A NEW TURN IN PEPTIDE PURIFICATION

TRACELESS PARALLEL PEPTIDE PURIFICATION BY A FIRST-IN-CLASS REDUCTIVELY CLEAVABLE LINKER SYSTEM FEATURING A SAFETY-RELEASE

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Introduction

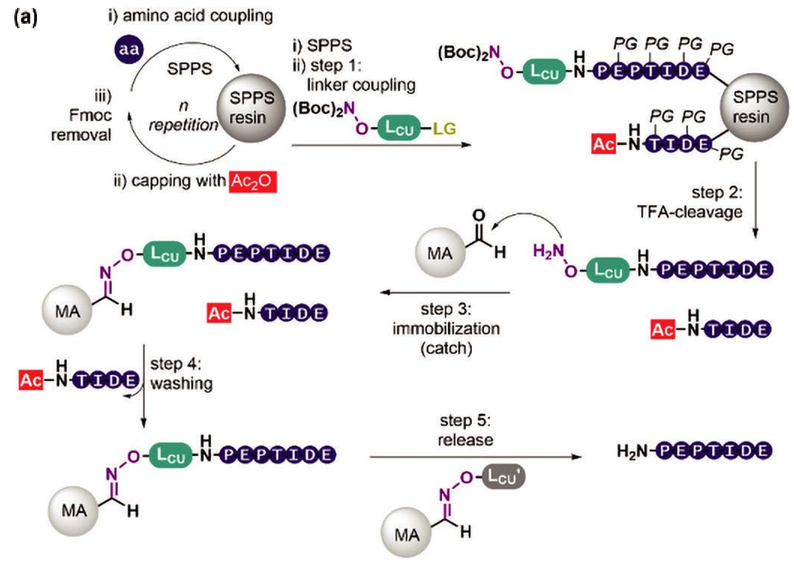
Solid phase peptide synthesis (SPPS) is the most commonly used method for peptide production and the current generation of automated peptide synthesisers are capable of producing dozens of different peptides in a single parallel run. However, the go-to purification method, reverse-phase high performance liquid chromatography (RP-HPLC) represents the bottleneck of peptide production, from research scale through to API development and manufacturing. Technological advances have increased the speed and resolution power of RP-HPLC, but the process remains linear, which is sub-optimal for peptide libraries and rapid process development. Catch-and-release (C&R) technology offers a potential solution for the associated difficulties of RP-HPLC, but as yet, it has not been widely adopted in peptide manufacturing due to a lack of a robust process and universal applicability. Presented herein is the development of a new linker system, resulting from a long-term collaboration between the German company Belyntic and leading peptide manufacturer Bachem, which introduces a truly broad spectrum C&R purification method termed *peptide easy clean* (PEC). This combined research effort resulted in a recently published Edge "Pick of the Week" article in Chemical Science [1].

Benefits:

- ***Increasing the speed of peptide development**: i) PEC is universally applicable. ii) Automated parallel purification is a possibility.*
- ***Facilitated purification of difficult peptides**: i PEC is an orthogonal approach to RP-HPLC, specifically removing capped peptide truncations. ii) The peptide catch step is tolerant of a wide range of conditions, enabling loading in organic solvents that are typically incompatible with traditional RP-HPLC. iii) The linker can facilitate solubilization of aggregation-prone peptides through disruption of secondary structures.*
- ***Improving drug stability and efficacy**: PEC is a modification platform for peptides. The innovative safety-release enables conjugation chemistries to explore the impossible through highly efficient solid-phase peptide modification.*

The PEC process

Prior to catch-and-release via PEC, peptides are formed on-resin using SPPS. Routine capping after every amino acid coupling step is essential for removal of truncated species arising from incomplete coupling. The PEC process removes these capped species, as they cannot be linker-functionalized in the final SPPS step. PEC is achieved in five main steps as shown in Scheme 1: 1) linker attachment, 2) TFA cleavage, 3) catch step, 4) washing away of impurities, 5) release of linker-free purified peptide.



Scheme 1 - PEC process overview (aa: amino acid; PG: protecting group; MA: modified agarose beads; LCU: cleavable linker unit; LG: leaving group).

The 2nd generation reductively cleavable linker enables safety-release and an efficient peptide modification

1. The first reductively cleavable linker for peptide purification

The previous generation PEC linker **1** contained a base-labile moiety (Figure 1) [2]. Despite impressive purification and recovery for most peptides, the basic release solution was problematic for certain amino acid sequences. Therefore, an alternative release mechanism was sought. Reductively cleavable linkers have been successfully employed in prodrug release [3], and also applied to oligo production [4]. To apply reductive cleavage to peptide C&R, a new linker with an oxyamine catch tag, para-nitrophenol leaving group, and a reductively cleavable para-azido-benzyl unit was synthesized (Figure 1, linker **2**).

