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A universal strategy for preparing protected C-terminal peptides on the solid phase through an intramolecular *click* chemistry-based handle†

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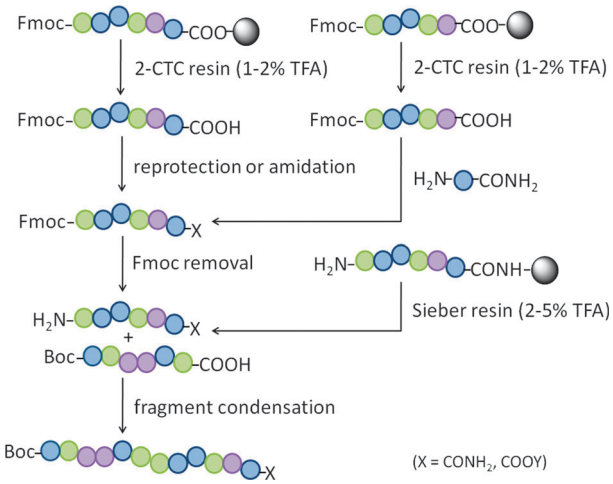
A new universal strategy exploits DKP formation in a dipeptide moiety whose C-terminal residue is blocked by a leaving group. It enables both synthesis of C-terminal protected peptides that are useful for convergent synthesis of large peptides and use of a C-terminal permanent protecting group that can be cleaved by catalytic hydrogenation to release the peptide.

The past decade has witnessed the so-called *peptide revolution*, in which peptides have been promoted from mere biochemical tools to real alternatives to small-molecule drugs.[1] Furthermore, peptides are becoming cornerstones of emerging fields such as drug delivery, nanotechnology and materials.[2] This is partly due to the explosion of solid-phase peptide synthesis (SPPS), first developed by Nobel Laureate Bruce Merrifield and then fine tuned by numerous research groups.[3] This method is now used to prepare all peptides used in research as well as commercial peptides comprising more than a few amino acids.

All synthetic strategies are based on the appropriate combination of protecting groups together with an efficient method for activating the carboxyl group prior to peptide coupling. While extensive studies have yielded myriad protecting groups for both the α-amino and the side-chain functions, which allow synthesis of cyclic and/or complex peptides, far less effort has been dedicated to the C-terminal function, which is blocked with the insoluble polymer resin. Removal of the C-terminal protecting group and concomitant liberation of the peptide from the resin afford a free C-terminal functional group, usually an acid or amide. However, a semi-permanent protecting group for the C-terminal function would be desirable, as it would enable cleavage of the peptide from the insoluble polymer resin to render the C-terminal function protected for further manipulation in solution.



Scheme 1 Classical convergent strategy for peptide synthesis.

Chemical synthesis of proteins and industrial preparation of peptides longer than 20–30 residues are performed by a convergent strategy in which different protected fragments are first constructed on the solid phase and subsequently coupled together in solution (Scheme 1).[4]

Protected peptides are currently prepared on a 2-Cl–Trityl–Cl (2-CTC) resin using an Fmoc–*t*Bu strategy. This resin enables liberation of the protected peptide using 1–2% TFA solution.[5] A drawback of this methodology is the preparation of C-terminal fragments whose C-terminal function requires to be blocked. In the case of C-terminal acid peptides, this requires reprotection of the C-terminal carboxyl group in solution, with a consequent risk of racemization and low yields. For C-terminal amide peptides, in addition to amidation or incorporation of the amide form of the last residue, superlabile amide peptide resins such as the Sieber-resin can be used.[6] However, liberation of protected peptides from these resins requires 2–5% TFA solution, which is not totally compatible with all side-chain protecting groups (*e.g.* the Trt of His).

Herein is described a new universal strategy for preparing C-terminal protected peptides on solid phase. The strategy was exemplified in two applications: (i) synthesis of C-terminal protected peptides useful for assembly of large peptides (convergent approach); and (ii) use of a permanent C-terminal protecting group that can be liberated by catalytic hydrogenation.

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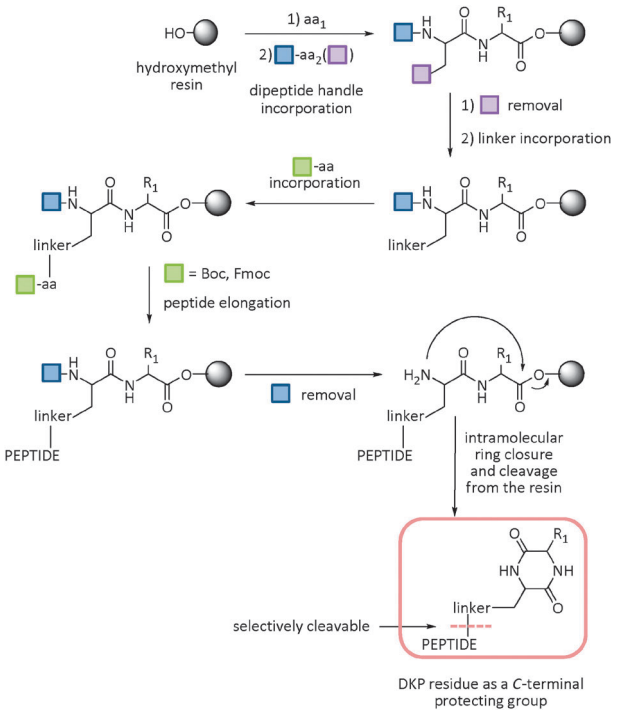


Scheme 2 Schematic representation of the universal DKP *click* handle strategy.

