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# Fmoc solid-phase synthesis of C-terminal modified peptides by formation of a backbone cyclic urethane moiety†

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**C-terminally modified peptides are of high significance due to the therapeutic properties that accompany various C-terminal functional groups and the ability to manipulate them for further applications. Thus, there is a great necessity for an effective solid phase technique for the synthesis of C-terminally modified peptides. Here, we report a universal solid phase strategy for the synthesis of various C-terminal modified peptides which is independent of the type of resins, linkers, and unnatural moieties typically needed for C-terminal modifications. The technique proceeds by the modification of C-terminal serine to a cyclic urethane moiety which results in the activation of the backbone amide chain for nucleophilic displacement by various nucleophiles to generate C-terminally modified acids, esters, *N*-aryl amides, and alcohols. This cyclic urethane technique (CUT) also provides a general strategy for synthesis of C-terminal protected peptides that can be used for convergent synthesis of large peptides. The C-terminal protecting groups are cleaved by facile hydrolysis to release the free peptide.**

C-terminal modified peptides such as acids, esters, *N*-aryl amides, alcohols, and amides have been extensively used in chemical synthesis of proteins, semi-synthesis, catalysis, and in various therapeutic applications.[1] These C-terminally modified peptides exhibit a wide range of biological activities; for instance, *N*-aryl amides enhance metabolic stability and biological activity.[2,3] Key processes of cell growth and differentiation embody ester groups at their C-terminus.[4] Moreover, peptide alcohols, such as peptaibols, are well known antibiotics.[5]

In general, C-terminal modified peptides are synthesized in solution after cleavage from a solid support.[6] For subsequent biological investigations, however, a series of peptide derivatives are often required, which calls for the development of flexible solid phase techniques. Modifications at the C-terminus of peptides are challenging due to the direct attachment of the C-terminus on the solid support. Therefore, these modifications are generally attainable by using special resins that are appropriately designed to allow anchoring of the C-terminus of the peptide and generation of a specific end group at the C-terminus upon cleavage.[7] Due to the therapeutic potential of C-terminally modified peptides, a growing number of studies have reported alternative methods such as the use of special linkers which rely on unique oxidative conditions to activate the linker for attack by various nucleophiles.[8] However, this approach is limited due to the undesirable oxidation of sensitive residues such as Cys, Met, and Trp. Other methods include side-chain or backbone amide anchoring approach, whereby the C-terminal carboxyl group is not involved as an attachment point to the resin,[9] and inverse solid-phase peptide synthesis (*viz.*, from N to C-terminus).[10] One major drawback of inverse solid-phase peptide approach is potential epimerization at all coupling stages due to repeated resin-bound carboxyl activation. Another technique is internal resin-capture approach, which also involves the use of special linkers or handles.[11–14] All of the abovementioned methods require special resins, unnatural linkers, or handles for the synthesis of C-terminally modified peptides. Here, we report the discovery of a novel general strategy that circumvents the use of special resins, unnatural linkers, or handles and affords peptides with various types of C-terminal modifications such as acids, esters, *N*-aryl amides, and alcohols.

Our methodology is based on the activation of a peptide backbone amide bond by increasing its nucleofugality. This renders the C–N bond susceptible to nucleophilic attack, affording peptides with various C-terminal modifications. Synthesis of C-terminally modified peptides by this strategy entails anchoring of a C-terminal serine residue with a selectively removable side-chain protecting group (Pg) to a solid support followed by the side-chain activation that results in the formation of a cyclic urethane moiety (Scheme 1A). Earlier attempts at the formation of this moiety on Fmoc/Boc protected peptides led to the formation of dehydroalanine.[15] Our group recently reported the application of such moiety in sequencing of peptides in solution.[16] Next, nucleophilic displacement of the cyclic urethane

