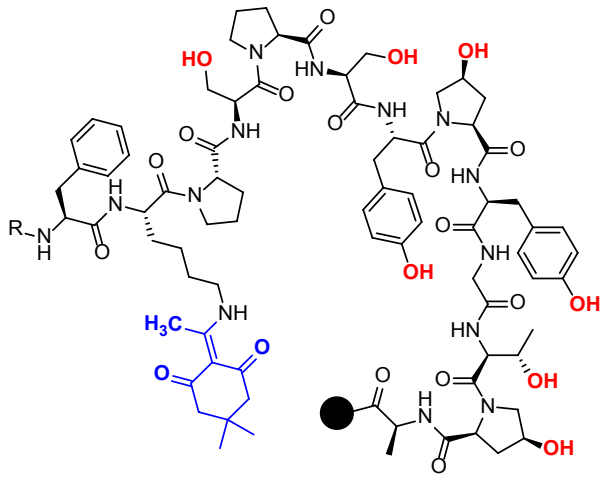
Minimal Protection Strategies for SPPS

Green Chemistry for Sustainable Peptide Manufacturing

AUGUST 2024



Minimally protected peptide small molecule conjugate.

Peptide manufacturing requires repetitive synthetic steps which makes synthesis on solid support advantageous. Solid-phase peptide synthesis (SPPS), the preferred method for preparing peptides greater than 5 amino acids, facilitates efficient couplings due to excess reagents at high concentrations to drive reactions to completion. Excess reagents and side products can be easily washed away and removed from the insoluble peptide chain by iterative filtration steps. All the synthesis steps occur in the same reaction vessel with no material transfer. As a result, intermediates do not require purification, isolation, or characterization.

Despite these advantages, SPPS approaches still require that the side chains of certain amino acids be protected from undesired reactivity during synthesis. The installation and removal of these protection groups results in a lower atom economy in the production process. Removal of the protection groups often requires large volumes of trifluoroacetic acid (TFA) or other strong acids which can result in lower yields and pose a significant risk to the environment. Over the past several decades, 9-fluorenylmethoxycarbonyl/t-butyl (Fmoc/t-Bu) solid-phase peptide synthesis (Fmoc-SPPS) has emerged as the dominant synthetic SPPS approach because the reaction conditions are milder and requires less TFA treatments compared to tert-butyloxycarbonyl/benzyl (t-Boc/Bzl) protection strategies. Fmoc-SPPS requires a single TFA treatment for the global removal of side chain protection groups (compared to t-Boc-SPPS at 1 TFA treatment per cycle/coupling), and does not need hydrogen fluoride (HF) or hydrogen bromide (HBr) during the cleavage step. While side chain protection reduces some common side reactions in SPPS, the removal of the protection groups can result in their reattachment to the peptide by electrophillic addition. If not sufficiently scavanged, protection group reattachment may become permanent and compromise the crude peptide purity.

In this study, we present a minimal protection-group strategy for the synthesis of a novel peptide-drug conjugate. Due to the acid-sensitivity of the small molecule moiety, avoiding strong acids like TFA was a key factor in yield optimization. In conventional SPPS, strong acids such as TFA are required to remove t-butyl ether protection from hydroxyl bearing side chains of serine, threonine, tyrosine, and hydroxyproline as well as to remove t-butyloxycarbonyl (t-Boc) protection from the amino bearing side chain of lysine. During the global deprotection step, removal of the t-butyl ethers create highly reactive t-butyl carbocation ion intermediates that result in unwanted peptide impurities by reattachment to the peptide. The introduction of chemical scavengers (e.g., ethanedithiol, thiolanisole, phenol, water) to the 'cleavage cocktail' can mitigate these side reactions, but the substances are also highly toxic to the environment and their scavenging efficiency vary among peptide sequences. Following the cleavage step, copious amounts of diethylether are required to isolate the peptide and remove excess scavengers.