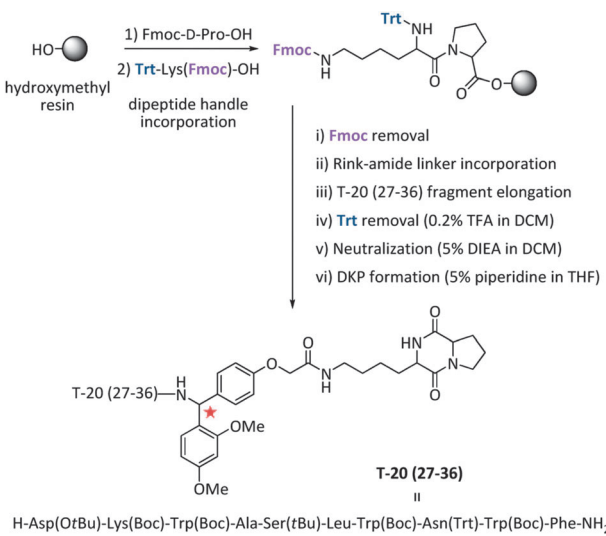
Click reactions were defined by K.B. Sharpless as "*those inspired in nature, that give very high yields, generate inoffensive side-products, are stereospecific, exhibit a large thermodynamic driving force, have simple reaction conditions...*"[7] In peptide chemistry, the reaction that fits this definition is diketopiperazine (DKP) formation, which often jeopardizes preparation of simple C-terminal acid peptides as well as complex ones that contain *N*-alkyl amino acids.[8] After pioneering works of Geysen *et al.*[9] and Atrash and Bradley,[10] we have further elaborated the DKP cleavage concept by incorporating a selectively cleavable linker group in the DKP *click* handle (Scheme 2).

The present strategy exploits DKP formation in dipeptide moieties whose C-terminus is blocked by a leaving group. As the DKP itself becomes part of the permanent protecting group of the C-terminal carboxyl function, the dipeptide should contain a function to facilitate its connection to the C-terminal fragment, such as the amino side-chain of Lys.‡ Considering that DKP formation is favored by the presence of an *N*-alkyl amino acid, which favors the *cis*-conformation, and an L/D combination of amino acids, which stabilizes the 6-member ring DKP,[11] a D-Pro as the other component was chosen.§ This D-Pro is attached directly to a hydroxyl resin (*e.g.* hydroxy Merrifield resin). Finally, the connection between the C-terminal fragment and the dipeptide moiety would be achieved through a bifunctional linker (*e.g.* Rink-amide, Wang-type or benzyl-type handles), which acts as a permanent protecting group, and liberates the peptide with the expected functionality (acid or amide) during the final global deprotection.

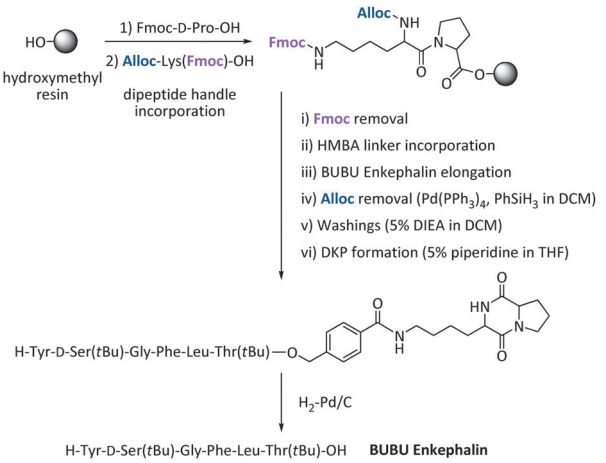
The cornerstone of this process is the protecting group of the α-amino of the penultimate amino acid (■—in Scheme 2), which dictates the conditions for releasing the protected peptide from the resin. Thus, if Trt is used, cleavage is accomplished by treatment with 0.1–0.3% TFA; if Alloc is used, then liberation of the protected peptide is carried out with Pd(0); and if pNZ is used, then cleavage is performed with Sn^{2+}.[12] At this stage, DKP formation does not take place. Moreover, during successive treatments with 5% DIEA in DCM, which enables washing/neutralization of the resin before liberation of the protected peptide, DKP formation is negligible. This feature is extremely important when Pd(0) is used, because extra washing facilitates removal of any Pd salts, which can contaminate the final product.