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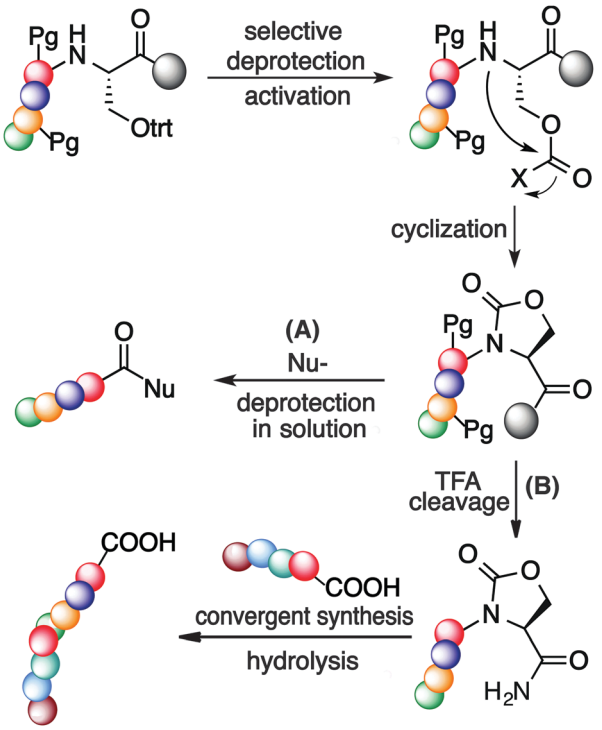



Scheme 1 Rationale for the development of cyclic urethane technique (CUT) for synthesis of C-terminal modified peptides: (A) application in synthesis of C-terminally modified peptides by nucleophilic displacement of cyclic urethane moiety. (B) Synthesis of protected C-terminal peptides using (CUT).

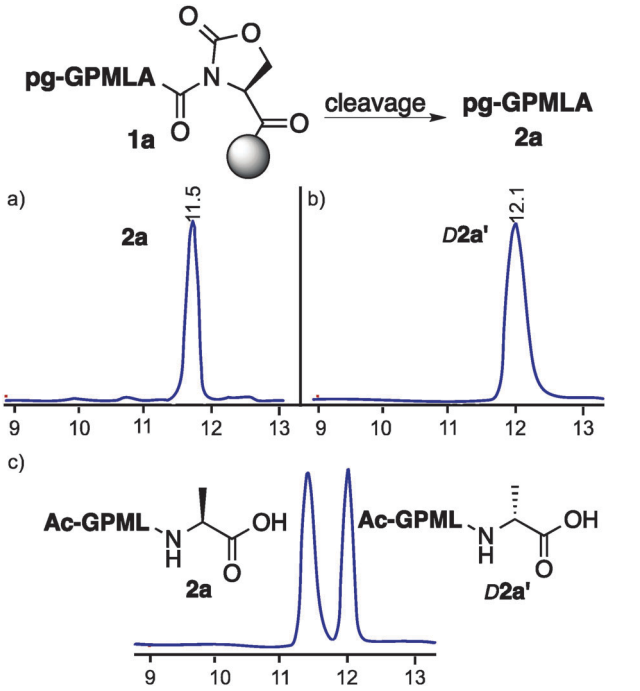



Fig. 1 HPLC traces of (a) purified peptide acid **2a** Ac-GPMLA-(L)-OH (b) purified diastereoisomer *D***2a**′ Ac-GPML-A-(D)-OH and (c) mixture of both diastereoisomers **2a** and *D***2a**′, indicating lack of detectable epimerization. Pg = Ac.

moiety by treatment with various nucleophiles releases the C-terminal modified peptides from the solid support (Scheme 1A). Importantly, only those resin-bound peptides that complete both activation and displacement steps are released. Thus, the released C-terminally modified peptides exhibit high purity, eliminating the need for extensive purification.

Another application of the cyclic urethane technique (CUT) is to generate a peptide with semi-permanent protection at the C-terminus upon cleavage from the solid support (Scheme 1B).[17] Such protection is of significance due to the potential use of the C-terminally protected peptides for the formation of large peptides *via* convergent synthesis. Finally, the Pg at the C-terminus can be easily removed by mild, hydrolytic conditions.

