Routine Protocol

Scavengers are King

Peptide cleavage and deprotection is a critical step at the end of SPPS. If not performed cautiously, many side products form due to the re-attachment of protecting groups that critically affect crude purity and yield, as well as final purity when using the PEC purification technology. Here we present optimized cleavage cocktails and show how to improve crude and product purity already by choice of amino acid building blocks.

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1. Introduction

The global deprotection and cleavage of a peptide from the synthesis resin with trifluoroacetic acid (TFA) is a very complicated but essential step for improving the crude purity. Numerous electrophiles form during the acidic deprotection of multiple functional side chains that can readily react – or re-attach – with peptidic nucleophiles on the full-length sequence to generate side products. Unfortunately, some amino acids, such as Cys or Trp, are primarily reactive towards electrophiles and typically require extensive screenings of useful cleavage mixtures. The two most encountered side products after insufficient TFA-cleavage are *t*Bu adducts (+56 Da) on the thiol-containing side chains of cysteines and remaining 2,2,4,6,7-pentamethyldihydrobenzofuran-sulfonyl (Pbf)-protecting group (+252 Da) on Arg residues.

Mixtures of nucleophilic additives (scavengers) with TFA are, therefore, crucial to suppress unwanted re-attachments or insufficient removals. The absence of such by-products has a substantial impact not only on crude purity but also on catch-and-release purifications because side reactions on the linker-modified target peptide remain in the product and lower the final purity.

In this case study, we investigate the effect of different scavenger compositions and the cleavage times on a peptide that is rich in Arg and Cys residues. Also, we exploit the potential of Fmoc-Cys(S*t*Bu) as a particular building block to significantly suppress *t*Bu adduct formation.

2. Method

Synthesis. We designed a peptide sequence containing 3 Cys and 3 Arg moieties (Table 1) to investigate the accumulation of *t*Bu-adducts and the remaining Pbf after TFA cleavage. One peptide was synthesized with classical Fmoc-Cys(Trt)-OH (#1) building block, and a second peptide with Fmoc-Cys(S*t*Bu)-OH (#2), both in 100 µmol scale on Rink Amide AM resin. The S*t*Bu-protection on Cys was selected to prevent *t*Bu addition to Cys during TFA-cleavage. Routine capping using acetic anhydride after each coupling step ensured the selective coupling of the PEC-Linker only on the full-length peptide. The peptides were modified with the PEC-Linker RC+ (4 eq.) using DIPEA (6 eq.) and Oxyma (4 eq.) in DMF for 2 h.

Table 1: Peptides usesd in this study.

ID	sequence
#1	H-GC^{Trt}REG FLRC^{Trt}L HRPTV C^{Trt}G-OH
#2	H-GC^{StBu}REG FLRC^{StBu}L HRPTV C^{StBu}G-OH

Design of TFA-cocktails. For all cocktails in this study, we avoided toxic phenol, which showed no positive effects in previous experiments. Besides, TIS was added as an additional scavenger to the scavenger pool because trityl-cations are caught more efficiently and irreversible by hydrides than by thiols.[1] We chose the scavengers with the highest potential and varied their content to study the effects of TFA, H_2O, PhSMe, EDT, DTT, and TIS:

- Cocktail 1 represents a basic version containing EDT as thiol and PhSMe:
  83 : 5 : 5 : 5 : 0 : 2
- Cocktail 2 contains EDT as thiol but no PhSMe:
  91 : 4 : 0 : 3 : 0 : 2
- Cocktail 3 contains DTT as thiol but no PhSMe:
  91 : 4 : 0 : 0 : 3 : 2
- Cocktail 4 is a variation of cocktail 3 with an increasing amount of DTT as thiol and less TFA:
  88 : 4 : 0 : 0 : 6 : 2

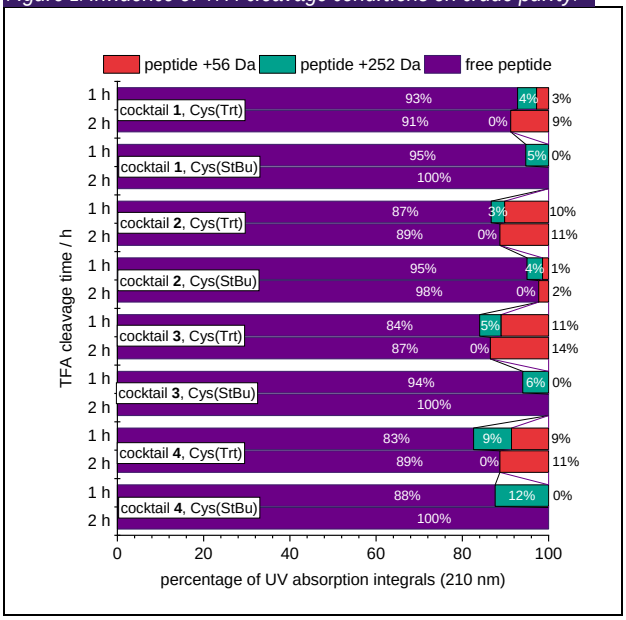
Investigation of the scavengers' effects. 8 x 10 µmol (45 mg resin) of the Cys(Trt) and 8 x 10 µmol (45 mg resin) of the Cys(S*t*Bu) containing linker-modified peptide on resin were added to fritted-cartridges. 1 mL of each cleavage cocktail was poured into two cartridges of each peptide to separate between one and two hours of shaking time. After ether precipitation, centrifugation, and removal of the ether supernatant for all peptides, precipitates were dissolved and purified by PEC.