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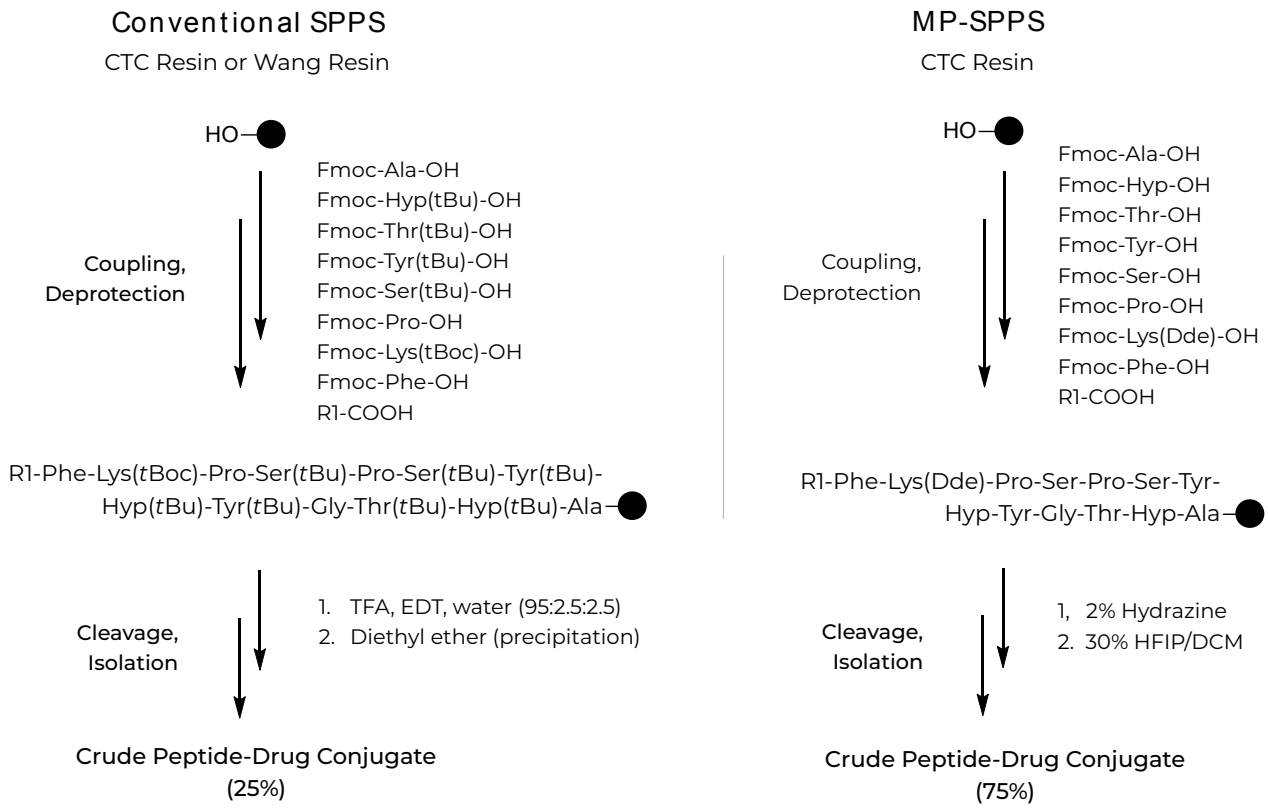
In an effort to mitigate product decomposition and reduce our reliance on hazardous chemicals such as TFA and diethylether, we investigated synthetic routes that don't rely on protection schemes requiring strong acids for their removal. To this end, we introduced a protection scheme that forgoes tert-butyl ether protection of serine, threonine, tyrosine, and hydroxyproline side chains. While hydroxyl side chains don't require protection during coupling steps in Fmoc-SPPS, protection of lysine side chain amines are needed to avoid reactivity toward activated incoming monomer amino acids (i.e., Fmoc-AA-Op). t-Boc side chain-protection for lysine is common in Fmoc-SPPS, but can be replaced with Dde (4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl), a protection group that is removed under non-acid and milder conditions (i.e., dilute hydrazine solution). Critically, omitting TFA from the manufacturing process makes protection of the hydroxyl groups and isolation with diethylether unnecessary.

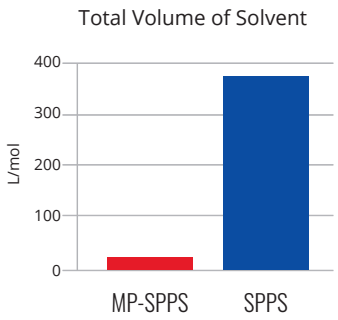


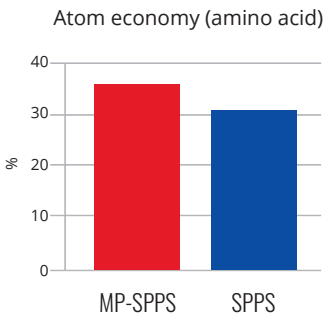
Comparison of Synthetic Schemes for SPPS and Minimal Protection SPPS (MP-SPPS).
In the conventional SPPS approach (left), all hydroxyl-bearing amino acids are protected with t-Bu ethers with the lysine side chain is t-Boc-protected. In the minimal protection (MP-SPPS, right), all of the hydroxyl sidechains remain unprotected throughout the synthesis with the lysine side chain Dde-protected.