To implement this strategy for the synthesis of a C-terminally modified peptide, a model peptide, Ac-GPMLAS-Rink was synthesized on Rink Amide resin using standard Fmoc SPPS with serine as the C-terminal residue. The trityl (trt) group from serine was selectively removed with TFA/DCM (1 : 3) to generate a free serine residue. Next, the hydroxymethyl group of serine was activated with *N*,*N*′-disuccinimidyl carbonate (DSC) followed by intramolecular nucleophilic attack of the amide nitrogen of the peptide backbone generated a five-membered cyclic urethane ring Ac-GPMLAOxd **1a** in 16 h (Fig. 1).[16]

After formation of the cyclic urethane moiety **1a**, nucleophilic release of the peptide acid Ac-GPMLA **2a** from the solid support was achieved by hydrolysis under basic conditions. The solvent was then removed, and the resulting peptide acid **2a** was analyzed by HPLC and LCMS. Various reaction conditions (time, solvent, and temperature) were optimized to achieve the maximum conversion (Table S1, ESI†). The extent of epimerization by formation of the cyclic urethane moiety and nucleophilic displacement was also investigated. Diastereomer, Ac-GPMLA-(D)-OH (*D***2a**′), was synthesized using the optimized conditions, and peptide acids **2a** and *D***2a**′ were subsequently analyzed by HPLC (Fig. 1). No detectable levels of epimerization were observed.

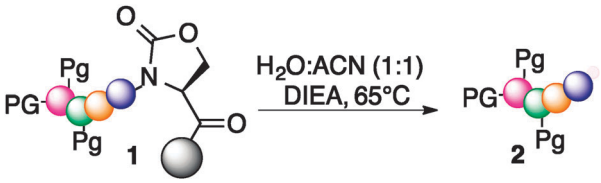
To evaluate the compatibility of various amino acids and protecting groups toward serine activation and its nucleophilic displacement, peptides with different sequences were evaluated (Table 1). The results indicated that all peptides underwent smooth activation to generate activated peptides **1b–1k** followed by hydrolysis to corresponding protected peptide acids **2b–2k** with varying HPLC conversions ranging from 80 to greater than 95% (entries 1–10, Table 1). Hydrolysis was carried out in water:ACN at 65 °C for 2–4 h.

Peptides with bulky amino acids preceding serine, FESQIS-Rink (**1e**) and TCDVS-Rink (**1k**), underwent smooth activation and nucleophilic release under the reaction conditions and generated **2e** and **2k** (entries 4 and 10, Table 1). The complete study with various peptides can be found in Table S2, ESI.† Moreover, the compatibility of CUT with different resins was confirmed by synthesizing VWRAS **1j** on Rink Amide, Wang, Chem Matrix, and Tentagel resins. This is in direct contrast to previously reported methods.[7]

Protected peptide acids have been extensively used for the synthesis of therapeutic macrocyclic peptides.[18] To demonstrate the application of CUT in the synthesis of macrocyclic

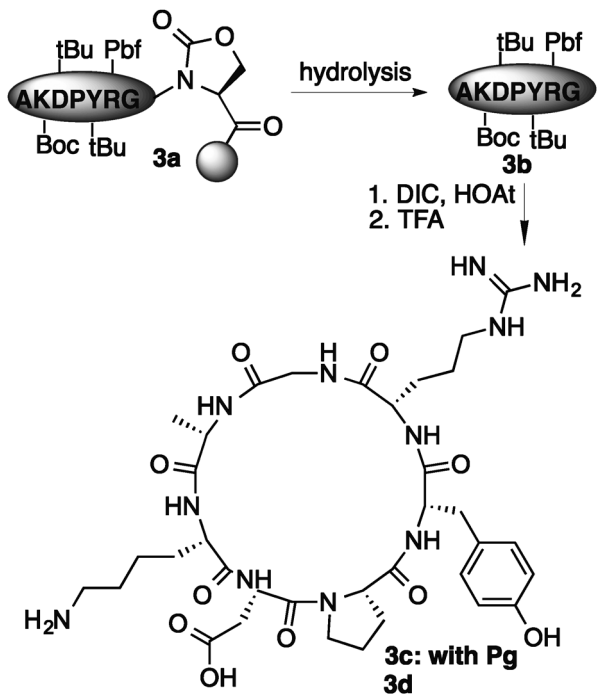
Table 1 Substrate scope of CUT in synthesis of peptide acids[a]