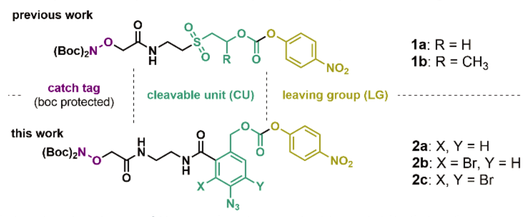
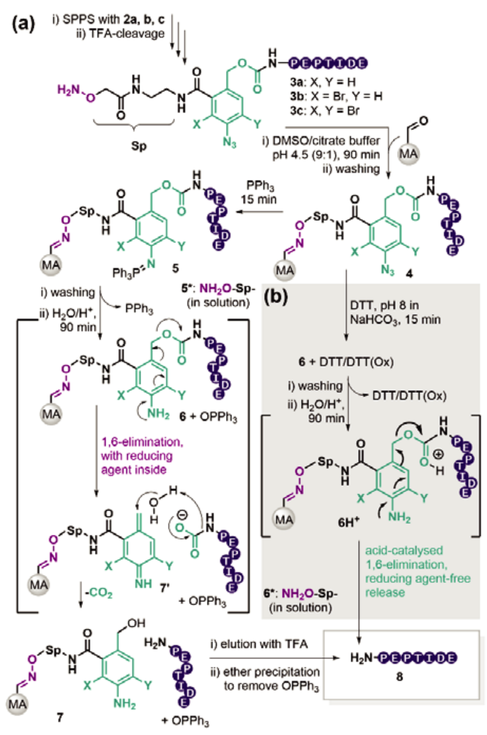


*Figure 1 - Molecular structures of the previously reported base-labile linker **1** and novel reductively cleavable linker molecule **2**, where X and Y = H or Br.*

After SPPS, attachment of the linker is performed in DMF, in the presence of Oxyma. The reaction is typically complete within 120 minutes and coupling/washing steps may be automated using a peptide synthesiser. Next, TFA cleavage is performed using standard protocols; the linker is stable towards standard cleavage scavengers such as H_2O, TIPS and EDT. After precipitation in ether and workup, the linker-tagged full-length peptide **3** and acetylated truncations remain. The crude mixture may then be dissolved in a wide variety of solvents for the catch step, but the optimum conditions are DMSO + 10 vol.% 0.1 M citric acid buffer containing 7 M guanidinium chloride (GdmCl, pH 3.5). Solvation using this mixture usually results in loading of the linker-tagged peptide within 90 minutes. Loading can be monitored by UPLC/HPLC, following the diminishing linker-modified peptide peak. The agarose resin is then washed to remove capped truncations.



Scheme 2 - (a) Application of reductively cleavable linker 2a, b, c in C&R purification of peptides with PPh_3 as reductive stimulus; MA: modified agarose beads, (b) Usage of dithiothreitol (DTT) as reductive stimulus.

The reductive release step is initiated by using PPh_3 (Staudinger reaction) or DTT to reduce the benzyl azide of species **4** to aniline. Acidolysis via nitrogen lone pair attack 1,6-elimination mechanism frees the target peptide as a carbamate that spontaneously decomposes to CO_2 and the desired peptide **8** (also releasing Ph_3PO if PPh_3 is used). The target peptide may be isolated from the acidic cleavage solution by standard ether precipitation.

2. Side-reaction-free purification

The reductively cleavable system was shown to be suitable for a panel of model peptides that were previously shown to undergo base-induced side-reactions, as depicted in table 1.

Name	Peptide sequence[a]	Expected base-induced side product
P1	H-**T**RYQAKPVNRSTPISTGKEG-OH	oxazolidinone (+26 Da)[b]
P2	H-RTGKLAPSFNGKSSQT**RE**IL-OH	citrulline (+1 Da)[b]
P3	H-DSAPNPVLDI**DG**EKLRTGTN-OH	aspartimide (-18 Da)[b]
P4	H-ARTKQTARKSTGGKA-OH	none

Table 1 - Set of peptides for reductive linker evaluation and results of PEC purification of peptides P1-4. [a] Bold underlined motif underwent base-induced side reactions earlier; [b] the mass deviation to the native peptide when side product is formed.

Gratifyingly, none of these side products occurred when peptides P1-P3 were recovered via PPh_3 reduction in combination with linker 2a (black traces of products in P1-3 Figure 2) and for P4, no side-products or co-eluting impurities remained after purification.

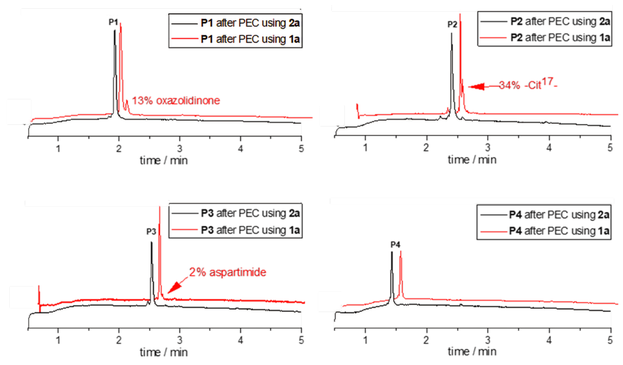


Figure 2 - PEC-purification of base-sensitive peptides P1-3 and Histone H3 (1-15) peptide P4.

