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Convenient synthesis of a head-to-tail cyclic peptide containing an expanded ring

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Abstract

Here we describe a rapid and efficient solid-phase synthesis of a 29-mer cyclic antibacterial peptide **1**, currently under pharmaceutical development. The linear peptide was assembled by standard Fmoc chemistry on an Fmoc-Asp(resin)-ODmab carrier. Intramolecular on-resin head-to-tail cyclization was enabled after selective deprotection of the Asp α-carboxy protecting group with 2% hydrazine–DMF at room temperature.

Keywords: cyclization; antibacterial peptide; solid-phase synthesis.

Head-to-tail cyclic peptides, important targets in peptide synthesis over decades, have attracted considerable interest in recent years.[1] The reason for this interest stems from the observation that constraining of the highly flexible linear peptides by cyclization induce or stabilize the bioactive conformation of peptides.[2,3] Furthermore, cyclic peptides are more resistant to proteolysis than their linear counterparts due to the lack of exopeptidase cleavage sites. To date, general and commercial synthesis of head-to-tail cyclic peptides has used only the allyl group (All) for temporary protection of the α-COOH group of resin-bound aspartic acid and glutamic acid.[4] This protection strategy requires the removal of the allyl group with Pd^0, a reaction that is inconvenient and sluggish at higher resin loads or for expanded ring sizes. To overcome this difficulty, recently the use of 4-{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]amino}benzyl ester (Dmab) as a new carboxy-protecting group in the synthesis of a model cyclic heptapeptide has been reported.[5] However, the benefits of the Dmab-protection strategy compared to the allyl group in small peptides are not apparent. A similar attempt with the trimethylsilylethyl ester as a carboxy protecting group was used in the synthesis of a cyclic heptapeptide.[6] A more recent approach to cyclic peptides that is compatible with Fmoc-chemistry is based on Kenner's 'safety-catch' linker, but, again, this method has only been successfully applied to the synthesis of a cyclic hexapeptide.[7] Principles of the chemical ligation method,[8] one of the most important recent developments in the

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total synthesis of proteins, have been used in thia zip cyclization of large (31 and 33 amino acid-residue) peptides.[9] However, in contrast to the conventional methods of peptide cyclization which require amide-bond formation via activation of a single unprotected carboxyl group, the thia zip cyclization method involves a series of intramolecular rearrangements of completely unprotected precursors. Restrictions of this method are the requirements for the decreasingly used Boc-chemistry and the availability of key functional groups which include an N^{α}-cysteine, a thioester, and at least one internal free thiol group in the peptide to be synthesized.

Following a search for a simple and convenient route to cyclic peptides on a scale required for drug development, and in a ring size compatible with high antibacterial activity, here we describe a fast and efficient solid-phase synthesis of a 29-mer head-to-tail cyclic peptide. A cyclic analog (cyclo[KGSYLPRPTPPRPIYNRNRPPTPRPLKVD]) **1** of the insect antibacterial peptide pyrrhocoricin[10] (VDKGSYLPRPTPPRPIYNRN) demonstrated a considerably broader activity spectrum than the parent native linear peptide.[11] The cyclic peptide kills model gram-negative and gram-positive strains in submicromolar and low micromolar concentrations. When we first made this cyclopeptide on small scale, we used the commercially available Fmoc-Asp(PEG-PS)-OAll resin (0.2 g; 0.16 mmol/g, PerSeptive Biosystem). In spite of multiple rounds of allyl removal with Pd^0, after resin cleavage the main product still contained the allyl protecting group. Nevertheless, the target cyclopeptide could be isolated after extensive purification on reversed-phase HPLC. After learning of the promising pharmacological profile of this analog, we decided to repeat the synthesis on a larger scale to obtain sufficient material for stability, toxicity, and in vivo efficacy studies. However, when 1 g of resin was used, this attempt failed completely due to the unsuccessful deprotection of allyl ester group. The only product that was obtained represented the allyl ester of the linear precursor as observed by RP-HPLC (Fig. 1) and documented by mass spectrometry. Similar results have been described by Valero and co-workers,[12] who observed substantial levels of by-products, most likely formed during Fmoc and OAll deprotections steps, when cyclization of head-to-tail peptides were attempted on resin amounts larger than 100 mg.

