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Synthesis of Large Cyclic Cystine-Knot Peptide by Orthogonal Coupling Strategy Using Unprotected Peptide Precursor

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Abstract A simple and direct method for the synthesis of large end-to-end, disulfide rich cyclic peptide using orthogonal coupling of unprotected N^{α}-cysteinyl thioester peptide is described.

Recently, several large end-to-end cyclic peptides with interesting biological activities have been isolated. Cyclopsychotride (CPT),[1] a 31 residue end-to-end cyclic peptide containing three disulfide bonds, was isolated from the tropical plant, *Psychotria Longibes*. CPT inhibits neurotensin (NT) binding to HT-29 cell surface receptors and also stimulates increased levels of cytosolic Ca^{2+} ion in two unrelated cell lines that do not express NT receptors. The disulfide bond connectivity of CPT is not known but based on sequence homology and cysteinyl placements, this cyclic peptide belongs to the Kalata and Circulin family of cyclic peptides with cystine-knot architecture.[2] The solution structure of Kalata B1 has been determined and its disulfide connectivity is 1-4, 2-5, and 3-6 (Fig. 1). The cystine-knot is a unique, closely packed sulfur-sulfur core arrangement with one of the disulfide bonds threading though the embedded loop formed by the two other disulfide bonds. An other conserved residue within the cystine-knot motif is a small amino acid such as Ser, Thr, Ala, or Gly just prior to the Cys-X-Cys sequence. Similar cystine-knot architecture has also been found in open-chain peptides, including in toxins, inhibitory polypeptides, and growth factors.[3]

The synthesis of CPT containing a cystine-knot motif and an end-to-end cyclic peptide would be complicated and challenging by the conventional approach using protected peptide schemes. It is simple and direct by the intramolecular orthogonal coupling strategy using an unprotected precursor peptide and exploiting an α-cysteinyl

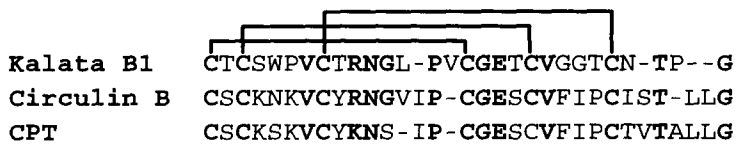

Fig.1 Amino acid sequences of Kalata B1, Circulin B and CPT.
Bold amino acids indicate conserved residues.

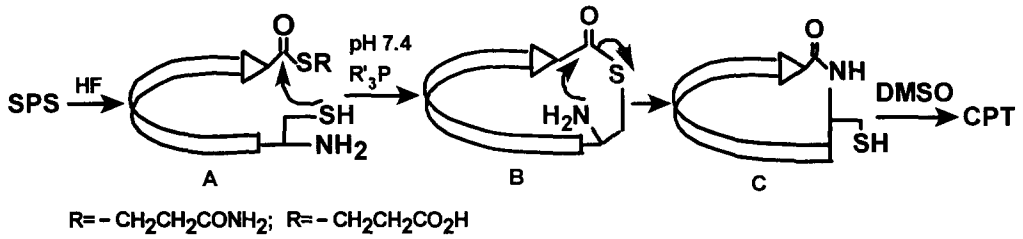

Fig.2. Cyclization of an unprotected peptide precusor (A) derived from solid-phase synthesis (SPS) through intramolecular transthioesterification to form a cyclic thioester (B), and then an S, N acyl migration (C) to form an end-to-end peptide with free thiols which are oxidized by DMSO to CPT.

at the amine and α-thioester at COOH termini.[4-6] In this scheme (Fig. 2), cyclization is mediated by intramolecular transthioesterification via the α-thiol with the α-thioester to form a thiolactone. A subsequent ring contraction through an S, N acyl migration forms the lactam. Recently we have carried out this scheme for orthogonal coupling of unprotected peptides[7] and cyclic peptides.[8] In this paper, we describe the synthesis of CPT by the orthogonal coupling strategy and confirm the disulfide connectivity. The linear precursor peptide containing an α-cysteine and α-thioester was prepared by solid-phase synthesis using Boc-benzyl chemistry [9] on a Boc-Gly-SCH_2CH_2CO-methylbenzhydrylamine (MBHA) resin.[10] Because there are six cysteines in this peptide and cyclization can be mediated through any one of these, the least hindered site for cyclization between Gly-31 and Cys-1 was chosen as the respective C^{α}-and N^{α}-terminal residues. To confirm the disulfide connectivity, we used a two-step disulfide bond forming strategy[11] which employed two sets of thiol protecting groups for cysteine. Base on the cysteinyl alignment with Kalata, acetamidomethyl (Acm) for Cys 3 and Cys 6 and methylbenzyl (Mbzl) for the other four were used. In the first stage, the four thiol protecting groups of MBzl were removed during high-HF cleavage, and the number of possible disulfide isomers after oxidation was reduced to 3 as compared to 15 when all six cysteines were oxidized simultaneously. At this stage the disulfide isomers could be isolated and their disulfide bond connectivities determined by a combination of partial acid hydrolysis and MS.[12] The third disulfide bond was then formed by I_2 oxidation.