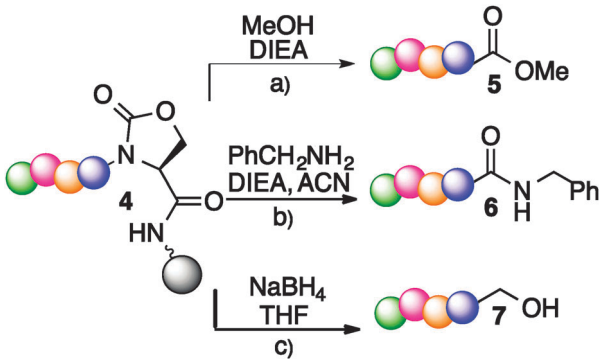

| Entry | Substrate | Peptide | PG | Conv.[b] (%) |
|---|---|---|---|---|
| 1 | **1b** | LFK(Boc)N(Trt)A | Ac | 95 |
| 2 | **1c** | LFK(Boc)N(Trt)A | $NH_2$ | 95 |
| 3[c] | **1d** | LFK(Boc)N(Trt)A | Fmoc | 80 |
| 4 | **1e** | FE(*t*Bu)S(*t*Bu)Q(Trt)I | $NH_2$ | 90 |
| 5 | **1f** | R(Pbf)D(*t*Bu)PMLG | Ac | 95 |
| 6 | **1g** | R(Pbf)D(*t*Bu)PMLG | $NH_2$ | 90 |
| 7 | **1h** | Y(*t*Bu)LFK(Boc)N(Trt)A | Ac | 95 |
| 8 | **1i** | Y(*t*Bu)LFK(Boc)N(Trt)A | $NH_2$ | 90 |
| 9 | **1j** | VWR(Pbf)A | Ac | 90 |
| 10 | **1k** | T(*t*Bu)C(*t*Bu)D(*t*Bu)V | Ac | 90 |

[a] Reaction conditions: peptide **1** (25 mg, 0.7 mm $g^{-1}$) on solid support was reacted with $H_2O$:ACN in DIEA (20 μL) at 65 °C for 2–4 h. [b] Conversion to peptide acid was calculated from the absorbance at 220 nm using HPLC. [c] Mixture of completely protected peptide and Fmoc deprotected peptide was observed.

peptides, AKDPYRGS-Rink AM was synthesized on solid support, followed by serine selective deprotection, activation **3a**, and cleavage with water to obtain the protected linear peptide acid AK(Boc)D(*t*Bu)PY(*t*Bu)R(Pbf)G-OH **3b** (Scheme 2). Next, the protected peptide acid **3b** was subjected to coupling reagents to obtain the desired head to tail macrocyclized peptide **3c**, followed by removal of the side-chain Pg to generate deprotected macrocyclic peptide cyc(AKDPYRG) **3d** which was analyzed by




Scheme 2 Application of CUT in the synthesis of macrocyclic peptide **3d**.




Scheme 3 CUT for synthesis of peptide esters, *N*-aryl amides and alcohols. peptide = **X**-AVGPPGVA-Oxd, **4**. where **X** = $NH_2$ or Ac.

