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A biomimetic strategy in the synthesis and fragmentation of cyclic protein

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Abstract

This paper describes a simple biomimetic strategy to prepare small cyclic proteins containing multiple disulfide bonds. Our strategy involves intramolecular acyl transfer reactions to assist both the synthesis and fragmentation of these highly constrained cyclic structures in aqueous solution. To illustrate our strategy, we synthesized the naturally occurring circulin B and cyclopsychotride (CPT), both consisting of 31 amino acid residues tightly packed in a cystine-knot motif with three disulfide bonds and an end-to-end cyclic form. The synthesis of these small cyclic proteins can be achieved by orthogonal ligation of free peptide thioester via the thia zip reaction, which involves a series of reversible thiol-thiolactone exchanges to arrive at an α-amino thiolactone, which then undergoes an irreversible, spontaneous ring contraction through an S,N-acyl migration to form the cyclic protein. A two-step disulfide formation strategy is employed for obtaining the desired disulfide-paired products. Partial acid hydrolysis through intramolecular acyl transfer of X-Ser, X-Thr, Asp-X, and Glu-X sequences is used to obtain the assignment of the circulins disulfide bond connectives. Both synthetic circulin B and CPT are identical to the natural products and, thus, the total synthesis confirms the disulfide connectivity of circulin B and CPT contain a cystine-knot motif of 1–4, 2–5, and 3–6. In general, our strategy, based on the convergence of chemical proteolysis and aminolysis of peptide bonds through acyl transfer, is biomimetic and provides a useful approach for the synthesis and characterization of large end-to-end cyclic peptides and small proteins.

Keywords: circulin; cyclic protein; Cyclopsychotride; cystine-knot motif; orthogonal ligation; partial acid hydrolysis; thia zip reaction; two-step disulfide formation

Although cyclic proteins are not known to occur naturally in animals, large end-to-end cyclic peptides have been found in plants. Circulin A, B (Gustafson et al., 1994), Cyclopsychotride (CPT) (Witherup et al., 1994), and Kalata (Gran, 1970, 1973) belong to a family of novel plant-derived macrocyclic peptides. These peptides, which consist of 28–31 amino acids with six cysteines, share a high degree of sequence homology, differing by only three or four amino acids (Fig. 1). Thus far, they represent the largest end-to-end cyclic peptides known to occur naturally, and can serve as prototypes of small cyclic proteins. Based on random screening, these small protein possess a diverse range of biological activities. Circulin A and B from *Chassalia parvifolia* (Rubiaceae) are reported to inhibit the replication and cytopathic effects of the human immunodeficiency virus (HIV). However, the mechanism of anti-HIV reactivity for circulins is not known at this time. CPT obtained from *Psychotria longipes* inhibits neurotensin (NT) binding to HT-29 cell surface receptors and also stimulates increased levels of cytosolic Ca^{2+} ions in two unrelated cell lines that do not express NT receptors. Kalata obtained from plant *Oldenlandia affinis* leaves is found to be uteroactive.

In practice, these cyclic proteins are likely to be antimicrobial cysteine-rich class of peptides such as the widely distributed defensin, because of their size and structure similarities. Defensins contain 29–38 amino aicds and three disulfide bonds, one of which links the N- and C-termini. Furthermore, defensins are active against enveloped virus including HIV. Thus, synthesis of circulins and CPT is of interest not only to validate their biological activities, but also to meet the challenge of preparing the novel structure of an end-to-end cyclic protein with three disulfide bonds.

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Abbreviations: Standard abbreviations are used for the amino acids and protecting groups [IUPAC-IUB Commission for Biochemical Nomenclature (1985) *J Biol Chem 260*:14]. Other abbreviations are as follows: TFA, trifluoroacetic acid; HF, hydrofluoric acid; DMSO, dimethylsulfoxide; DMF, dimethylformamide; DCM, dichloromethane; DCC, *N,N'*-dicyclohexylcarbodiimide; DIC, *N,N*-diisopropylcarbodiimide; HOBt, *N*-hydroxybenzotriazole; GSH, glutathione, reduced; GSSG, glutathione, oxidized; DIEA, *N,N*-diisopropylethylamine; TCEP, tris(carboxyethyl)phosphine; BOP, benzotrizol-1-yl-oxy-tris(dimethylamino)phosphoniumhexafluorophosphate; MBHA, methylbenzhydrylamine; RP-HPLC, reverse-phase high-performance liquid chromatography; MALDI/MS, matrix-assisted laser desorption ionization mass spectrometry.

