Free C-Terminal Resin-Bound Peptides: Reversal of Peptide Orientation via A Cyclization/Cleavage Protocol

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Recently, a number of new drug discovery methods have been reported involving the synthesis and screening of combinatorial biopolymers libraries.[1] These methods include the screening of oligomers individually or as mixtures in solution,[2,3] or on solid supports such as pins,[4] beads,[5] glass surfaces,[6] cellulose,[7] cotton,[8] or phage particles.[9] Chemical methods of synthesis have the advantage that they may incorporate D-amino acids, unnatural amino acids, peptidomimetics[10] and cyclic analogs[11] using routine peptide chemistries; in addition, they allow us to prepare oligomers with novel backbones.[12–13] Methods that generate solid-support-bound oligomers can allow the rapid identification of binding ligands from a diverse library.[5,6] However, in the case of peptides, only the N-terminus is available for binding, as the C-terminus is necessarily attached to the solid phase surface. We now report a novel strategy for reversal of peptide orientation, enabling C-terminal oligomer display via a cyclization/cleavage strategy.

To demonstrate this method, we required the synthesis of two different types of resins: "cleavable" and "noncleavable". These resins were prepared by standard methods[14] according to Schemes 1a and 2a, respectively. The cleavable resin allowed for chemical characterization of the successfully cyclized/cleaved peptide, while the noncleavable resin retained the reversed peptide for the purposes of enzymatic characterization and ultimately biological screening.

The cyclization/cleavage strategy was characterized chemically by methodology shown in Scheme 1b. A peptide was synthesized on the cleavable resin **5** by esterification with Fmoc-Trp using DIC, HOBt, and DMAP followed by elongation of the rest of the peptide on an automated synthesizer (ABI Model 431) to give resin **8**. The *o*-nitrobenzyl group was then removed by photolysis, freeing the succinic acid unit which is condensed with the amino-terminus of peptide upon cyclizing with 10 equiv of BOP and 5 equiv of DIEA in DMF.[15] *Only peptide that successfully cyclizes will be succinylated.* Therefore, the efficiency of cyclization can be determined from the ratio of succinylated to non-succinylated peptide upon cleavage. Cleavage of resin **10** with TFA (which also removes side chain protecting groups) followed by reverse phase HPLC analysis revealed the succinylated peptide **11** as the major peptide component at a retention time of 13.9 min.[18] Comparison with the unsuccinylated peptide **9** (retention time, 12.9 min), prepared by cleavage of resin **8** (before deprotection/cyclization), demonstrated that the cyclization occurred with greater than 90% efficiency. Because of the known sequence dependence of macrocyclizations, this efficiency is not expected in all cases. However, a screening approach can tolerate some variation.

Biological availability of the peptide for bead screening was demonstrated as shown in Scheme 2b. The noncleavable resin **7** was loaded with peptide, deprotected, cyclized, and C-terminally cleaved (and side chain deprotected).[19] Two separate sequence-containing resins **14a** and **14b** were prepared.[20] The **14a** sequence was chosen because it is an efficient substrate for carboxypeptidase Y (CPY).[20] Treatment of **14a** with CPY in aqueous buffer[22] led to sequential release of the amino acids Leu, Phe, and Gly, respectively, over a 5-h period. A 10-fold increase in enzyme concentration results in complete release of the first four amino acids (Leu, Phe, and two Gly) and even some cleavage of Tyr and Glu. This evidence confirmed the reversed direction of the peptide and demonstrated the accessibility of the resin-bound peptide to the enzyme active site. Application of this methodology to the endothelin C-terminal peptide[23] **14b** also confirmed the reverse direction of this peptide.

In summary, we have demonstrated by both chemical and enzymatic methods that resin beads can be prepared containing peptides which are attached in a reversed orientation anchored

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(14) Synthetic details of the resin preparation may be found in the supplementary materials section.