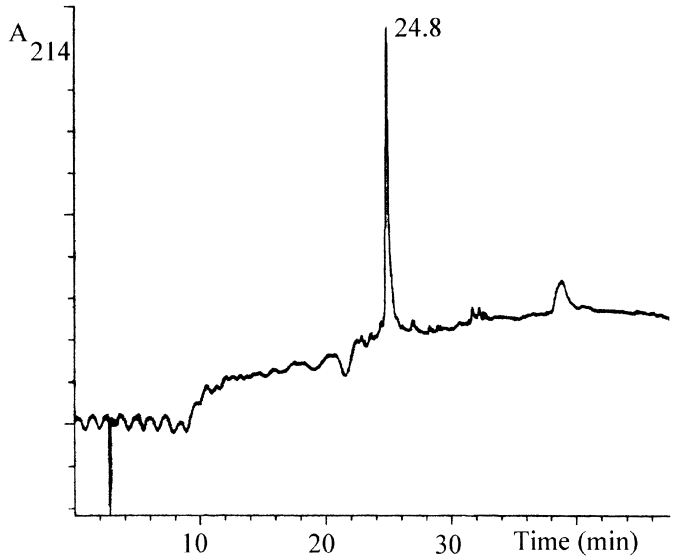
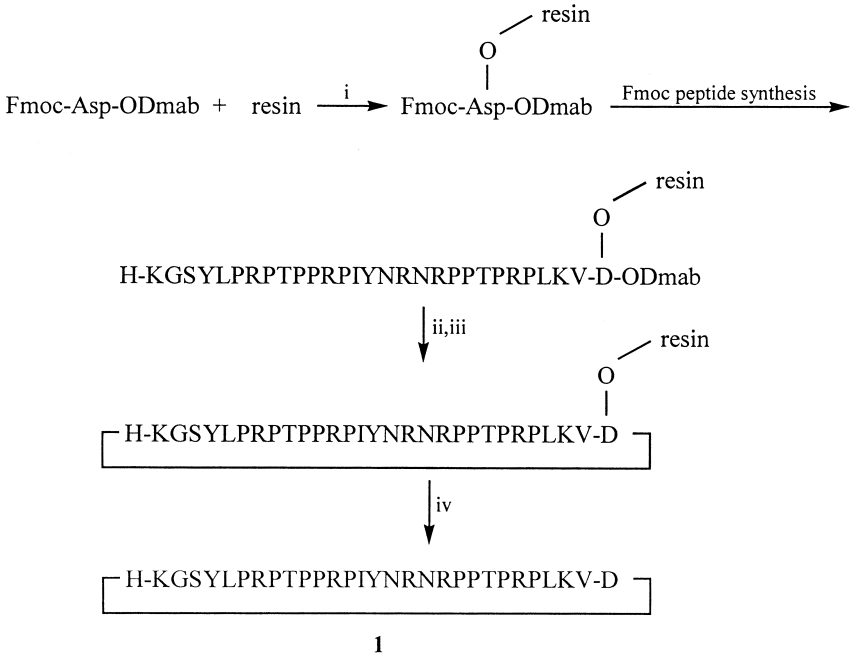


Figure 1. RP-HPLC of the crude product obtained in the large scale synthesis of cyclic peptide (**1**) using Fmoc-Asp(PEG-PS)-OAll resin. The single peak was characterized as the allyl ester of the linear precursor