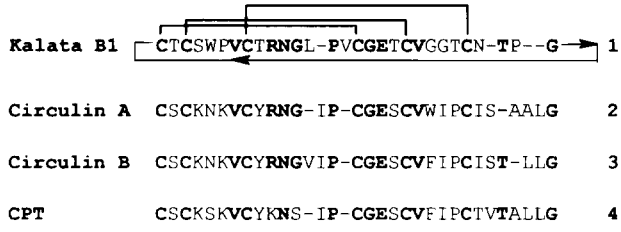

Fig. 1. Amino acid sequences of Kalata B1, circulin A, B, and CPT. Bold amino acids indicate conserved residues. All peptides have an end-to-end cyclic structure, as shown in Kalata B1.

Synthesis of circulins by the conventional approach using protected peptide segments would normally require an elaborate scheme of multitiered protecting groups because of the presence of their four covalently linked bonds. However, our laboratory has recently developed a more efficient and simpler approach to their synthesis through intramolecular orthogonal ligation in aqueous solutions using unprotected peptide precursors without protection and activation steps (Tam & Lu, 1997; Zhang & Tam, 1997). Orthogonal ligation is derived on thioester chemistry to form an amide bond (Wieland et al., 1953; Dawson et al., 1994; Tam et al., 1995). The mechanism for such an amide bond formation is mediated by entropic-favored intramolecular acyl transfer reactions (Wieland et al., 1953; Kemp & Galakatos, 1986). When there are multiple Cys present in the linear precursor peptide sequence, the cyclization proceeds through the thia zip mechanism involving a series of thiol-thiolactone exchanges via side-chain thiols. Eventually, an α-amino thiolactone intermediate is formed that links the thiol at the N^{α}-amine with the C^{α}-carboxyl moiety as a large thiolactone (Fig. 2). A spontaneous ring contraction of the amino thiolactone through an S,N acyl-migration forms the end-to-end cyclic peptide. The reaction is highly regioselective and would be suitable for the synthesis of small cyclic protein such as circulins.

The solution structure of Kalata B1 (Saether et al., 1995) has been determined, and its disulfide connectivity is a cystine-knot motif of 1–4, 2–5, and 3–6 (Fig. 1). The chemical structure of CPT has been established by chemical synthesis and formed to contain similar cystine-knot motif (Tam & Lu, 1997). Based on sequence homology as well as their cysteinyl placements with Kalata and CPT, circulins are believed to contain similar cystine-knot motif. A 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 (Pallaghy et al., 1994). Small amino acids such as Ser, Thr, Ala, or Gly just prior to the Cys-X-Cys sequence are highly conserved to accommodate the cystine-knot motif. Similar cystine-knot architectures have also been found in open-chain peptides, including trypsin inhibitors from the squash family (Heitz et al., 1989; Holak et al., 1989; Nielsen et al., 1994), carboxypeptidase inhibitor (Rees & Lips-

