DELETION SEQUENCES IN FMOC SPPS - ROOT CAUSE ANALYSIS AND PREVENTION STRATEGIES

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INTRODUCTION

The formation of deletion sequences is one of the most serious problems encountered in solid-phase peptide synthesis (SPPS). Deletion sequences only differ from the desired target peptide by the lack of one or few amino acids and are typically challenging to separate during purification especially of larger peptides.
Fig. 1 illustrates the typical finding that even after careful 2-dimensional HPLC purification of a crude >40mer peptide, deletion sequences are not separated, while most other impurities are.
Often deletion sequences elute within the range of the product peak and their substantial abundance is only revealed through peak purity LC-MS analysis, as shown in Fig. 2a and 2b.
Herein, we present causes for and experiments regarding the formation of deletion sequences as well as possible prevention strategies.

Fig. 1: Challenging purification of a synthetic >40mer peptide (U-HPLC analyses)

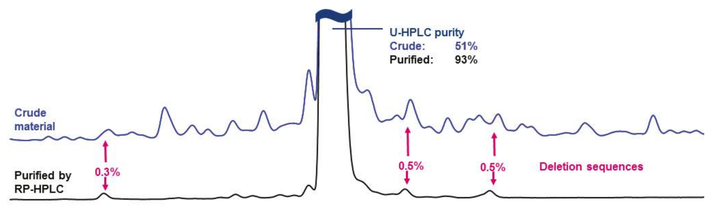


Fig. 2a: Peak purity analysis of a purified 19mer peptide originating from SPPS

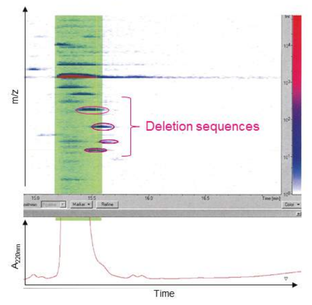
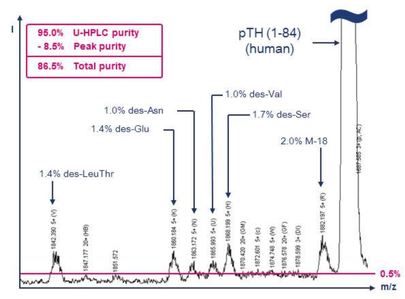
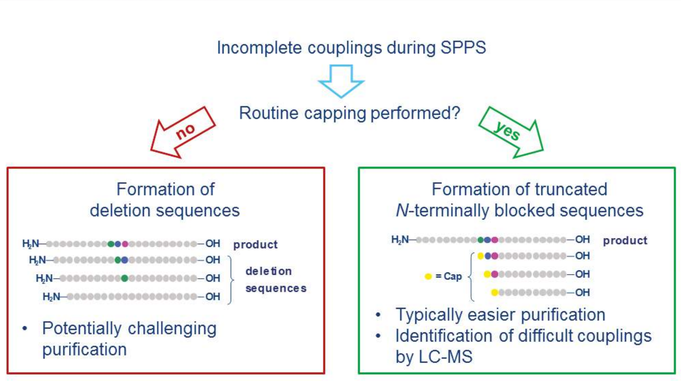


Fig. 2b: Peak purity analysis of purified pTH(1-84) originating from SPPS



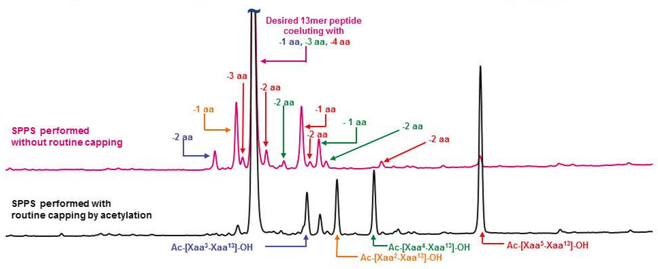
ROOT CAUSES AND PREVENTIONS STRATEGIES

INCOMPLETE COUPLINGS



Deletion sequences resulting from couplings that cannot be driven to completion can be "converted" to truncated sequences by quantitative capping. As shown in Fig. 3, truncated sequences are usually better separated than deletion sequences, allowing an easier purification.