The unprotected peptide thioester ($[M+H]^+$, m/z 3486.7 found, 3485.07 calcd.) was cleaved from the resin support by HF and allowed to refold and cyclize in descending concentrations of an 8M urea solution containing 0.1M Tris HCl, pH 7.4 and a 10 fold excess of water-soluble Tris (2-carboxyethyl) phosphine to prevent polymeric disulfide formation. The initial 8M concentration of urea allowed the dissociation of small organic molecules and aromatic scavengers such as anisole and p-cresol from the peptide. Subsequent lower urea concentrations (< 2M) allowed refolding and cyclization. The progress of the end-to-end cyclization was monitored by HPLC and MS and the whole process was complete within 24 h. A single cyclic peptide with four reduced thiols in > 80 % yield was found ($[M+H]^+$, m/z 3380 found, 3379.07 calcd.). Disulfide formation was achieved by DMSO [13] and was complete in 10 h ($[M+H]^+$, m/z 3376 found, 3376.04 calcd.) to give three disulfide isomers. Each isomer was isolated by HPLC and their disulfide connectivities were determined by partial acid hydrolysis in 250 mM oxalic acid at 100° C for 5 h. Disulfide-containing peptides in the chromatographic fractions of the hydrolysate were

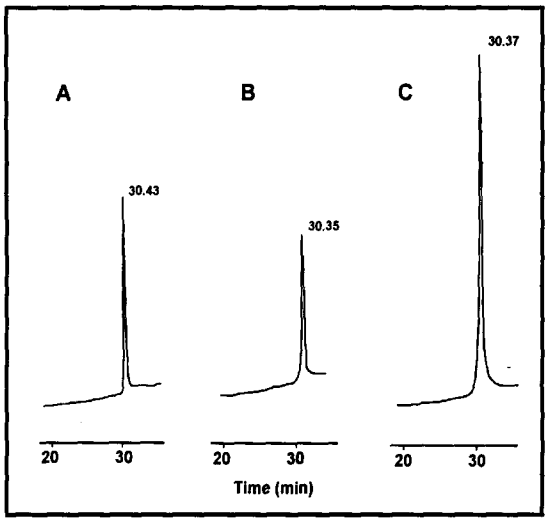

Fig. 3. Comparision of native and synthetic CPT. (A) Native CPT. (B) Synthetic CPT. (C) Coelution of native and synthetic CPT. HPLC profile on Vydac Column (C_{18} 4.6 x 250 mm). Eluent A: 0.045% TFA/5% CH_3CN/H_2O; B: 0.04% TFA/60% CH_3CN/H_2O. Gradient from 0-90% B at 1.0ml/min. Detection: 225 nm.

analyzed by matrix-assisted desorption/ ionization mass spectrometry (MALDI-MS) to determine disulfide connectivities. The expected disulfide isomer of Cys-1,4; 2,5 was obtained in 30% yield. The third disulfide was formed by I_2/MeOH under nitrogen to remove the Acm protecting groups of Cys-3,6 to give CPT ($[M+H]^+$, m/z 3232.2 found, 3230.89 calcd.). The synthetic material was identical to naturally isolated CPT both chemically and biochemically (data not included). Furthermore, synthetic and naturally isolated CPT eluted as a single peak under several RP-HPLC conditions (Fig.3). These results support the contention that CPT has a disulfide connectivity similar to Kalata B1.

Two separate syntheses of CPT with Cys 2,5 protected by Acm and without the Acm protecting group on any thiols were also performed to improve the overall yield. End-to-end cyclization was essentially achieved under one-pot reaction from the crude peptide after peptide cleavage from resin support. Similar to the previous synthesis, multiple disulfide isomers were obtained to give a final unoptimized yield of about 10%. The low yield in these syntheses could be attributed to two reasons. First, during the synthesis, the thioester bond attached to the MBHA-resin was not completely stable. There was also partial aminolysis and hydrolysis of the thioester bond that accumulated during the repetitive chain assembly and resulted in ~20% loss. Second, the close packing of the cystine-knot motif in an end-to-end cyclic peptide produced different disulfide isomers that severely undermined the yield of the overall process. The yield could be improved by recyclizing the incorrecting formed disulfide isomers. Our results show that the cyclization of CPT under denaturing condition through intramolecular

transthioesterification is convenient and efficient considering that the number of ring atoms of CPT exceeds 90.

Thioesters have been used for the synthesis of lactones in microlides, [14,15] but only recently extended for lactam formation.[8b] In our study, it is likely that the cyclization was assisted by the favorable placement of these free internal cysteines which are capable of forming smaller thiolactone intermediates and thiol-thiolactone exchanges through ring expansion via a novel thia zip reaction. A final thiolactone is thus produced prior to the S, N acyl migration to yield the stable end-to-end cyclic peptides.

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