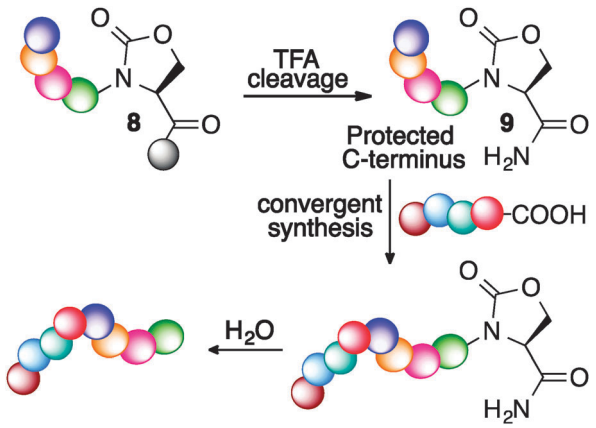
NMR, HPLC, and MS (Scheme 2 and Fig. S1, ESI†). In comparison, peptide acid AKDPYRG-OH generated by using Wang resin[19] gave an undesired double cyclized product, which can arise from side-chain to head or side-chain to side-chain reactions (Fig. S2, ESI†).

Next, we applied CUT for the synthesis of protected peptides with various C-terminal modifications such as esters, *N*-aryl amides, and alcohols (Scheme 3). To obtain the modified peptide, the corresponding nucleophile was added to a solid support with the cyclic urethane moiety at the C-terminus, where it underwent nucleophilic displacement and generated the C-terminally modified peptide in solution.

The peptide methyl ester, AVGPPGVA-OMe **5** was obtained by treatment of activated peptide **4** with methanol in the presence of a base (a, Scheme 3). This technique was also utilized for the synthesis of peptide methyl ester PFFFOMe, which was then used as a catalyst for an asymmetric aldol reaction (Fig. S3, ESI†); the presence of such methyl ester group is known to be critical for catalytic activity of peptide PFFFOMe.[20] Next, benzylamine was used as a nucleophile to obtain *N*-aryl amide peptide **6** (b, Scheme 3). The presence of an aryl amino group at a peptide's C-terminus enhances its biological activity, metabolic stability, and hydrophobicity.[2,3] CUT was also applied for the synthesis of biologically active peptide **7** with a C-terminal alcohol group,[5] by treatment of activated peptide **4** with a solution of sodium borohydride in THF (c, Scheme 3).

We further demonstrated the application of CUT for synthesis of peptides with a semi-permanent protecting group (Pg) at the C-terminus (Scheme 4), which can be used for various manipulations in solution. The use of CUT for synthesis of protected C-termini peptides is distinguishable from current methods that require reprotection of the C-terminal carboxyl group in solution with a consequent risk of racemization and low yields.[21] Another method utilizes click chemistry-based handles and is limited by the use of special linkers and harsh deprotection conditions.[21] Our method does not require any special linkers or handles, and it employs mild conditions for removal of Pg at the C-terminus.

For the synthesis of peptides with a semi-permanent Pg at the C-terminus, activated peptide Fmoc-AR(Pbf)FPPFR(Pbf)AOxd **8** on a solid support was cleaved from the resin by using the TFA cleavage cocktail to generate peptide Fmoc-ARFPPFRAOxd **9** with




Scheme 4 CUT technique for synthesis of protected C-terminus peptide Fmoc-ARFPPFRA-Oxd **9**.

Oxd as a Pg at the C-terminus (Scheme 4 and Fig. S4, ESI†). The C-terminally protected peptide Fmoc-ARFPPFRAOxd **9** can be used for the synthesis of larger peptides by a convergent approach. Subsequently, the Oxd protecting group at the C-terminus of the resulting large peptide can be easily removed under basic hydrolysis conditions.

A universal solid phase strategy has been effectively developed and applied for the synthesis of various C-terminal modified peptides of therapeutic importance such as acids, esters, *N*-aryl amides and alcohols. This approach is resin-, linker-, and handle-independent, thus circumventing limitations inherent to earlier methods. The main advantage of the approach described is that the elongation of the peptide chain can be carried out by standard methods and only activated peptide chains are released from the resin, thus delivering highly pure C-terminal modified peptides in solution. Moreover, the modified peptides are released from the resin without racemization. Another significant advantage of CUT is that it generates peptides with a protecting group at the C-terminus, which can undergo various modifications in solution after release from solid support (*e.g.*, the synthesis of large peptides using a convergent strategy). Finally, the C-terminal protecting group is easily removed under mild, basic hydrolytic conditions to generate a free peptide.

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