To evaluate our approach, we prepared the target peptide-drug conjugate by traditional and minimal protection strategies. The peptides were synthesized on 2-chlorotrityl resin with identical coupling and Fmoc-deprotection conditions for each. In the conventional approach, the resin-bound protected sequence (R1-Phe-Lys(tBoc)-Pro-Ser(tBu)-Pro-Ser(tBu)-Tyr(tBu)-Hyp(tBu)-Tyr(tBu)-Gly-Thr(tBu)-Hyp(tBu)-Ala-resin) was generated and then cleaved, globally deprotected, and isolated with TFA, EDT, water (95:2.5:2.5) followed by diethyl ether (precipitation). In the minimal protection approach, the protected intermediate R1-Phe-Lys(Dde)-Pro-Ser-Pro-Ser-Tyr-Hyp-Tyr-Gly-Thr-Hyp-Ala-resin was prepared, the Dde group was removed by 2% hydrazine solution, and the peptide was cleaved from resin with 30% hexafluoroisopropanol in DCM.

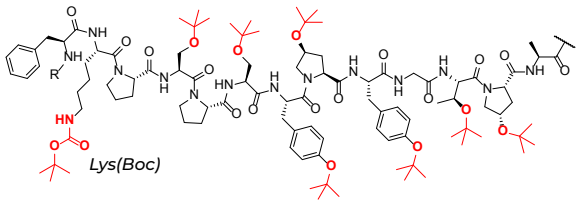
Comparison of Solvent Consumption and Atom Economy

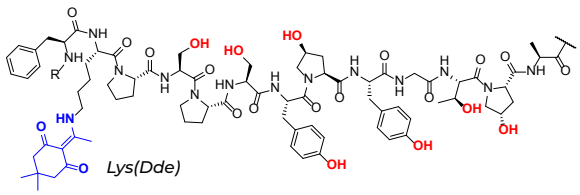
	SPPS	MP-SPPS
Atom economy (amino acid)	30.6%	35.6%
Trifluoroacetic acid (TFA)	30 L/mol	0
Dichloromethane (DCM)	0	17 L/mol
Hexafluoroisopropanol (HFIP)	0	5 L/mol
Diethylether (Et2O)	350 L/mol	0
Total Solvent	380 L/mol	22 L/mol
Crude Purity	< 25%	75%

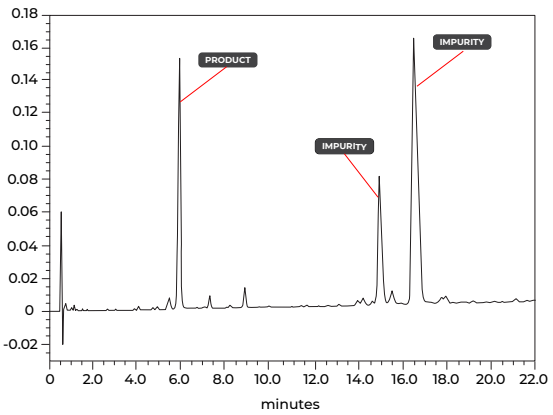


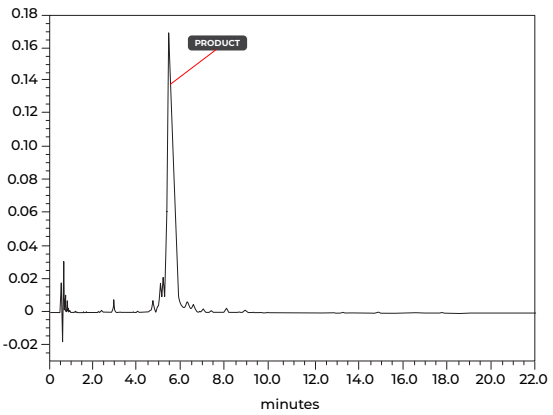


Removal of diethylether and TFA from the process results in a significant reduction in overall solvent utilization, from 380L to 22L/mol. In addition, omission of hydroxyl protection from serine, threonine, tyrosine, and hydroxyproline results in greater atom economy, from 30 to 35%. The increase in the crude purity from 25 to 75%, however, can be attributed to the small molecule degradation that occurs in the conventional methodology. Critically, removing highly hazardous chemicals like TFA and diethylether from the manufacturing process, establishes a green chemistry milestone for CPC Scientific in our effort towards a more sustainable future. Additional studies are underway to evaluate the feasibility of using other amino acid without side chain protection (e.g., arginine, histidine) for different target sequences and will be reported in future whitepapers.