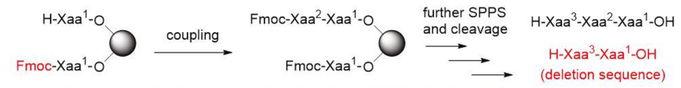
Fig. 3: U-HPLC analyses of crude 13mer peptides from SPPS with (bottom) or without routine capping (top)



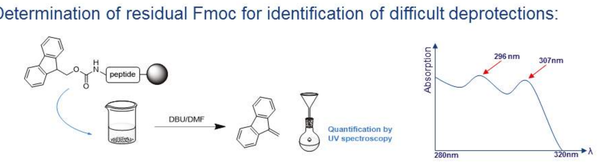
Prevention strategy:
Perform capping steps routinely or at least after difficult couplings

INCOMPLETE FMOC DEPROTECTION

- Incomplete Fmoc deprotection leads to deletions:



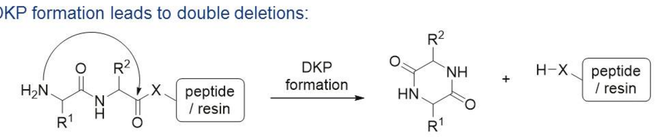
- Determination of residual Fmoc for identification of difficult deprotections:



Prevention strategy:
Identify difficult Fmoc deprotections and optimize the respective conditions, e.g. by prolongation, addition of DBU, change of solvent

DIKETOPIPERAZINE FORMATION

- DKP formation leads to double deletions:



Favored for:
- *N*-terminal H-Yaa-Pro, H-Yaa-ΨPro or H-Yaa-(*N*-alkyl)Zaa
- X = O, but also observed for X = NH

Prevention strategies:
- Avoid *N*-terminal sequences prone to DKP formation
- Avoid basic or harsh acidic conditions when *N*-terminal sequences prone to DKP formation are present
- Use resins with bulky linker groups (e.g. 2-chlorotrityl resin)

INCOMPLETE RESIN CAPPING

- Alcohol groups remaining after incomplete resin loading and capping e.g. on Wang resin might lead to formation of *C*-terminal deletion sequences (as experimentally also shown in Fig. 4):

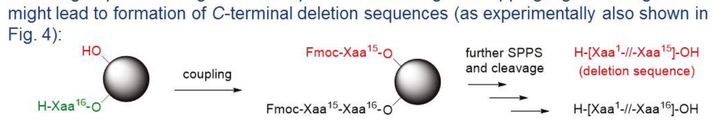
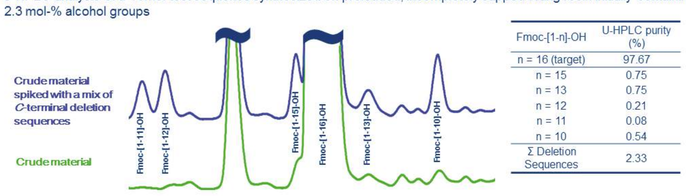


Fig. 4: U-HPLC analysis of a 16mer test sequence synthesized on preloaded, incompletely capped Wang resin initially containing 2.3 mol-% alcohol groups

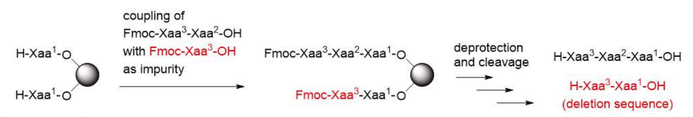


Fmoc-[1-n]-OH	U-HPLC purity (%)
n = 16 (target)	97.67
n = 15	0.75
n = 13	0.75
n = 12	0.21
n = 11	0.08
n = 10	0.54
Σ Deletion Sequences	2.33

Prevention strategies:
- Ensure complete capping of starting resin by MAS-NMR analysis[1] or test syntheses
- Use 2-chlorotrityl resin (2-chlorotrityl alcohol is less prone to acylation than e.g. the alcohol group of the Wang linker)

IMPURE BUILDING BLOCKS

- Impurities in dipeptide derivatives might lead to deletions:

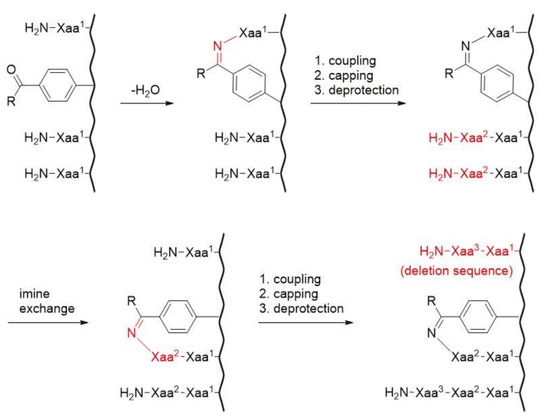


Prevention strategy:
Strict quality control of dipeptide derivatives used for SPPS

PRESENCE OF ALDEHYDES OR KETONES

Kent found that resin-bound aldehyde groups cause the formation of deletion sequences during Boc-SPPS.[2] Fig. 5 shows the proposed mechanism according to which *N*-termini of peptide chains reversibly form imines with resin-bound aldehydes and are thereby temporarily blocked from acylation during coupling or capping steps. Through this mechanism, the presence of one aldehyde or ketone group may result in multiple deletions sequences. Such impurities can be incorporated into the polymer backbone during polymerization due to impure starting materials or introduced during linker grafting.[3] Furthermore, polystyrene can be degraded by oxidation processes leading to the presence of aldehyde or ketone groups in the polymer.[4]