(15) Abbreviations: Fmoc, 9-fluorenylmethoxycarbonyl; *t*-Boc, *tert*-butoxycarbonyl; HMPA, (hydroxymethyl)-*p*-phenoxyacetic acid; HOPfp, pentafluorophenol; DMF, dimethylformamide; TFA, trifluoroacetic acid; DIC, diisopropylcarbodiimide, HOBt, 1-hydroxybenzotriazole; DMAP, (dimethylamino)pyridine; HPLC, high-pressure liquid chromatography.

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(17) Commercial source: HMPA-OPfp, Millipore.

(18) Peptide identity was confirmed by FAB-MS (for compound **11**, calcd MS (M + H) 978.5, found 978.6), amino acid analysis, and comparison with authentic peptide prepared by standard methods.

(19) It should be emphasized that in the case of the noncleavable resin, only cyclized peptide, ultimately C-terminal peptide, will remain attached to the resin after efficient cleavage with TFA. Uncyclized peptide is cleaved from the resin and removed during subsequent washings.

(20) The efficiency of cyclization for the noncleavable resin can be calculated from amino acid analysis of the resin before and after TFA cleavage. Resin **14a** is estimated to cyclize with an 80% efficiency, while **14b** gives a 55% yield.

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(22) Experimental details of the enzymatic analysis including graphical presentation of amino acid release are presented in the supplementary material.

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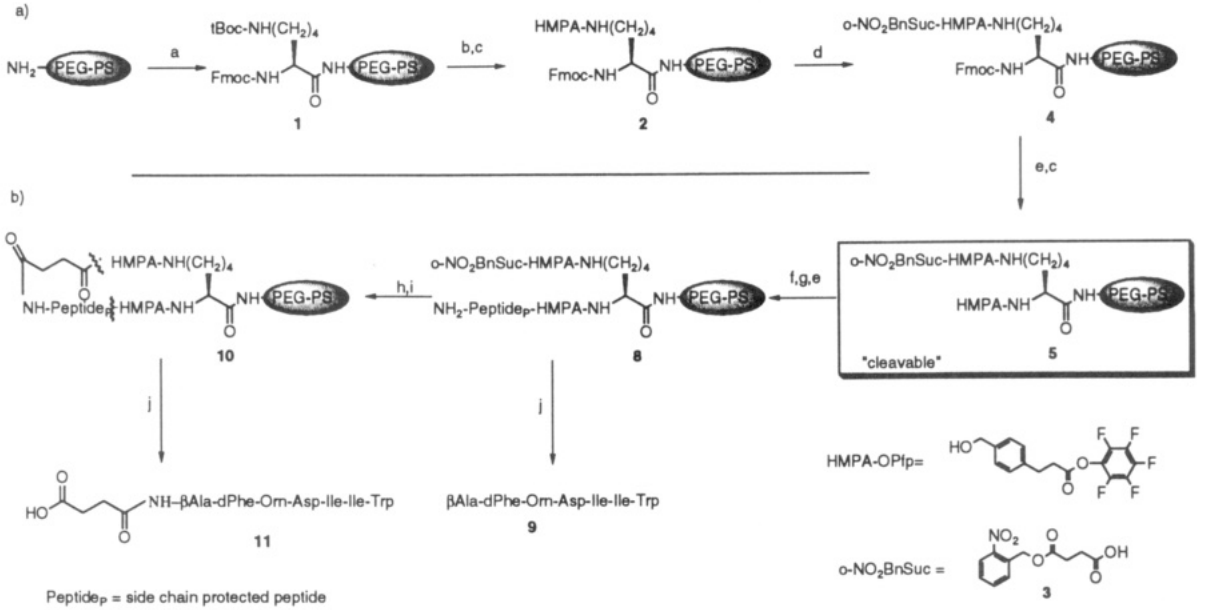
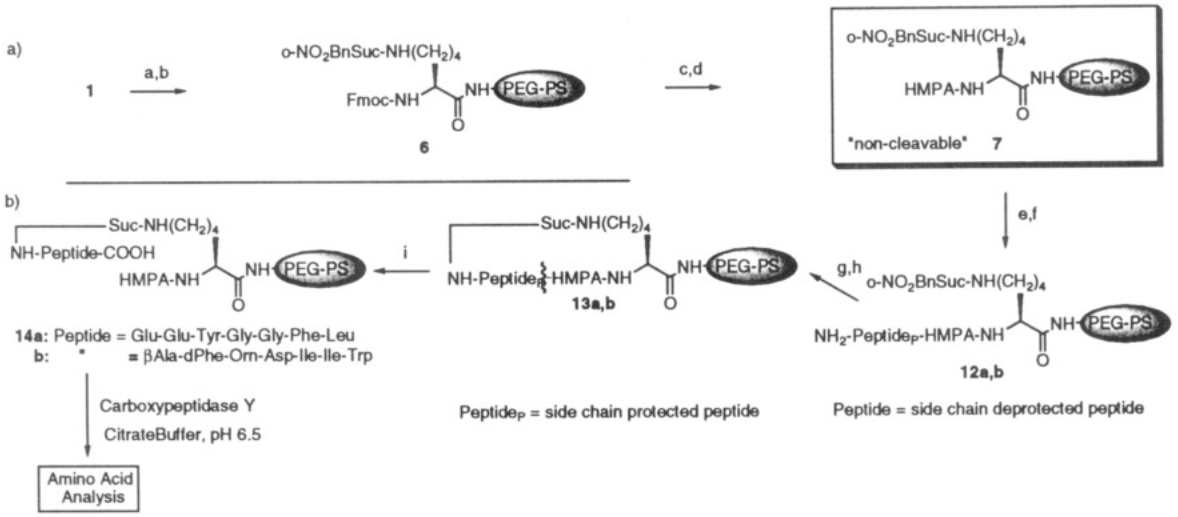
Scheme 1. Synthetic Preparation of the Cleavable Resin and Its Chemical Analysis[a]