To increase the stability of the linker towards TFA, the benzyl group was brominated at either the 2-, or 2,4-positions. Solution phase assessment indicated a marked improvement in stability of the linkers. The stability enhancement was mirrored when employing the bromo-linkers to purification of peptides P1-P4; peptide recovery was effectively doubled with mono-brominated compared to non-brominated linker, whilst retaining the high observed purities (Table 2).

No.	crude purity[c]	Purity[c] after PEC using linker				recovery[d] using linker			
		1a	2a	2b	2c	1a	2a	2b	2c
P1	67%	77%	91%	91%	91%	74%	40%	74%	44%
P2	42%	67%	83%	89%	92%	60%	40%	70%	42%
P3	75%	84%	88%	87%	88%	46%	23%	56%	32%
P4	36%	92%	89%	96%	91%[c]	32%	22%	58%	29%[e]

Table 2 - Results of PEC purification of peptides P1-4.

3. Brominated reductively cleavable linker enables safety-release and an efficient peptide modification platform

The additional stability conferred the possibility of safety-catch applications. The mono-bromo linker provided sufficient stability of the aniline species for washout of DTT/DTT(Ox.) while allowing the peptide release by a final acid treatment. This opens up the possibility for on-resin modification of the peptide in neutral to basic pH, such as alkylation, oxidation or dye-labeling, including modifications not traditionally possible.

Furthermore, the presence of the linker on the full-length peptide can improve the solubility of hydrophobic and aggregation-prone peptides in organic solvents, such as DMSO (dimethyl sulfoxide) or HFIP (hexafluoro-2-propanol). This can aid handling of the crude peptide by preventing self-assembly and formation of aggregates. This feature affords the PEC linker a second application, a modification platform.

4. Rapid parallel purification of peptide libraries

The PEC purification process is an advantageous platform to perform parallel purification, and subsequently accelerate the development of peptide libraries. PEC has been applied for the purification of a set of peptides. A panel of 20 clinically relevant peptides with a length between 9-20 amino acids [5] were cleaved and purified in parallel within 6 hours. Mean purity determined by UPLC was 89% (Figure 3a) with an average recovery of 72%. The majority of the purified peptides (14 of 20) had a final purity of over 90%.

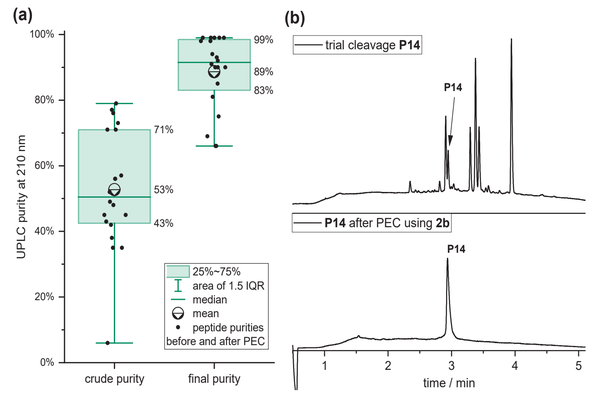


Figure 1 - (a) Box-diagram of crude vs. final purities of the 20 peptide set purified in parallel. IQR: interquartile range. (b) UPLC chromatograms before (above) and after (below) the PEC process ii) of peptide P14.

Impressively, the purity of peptide P14 (sequence: H-KVGYTERQRWDFLSEASIM-NH_2) improved from 6% to 99%, potentially difficult to achieve by HPLC due to closely eluting impurities (Figure 3b). This example demonstrates the potential of this technology for the rapid development of peptide APIs and for purification of research-grade material.

5. Summary

As a result of a collaboration between Belyntic and Bachem, a first-in-class reductively cleavable linker for C&R peptide purification was developed. The system overcame limitations in previous iterations of the linker and resulted in both high purity and recovery in a single step. The system was applied successfully for parallel purification of 20 peptides within 6 hours.

Bromination on the *p*-azido-benzyl core led to increased TFA-stability and enabled a safety-release mechanism. The linker 2b enabled a contamination-free traceless release, where the reductive agent could be washed out prior to acid-catalysed liberation of desired peptides by 1,6-elimination (safety release).

The addition of the PEC technology to the portfolio of Bachem for peptide manufacturing, provides access to peptides of extreme hydrophobicity that are difficult or impossible to purify by traditional RP-HPLC, rapid development of API or research-grade materials through parallel purification and unusual peptide modifications taking advantage of the solid support.

With the expertise of Bachem in the development and manufacturing of peptide APIs and in collaboration with our industry partner Belyntic, we once more underline our position as Leading Partner in TIDES - Peptides and Oligonucleotides. This technology will enable Bachem to develop high quality peptide APIs with better stability, efficacy and improved delivery properties for customers. These peptides and collaborations will render possible therapeutic applications that have been yet unreachable.

References

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