Dmab protection of the carboxyl groups of aspartic and glutamic acids is quasi-orthogonal to the Fmoc/*t*Bu strategy and offers a simple, generally applicable and convenient route to large scale synthesis of head-to-tail cyclic peptides. Scheme 1 shows the synthetic approach to our 29-mer cyclic peptide **1**. Macroporous Kieselguhr-supported polydimethylacrylamide resin (Pepsyn K, Novabiochem) was used in our synthesis, principally due to its low loading and its amenability to peptide cyclization due to resin-induced pseudo-dilution effects. The Fmoc-Asp(resin)-ODmab was prepared according to the following protocol: 1.77 g (0.20 mmol) Pepsyn K resin was suspended in ethylene diamine overnight and then washed well with DMF. The resulting support was transferred to a continuous flow reaction column and the free amino groups were acylated by 1 h treatment with 4 molar equivalents of the symmetrical anhydride of a hydroxymethylphenoxy acetic acid linker. The resin was washed well with DMF and then acylated for 1 h 30 min with 3 molar equivalents of the symmetrical anhydride of Fmoc-Asp-ODmab (Novabiochem) in the presence of 0.3 molar equivalents of 4-dimethylamino-pyridine (DMAP) catalyst. 1 g of the resin with an initial load of 0.11 mmol/g was then used in a synthesis of the linear precursor peptide on a Milligen 9050 continuous-flow automated synthesizer. Standard Fmoc-chemistry was used throughout.[13] Couplings were made with 4-molar excess of the acylating amino acids activated by HATU (1-hydroxy-7-azabenzotriazole uronium salt), as recommended for the synthesis of complex peptides.[14] After completion of the peptide assembly, the resin was treated with 2% hydrazine–DMF for 3 min. The treatment was repeated twice, and the resin was thoroughly washed with DMF. For the intramolecular cyclization we used 2 molar equivalents of benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) in the presence of *N*,*N*-diisopropyl-ethylamine (DIEA), and 1-hydroxybenzotriazole (HOBt) for 48 h. Since the activation strategy



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Scheme 1. Synthesis of the 29-mer cyclic peptide. Reagents and conditions: (i) derivatization of the resin with Fmoc-Asp-ODmab as symmetrical anhydride, DMAP, DMF; (ii) 2% hydrazine–DMF; (iii) DIEA, PyBOP/HOBt; (iv) trifluoroacetic acid, water, thioanisol (90:5:5; v/v/v)

was unchanged compared to reported cyclization reactions,[1] we consider the potential level of racemization to be similar to that general observed earlier. The final deprotection-cleavage step from solid support employed a mixture of trifluoroacetic acid, water and thioanisol (90:5:5; v/v/v) and afforded a crude cyclic peptide **1** in good yield (75 mg, 20%) (Fig. 2, panel A). The peptide was purified by reverse-phase high performance liquid chromatography (RP-HPLC).† Fig. 2, panel B shows the analytical chromatogram of the purified peptide (yield: 4 mg), and panel C demonstrates the corresponding mass spectrum ([M+H]$^+$, calcd 3364; found 3364).‡ The repetition of the synthesis using 0.8 g of the resin yielded 4.4 mg of the highly purified cyclic peptide **1**.