Fig. 5: Proposed mechanism for the formation of deletion sequences during SPPS due to the presence of resin-bound aldehyde or ketone groups



We performed test syntheses in order to elucidate whether resin-bound aldehydes or ketones can also cause deletions in Fmoc-SPPS. While a partial resin loading with biaryl ketones did not lead to an increase in deletion sequence formation (Fig. 6), a loading with a more reactive benzaldehyde derivative showed a substantial increase in deletion sequence formation compared to a reference synthesis (Fig. 7).

Fig. 6: U-HPLC analyses of a test synthesis on a resin partially loaded with biaryl ketones (top) compared to a reference synthesis on a resin without an intentionally introduced ketone impurity (bottom)

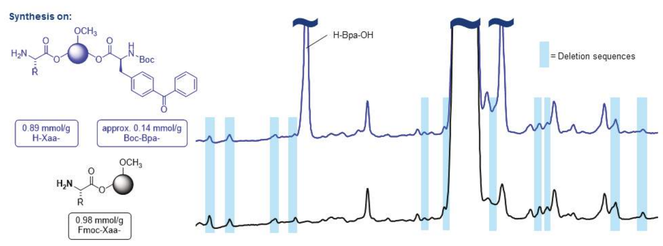
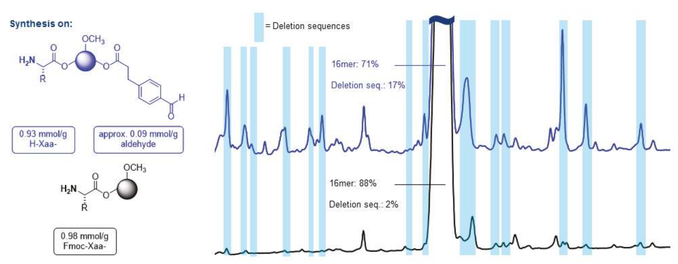
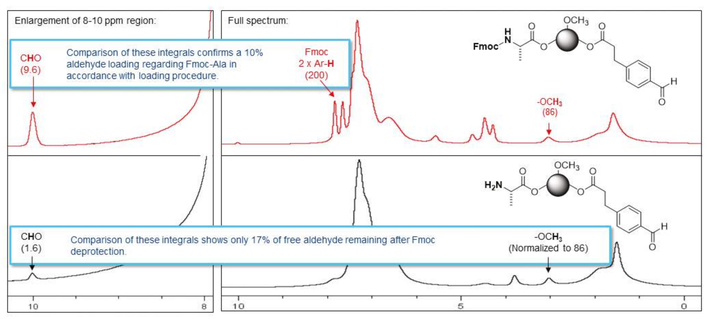


Fig. 7: U-HPLC analyses of a test synthesis on a resin partially loaded with a benzaldehyde derivative (top) compared to a reference synthesis on a resin without an intentionally introduced aldehyde impurity (bottom)



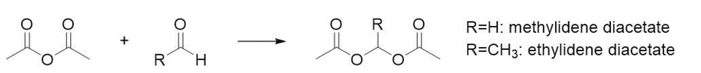
As shown in Fig. 8 direct detection of resin-bound aldehydes is feasible through ^{1}H-MAS-NMR. However, the presence of amines hinders aldehyde quantification, most likely due to imine formation between aldehyde and amine.

Fig. 8: ^{1}H-MAS-NMR analysis of an amino acid 2-chlorotrityl resin partially loaded with a benzaldehyde derivative before (top) and after Fmoc deprotection (bottom)



Besides resin-bound aldehydes, such impurities in reagents or building blocks could also cause deletions by temporary imine formation with free *N*-termini, e.g.:

Aldehyde impurities in Ac_2O:
- Aldehydes are formed as impurities during the production of Ac_2O or AcOH[5]
- Aldehydes react with Ac_2O:



Prevention strategy:
Ensure the resin, solvents, reagents and reactants used for SPPS are free of aldehyde impurities

CONCLUSIONS

- Deletion sequences in SPPS can be formed due to various causes.
- During SPPS, preventing the formation of deletion sequences is crucial as their later separation is potentially challenging.
- Depending on the identified root cause, different prevention strategies have to be applied.
- Careful control of used starting materials and/or of the conditions for SPPS is necessary in order to prevent the formation of deletion sequences.

REFERENCES

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[4] Yousif, E.; Haddad, R. *SpringerPlus* **2013**, *2* (1), 398.
[5] Torrence, G. P. et al. EP 0487284 B1, **1995**.