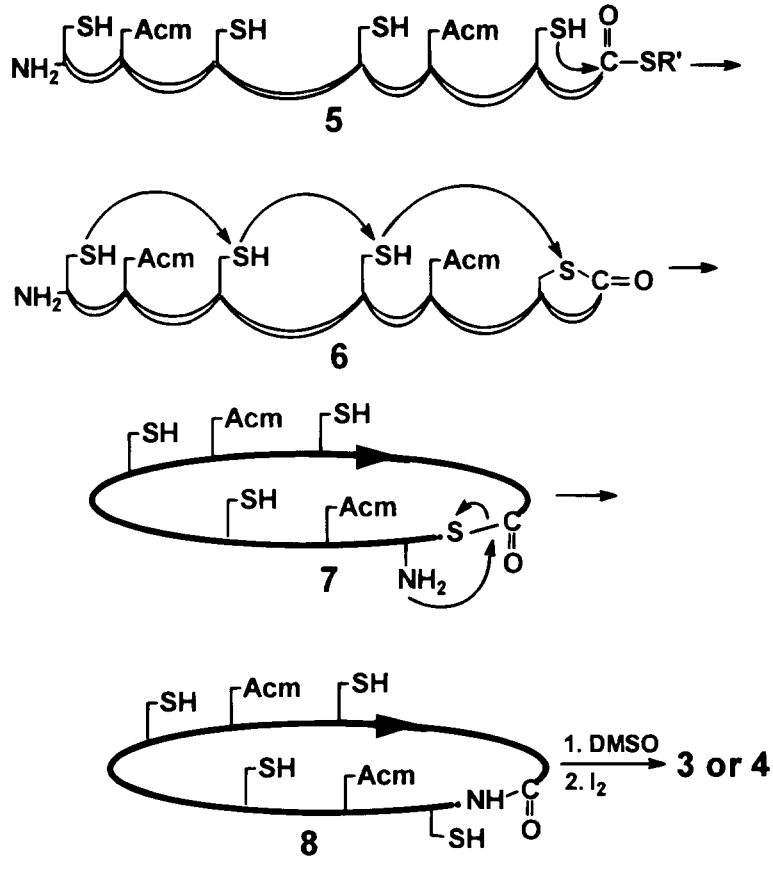

Fig. 2. Thia zip cyclization of free peptide thioester precursor **5** derived from solid-phase synthesis to form the α-amino thiolactone **7** and then an S,N-acyl migration to form an end-to-end cyclic peptide **8**.

comb, 1980) and growth factors (Daopin et al., 1992; Schlunegger & Grutter, 1992).

The cystine-knot motif coupled with an end-to-end cyclic structure produces a challenge in the identification of the connectivity of these disulfide bonds in the synthetic products. Disulfide bonds in proteins have traditionally been located by cleavage of a protein with a highly specific reagent or an enzyme to yield segments containing half-cystinyl residues; these traditional methods often fail in small protein (Lee & Shively, 1990), because as many as four independent cleavage sites are required and the cleavage reactions do not give peptide segments to provide useful information about the locations of the disulfide bonds. Such a situation is encountered in the circulins. Gray (1993a, 1993b) recently reported a method based on kinetic reduction of disulfides coupled with microsequencing for identifying cystine pair in proteins. This method will not be applicable for cyclic proteins that lack free amino ends. An attractive solution to overcome our problem is through partial acid hydrolysis (Zhou & Smith, 1990). Because all amide bonds are susceptible to acid hydrolysis, although at different rates, partial acid hydrolysis may provide a general solution to the identification of these highly constrained end-to-end cyclic proteins with multiple disulfide bonds.

Acid hydrolysis of proteins is often assumed to be random. However, there is a wealth of information regarding the acid susceptibility of specific peptide bonds (Light, 1967; Schultz, 1967; Zhou & Smith, 1990), particularly those that can undergo acyl-transfer reactions through either an ester or anhydride intermediate. Perhaps the best known is the Asp–Pro bond, which is cleaved under mildly acidic conditions when other peptide bonds are largely unaffected. Other bonds such as Gly–Ser, Asn–Gly, and His–Asn are also known to be susceptible to N,O-acyl rearrangement. Thus, we can exploit the limitation of the end-to-end cyclic protein and its rigidity to our advantage to provide a model that tests the chemical basis of the acid-catalyzed fragmentation reactions and establishes primary cleavage sites useful for partial acid hydrolysis as a method for obtaining specific peptide segments.