Analysis. UPLC-UV 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). As the mobile phase, mixtures of water (A) and MeCN (B) with 0.1% TFA were used. The peaks in the UV chromatograms were integrated and assigned by their corresponding ESI-MS spectra.

3. Discussion

Comparison between TFA cocktails. Figure 1 shows the ratios of the free peptides, peptides with *t*Bu-adducts (+ 56 Da), and peptides with remaining Pbf-protecting groups (+ 252 Da) after treatment with the four different TFA-cocktails after one and two hours.

Figure 1: Influence of TFA-cleavage conditions on crude purity.



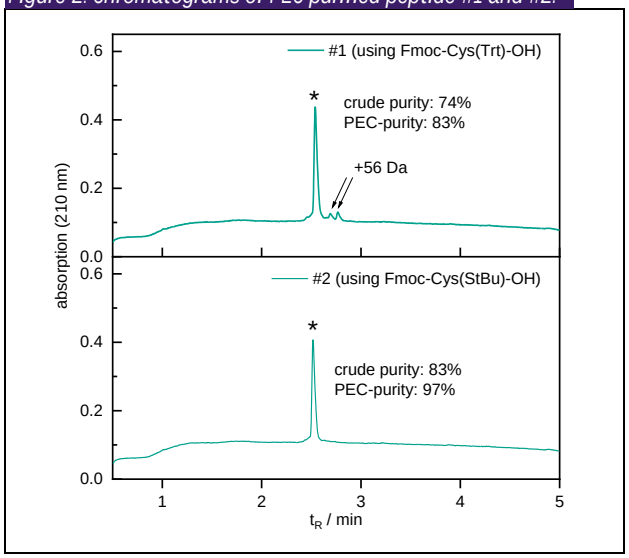
A closer look at the results obtained for peptide #1 (with Cys(Trt) building block) reveals that two hours cleavage time is necessary to remove Pbf. Comparison of the Pbf-levels after one hour indicates that higher amounts of TFA (e.g., Cocktail 4 vs. Cocktail 3) or the usage of PhSMe can accelerate the removal of Pbf. The situation for *t*Bu adducts, however, looks different as the amount of this impurity increases over time.

These results unravel a significant objection for catch-and-release purifications because impurities generated through incomplete deprotection or re-attached protecting groups will be carried through the process and contribute significantly to the product quality. In this case, for instance, an ideal cleavage time for complete removal of Pbf has, at the same time, adverse effects in *t*Bu related impurities.

Influence of Cys(StBu) building block. We opted for another strategy by re-thinking this dilemma using modified amino acid building blocks. Reduction-labile protecting groups such as S*t*Bu are ideally suited because deprotection and cleavage of the RC+ Linker happen simultaneously.

We, therefore, tested all four cocktails on the model peptide #2 where all Cys(Trt) were exchanged by Cys(S*t*Bu). The occurrence of *t*Bu re-attachments was suppressed quantitatively also for the longer deprotection times, allowing for side-product free global deprotection.

Figure 2: Chromatograms of PEC purified peptide #1 and #2.



This measure ultimately result in a significantly better purity, as shown in the chromatograms of the purified peptide #1 and #2 using odorless cocktail **4**. Not only the crude purity is increased, but also the final purity exceeds the 95% level. This improvement is substantial in terms of target use of PEC in higher purity mission.

4. Results at a glance

- apply optimized TFA-cleavage cocktails to improve crude purities and boost PEC-purities
- we recommend TFA/H_2O/PhSMe/EDT/TIS (83:5:5:5:2) or the odorless variant TFA/H_2O/DTT/TIS (88:4:6:2)
- suppress the number of tBu adducts in Cys-peptides using Cys(StBu) building block

5. References

[1] Pearson D. A.; Blanchette M.; Baker M. L.; Guindon C. A. *Tetrahedron Lett.* **1989**, 30, 2739-2742.

About Belyntic

Belyntic GmbH is a chemistry-for-healthcare enterprise focusing on the purification of biopolymers, especially peptides. Belyntic offers the world's first broadly applicable peptide purification kits as well as development and implementation services of its proprietary Peptide Easy Clean (PEC) technology.

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