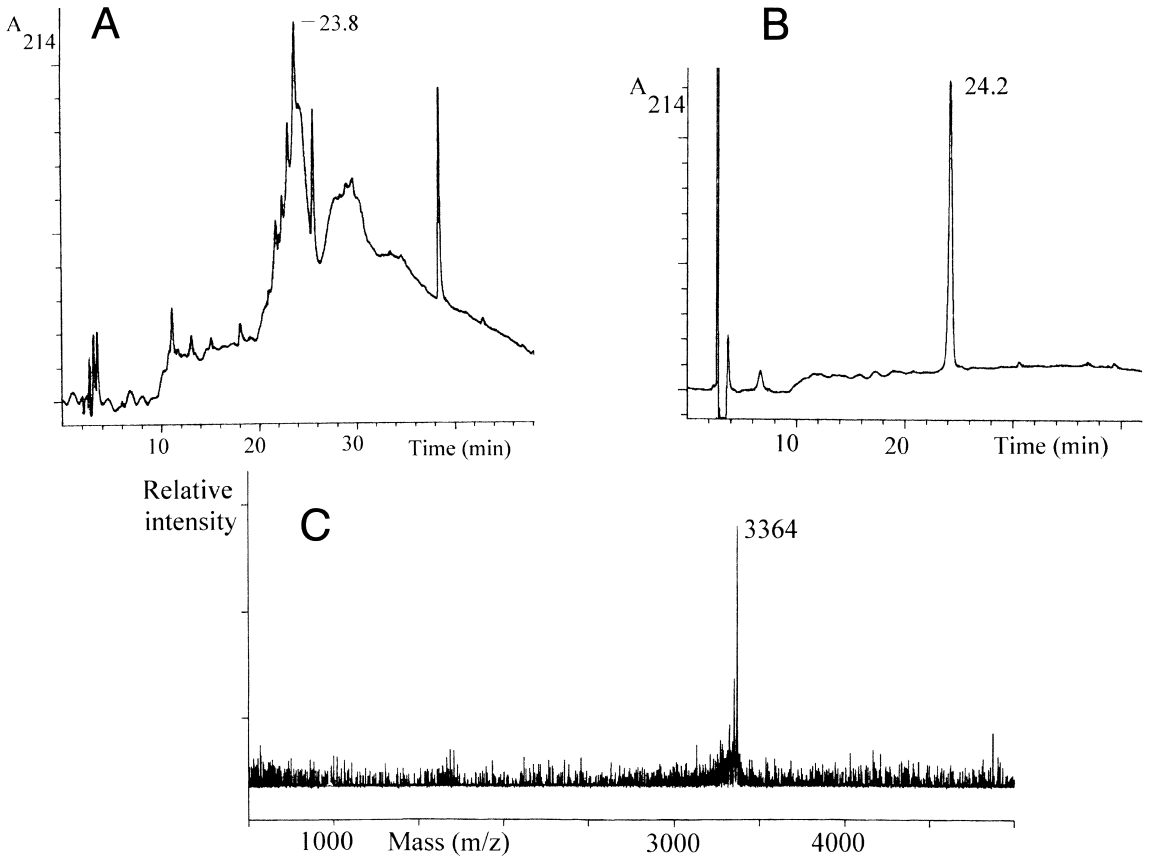

Figure 2. Reversed-phase chromatogram of the (A) crude product obtained in the large scale synthesis of cyclic peptide (**1**) using Fmoc-Asp(Pepsyn K)-ODmab resin; (B) after RP-HPLC purification; and (C) the corresponding MALDI mass spectrum

† A Phenomenex (Jupiter) 5 μm C_{18} 300 A (4×250 mm) column was used at a flow rate 1 mL min^{-1} for analytical runs, and a Phenomenex (Jupiter) 10 μm C_{18} 300 A (21.2×250 mm) column was used at a flow rate 10 mL min^{-1} for preparative runs. The analytical runs consisted of an isocratic elution of 5% solvent B (0.1% trifluoroacetic acid in acetonitrile) followed by a linear gradient from 5% to 65% solvent B in solvent A (0.1% trifluoroacetic acid in water) for 45 min, and 90 min for preparative runs, respectively.

‡ The final products were characterized by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) at the Wistar Institute Protein Microchemistry Laboratory on a Voyager Biospectrometry Workstation by standard methods.

In summary, we have demonstrated that the Dmab group can be effectively used as a temporary α-carboxyl protecting group for automated solid-phase synthesis of expanded cyclic peptides in good yields. Commercially available resin pre-loaded with Fmoc-Asp-ODmab would further reduce the synthesis time. Taken together the synthetic route outlined here represents reliable and preferred method of choice for the synthesis of head-to-tail cyclic peptides.

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