Crude HPLC Comparison of MP-SPPS vs. Conventional SPPS. RP-HPLC of crude peptide synthesized by conventional SPPS (left) and minimal protection (MP)-SPPS (right). Late eluting impurities are a result of decomposition of the small molecular conjugate. HPLC conditions: water : acetonitrile (biphasic solvent gradient, 0.1% TFA, wavelength = 220 nm.

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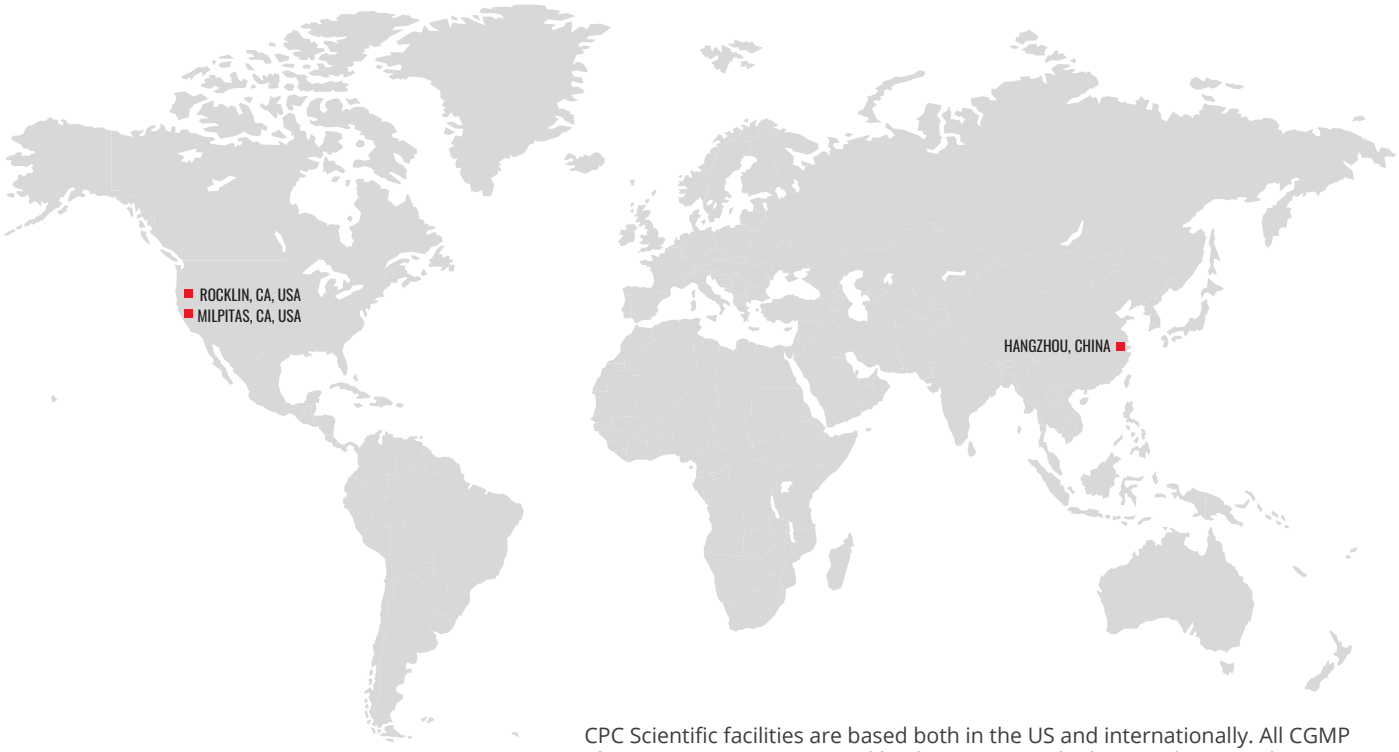

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