[a] (a) Fmoc-Lys(tBoc)OH, DIC, HOBt, CH_2Cl_2; (b) 50% TFA/CH_2CL_2; (c) 1 equiv of HMPA-OPfp, 1 equiv of DIEA, DMF; (d) **3**, 1 equiv of DIC, 1 equiv of HOBt, 0.1 equiv of DMAP, DMF; (e) 20% piperidine/DMF 5 equiv of Fmoc-Trp, 5 equiv of HOBt, 5 equiv of DMAP, DMF; (g) ABI synthesis; (h) hv at 360 nm, 5% NH_4OH, 1.5 h; (i) 10 equiv of BOP, 5 equiv of DIEA, DMF, 2h; (j) 5% anisole, 5% ethanedithiol, 90% TFA, 1.5 h.

Scheme 2. Noncleavable Peptide Resin Synthesis, Cyclization/Cleavage, and Enzymatic Analysis[a]



[a] (a)50% TFA/CH_2Cl_2; (b) **3**, 1 equiv of DIC, 1 equiv of HOBt, DMF; (c) 20% piperidine/DMF; (d) 1 equiv HMPA-OPfp, 1 equiv of DIEA, DMF; (e) 5 equiv of Fmoc-Leu (or Trp), 5 eq DIC, 5 eq HOBt, 5 equiv of DMAP, DMF; (f) ABI peptide synthesis; (g) *hv* at 360 nm, 5% NH_4OH, 1.5 h; (h) 10 equiv of BOP, 5 eq DIEA, DMF, 2 h; (i) 5% anisole, 5% ethane dithiol, 90% TFA, 1.5 h.

via their N-termini. Although peptide reversal is dependent on the cyclization, which can be quite sequence sensitive, this method has the advantage that only cyclized peptide will remain attached to the bead, while uncyclized peptide is released during the cleavage conditions. This methodology, in combination with resin splitting techniques[24,25] will enable production of libraries of resin-bound peptides with free C-termini which should prove to be useful in screening biological targets of pharmaceutical relevance.

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Supplementary Material Available: Experimental procedures for resin preparation with analytical data and enzymatic analysis including graphical presentation of time course for release of amino acids from the resins is presented (8 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.