The absence of DKP formation during treatment of the resin with DIEA is intriguing because, as it is well-known, in a Boc/Bzl strategy any C-terminal sequence Aa–Pro can easily render quantitative DKP formation after removal of the Boc group of the Aa residue and subsequent neutralization with 5% DIEA in DCM.[13] According to Goolcharran and Borchardt,[14] the differences observed in the rate of DKP formation can be explained by various factors: differences in the pK_a values of the terminal α-amino groups of the analogs, the steric bulk, the ability of the Aa–Pro peptide bond to undergo *cis–trans* isomerization, and/or the conformational stability of the resulting DKPs. In our case, the presence of the target peptide and the linker as the side-chain substituent of one of the DKP components can affect said parameters—namely, by adding bulkiness to the system and decreasing the polarity of the medium, thereby resulting in the need for a stronger base; hampering *cis–trans* isomerization of the Aa–Pro bond; and/or compromising the conformational stability of the resulting DKP.

As a model to test our strategy, the fragment H-(17–36)-NH_2 of the drug Fuzeon® (T-20) was chosen. The fragment is synthesized in solution, by coupling of fragments Fmoc-(17–25)-OH and H-(26–36)-NH_2. The former is prepared using a 2-CTC resin, and the latter, by coupling of H–Phe–NH_2 to pre-formed Fmoc-(26–35)-OH (also obtained using 2-CTC resin) in solution.[15] Scheme 3 shows the one-pot SPPS of C-terminal protected fragment H-(27-36)-DKP_{handle}, using the new DKP *click* handle strategy. The dipeptide Lys-D-Pro, the Fmoc-Rink-amide linker, as well as the 10 Fmoc-amino acids of the



Scheme 3 Synthesis of C-terminal protected T-20 fragment H-(27–36)-DKP_{handle} using the DKP *click* handle strategy.



Scheme 4 Synthesis of BUBU Enkephalin using the DKP *click* handle strategy.

fragment, were all incorporated smoothly using conventional SPPS protocols (see ESI†). After elimination of the Fmoc group from the last amino acid, the Trt group was removed. The peptidyl-resin was neutralized with 5% DIEA in DCM, and then the protected peptide was liberated by treatment with 5% piperidine in THF¶, which was removed under reduced pressure. The protected peptide was then washed with pre-cooled Et_2O. The protected T-20 fragment H-(27–36)-DKP_{handle} was ready to be coupled with the fragment Boc-(17–26)-OH, which was prepared using the 2-CTC resin, saving the two steps of the classical convergent approach (incorporation of H–Phe–NH_2 and removal of Fmoc in solution). Coupling of the fragments using DIPCDI/HOBt, followed by global deprotection with TFA–DMB(1,3-dimethoxybenzene)–TIS (92.5 : 5 : 2.5), afforded the desired product, unprotected H-(17–36)-NH_2, in a good purity (see ESI†).

Incorporation of a benzyl-type protecting group as a selectively cleavable linker enables peptide synthesis through *a totally acid-free* strategy, which has been exemplified by the synthesis of BUBU Enkephalin, a highly potent and selective δ-opioid agonist whose D-Ser^2 and Thr^6 hydroxyl groups are *tert*-butylated.‖ In this *totally acid-free* strategy, the α-amino function (■—in Scheme 2) of Lys was protected with the Alloc group (Scheme 4). After removal of Fmoc, 4-hydroxymethylbenzoic acid (HMBA) was incorporated and the peptide sequence was elongated (see ESI†). After cleavage of the protected peptide from the resin, the benzyl-type protecting group was removed by H_2–Pd/C.

In conclusion, a new concept for protection of the C-terminus of peptides has been developed. It overcomes some of the drawbacks associated with SPPS, such as preparation of the C-terminal fragment in a convergent strategy. Furthermore, it introduces more flexibility into SPPS, by enabling the use of benzyl-type protecting groups, which can be removed by catalytic hydrogenation. Finally, it may enable better control in the solid-phase strategy, by allowing further manipulation of the molecule in solution after release. We are presently extending this strategy to side-chain anchoring, which can involve other functional groups (*e.g.* hydroxyl, thiol and carboxylic acid).

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Notes and references

‡ Lysine was chosen due to its lower cost, but other amino acids with amino side-chains were also used (see ESI†).
§ Although D-Pro is an optimal component of the dipeptide part of the handle, it can be replaced with L-Pro and other *N*-alkyl amino acids (results not shown), which give similar results. Furthermore, γ-amino-Pro, which contains the two main features needed for this strategy (an *N*-alkyl amino acid and an amino side-chain), can be used.
¶ Pyrrolidine can be used instead of piperidine.
‖ BUBU Enkephalin was used to demonstrate the feasibility of this strategy; however, this peptide can be synthesized using the 2-CTC resin.

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