1:9) and washings of the SPPS resin after each coupling cycle with DMSO/EtOAc (2:8). Our final crude purity of 88% (Figure 1, A) is higher than the published results of Bivalirudin manufacturing using toxic DMF with the purity of 74%.[1,2]

Figure 1: Chromatograms of crude and purified Bivalirudin



Preventing waste using PEC

PEC saves around 75% of organic solvents in an orthogonal purification approach with HPLC (Link). Therefore, we further explored the compatibility of PEC with green solvents for SPPS to showcase an overall green manufacturing process. Moreover, we investigated the TFA catch as a precipitation substitute to avoid the use of hazardous ether.

Compatibility of PEC with green SPPS. Initially, we used the standard PEC process, including precipitation of the crude mixture after SPPS. As anticipated, PEC purification removed the truncated sequence's Ac(pro^4-Leu^{20}) present in crude Bivalirudin to increase the purity from 88 to 93% (Figure 1, B). These results demonstrate the compatibility of the green solvents used in SPPS and low-solvent consuming catch-and-release purification.

In ongoing trials, we tune solvent mixtures to prevent deletions and side reactions such as aspartimide-formation and create API-grade peptides with a single PEC purification.

TFA catch & PEC purification. As TFA dissolves virtually all peptides and the oxime ligation reaction proved efficient in highly acidic conditions, we probed a direct TFA-cocktail immobilization ("TFA catch") to eliminate the post-SPPS precipitation step and minimize the use of ether. This procedure could allow automated transfer from synthesis to low-pressure PEC purification in conventional SPPS setups to significantly improve productivity.

Thiols like DTT (Dithiothreitol) and EDT (1,2-Ethandithiol) are avoided in the cocktail to prevent the unwanted blockage of aldehyde groups on the purification beads. The cleavage of the PEC-Linker coupled peptide was performed with TFA/H_2O/TIS (95:2.5:2.5) and, subsequently, the peptide was directly immobilized on activated PMA beads. Peptide immobilization was initially incomplete due to the protonation of oxyamine in the TFA cocktail. Neutralizing the TFA cocktail with pyridine and diluting with the organic solvent CH_3CN has drawn immobilization to completion. With 93%, the final purity was identical to the standard PEC purification method (Figure 1, C).

4. Results at a glance

- Improve the crude purity with DMF-free SPPS
- Reduce waste by 75% using orthogonal PEC purification
- Use the TFA catch method to enable precipitation- and ether-free transfer from synthesis to purification

References

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[2] Jadhav, S. et al. Green Chemistry 2021, 23, p. 3312
[3] Martin V., et al. Green Chemistry 2021, 23, p. 3295

About Belyntic

Belyntic GmbH is a chemistry-for-healthcare enterprise focusing on the purification and modification of peptides. Belyntic offers the world's first broadly applicable peptide purification kits and custom solutions of its proprietary Peptide Easy Clean (PEC) technology.

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