In this paper, we describe the synthesis of circulin B and CPT by orthogonal ligation and the confirmation of their disulfide connectivity by partial acid hydrolysis. Interestingly, our findings show that the synthetic strategy for macrocyclization in forming an end-to-end peptide bond in aqueous basic solutions and the fragmentation of peptide bonds in dilute aqueous acidic conditions are governed, to a large extent, by similar mechanisms. Both methods primarily use intramolecular acyl transfers in making or breaking peptide bonds, analogous to the protein splicing mechanism occurring in biological systems (Hirata et al., 1990; Kane et al., 1990; Xu et al., 1993; Cooper & Stevens, 1995). Thus, the convergence of mechanisms in the chemical proteolysis and aminolysis of peptide bonds to produce activated intermediates through intramolecular acyl transfer reactions involving side-chain nucleophiles such as Cys, Ser, and Asp may provide useful methods for the biomimetic synthesis (Liu & Tam, 1994; Tam et al., 1995) and fragmentation of large end-to-end cyclic peptides and small proteins.

Results and discussion

Synthetic strategy

Our synthetic strategy involves three parts (Fig. 3). The first part involves the stepwise solid-phase synthesis of the unprotected peptide precursor as a COOH-thioester. The second part involves the thia zip cyclization for the direct formation an end-to-end cyclic peptide in its unprotected form without the use of an extraneous activation agent. This material can then be used directly without purification for the part three of disulfide formation (Tam & Lu, 1997). In this work, however, the cyclized but two-disulfide paired peptides were purified so that the disulfide connectivity of these isomers could be determined. The third part involves the disulfide formation to form the cystine-knot motif. We also describes a two-step disulfide formation of the six cysteines to reduce the number of misformed disulfide isomers.

Synthesis of the linear precursor

The orthogonal cyclization scheme requires that the N-terminal thiol captures the COOH-terminal thioester to give the N- to C-terminal thiolactone intermediate (Zhang & Tam, 1997). Thus, the precursor peptide should contain an N-terminal cysteine and a C-terminal thioester. Because there are six cysteines in circulin and cyclization can be mediated through any one of these, the least-hindered site for cyclization between Cys-1 and Gly-31 was chosen as the respective N- and C-terminal residues. The peptide was prepared by solid-phase synthesis using Boc-benzyl chemistry (Merrifield, 1963, 1986) on a Boc-Gly-SCH_2CH_2CO-MBHA resin. The thioester resin was prepared according to Hojo and Aimoto (1991) by converting the C-terminal Boc-Gly-OH to its three-thiopropionic acid ester and then attaching it onto the MBHA resin by DIC/HOBt. After assembling the sequence on the resin, the peptide was cleaved from the resin support by high-HF (anisole/HF, 1:9, v/v). The thioester resin used in our synthesis was not very stable to the peptide assemblage steps. As a result, partial aminolysis and hydrolysis of the thioester bond occurred, which resulted in ~20% loss for the synthesis of the 31-residue circulin thioester precursors.

Thia zip cyclization

The thia zip reaction involves a series of thiol-assisted intramolecular rearrangements in a cysteine-rich peptide to achieve end-to-end cyclization (Fig. 2). Key elements in this reaction require a peptide with an N^{α}-cysteine, a COOH-thioester, and at least one internal thiol in its sequence. The zip reaction is initiated by an intramolecular transthioesterification through an internal thiol with the α-thioester to form a thiolactone under entropic-driven ring-chain tautomeric equilibrium in aqueous buffered solutions at pH > 7 (Fig. 2). Successive ring expansions through reversible thiol-thiolactone exchanges in the direction of the amino terminus finally lead to a large α-amino thiolactone that undergoes an irreversible and spontaneous ring contraction through an S- to N-acyl isomerization to form the end-to-end lactam. The irreversible S- to N-acyl isomerization is likely the driving force for the efficiency of the thia zip reaction. The α-amino thiolactone of the circulins contains 100 ring atoms. Without the assistance of internal thiols in reducing the ring sizes of the intermediate thiolactones, one-step formation of such large thiolactones is expected to be slow. Indeed, detailed kinetic study shows that zip assisted cyclization under the strongly denaturing condition of 8 M urea is complete in 6 h and is 100-fold faster than the corresponding unassisted one-step N to C cyclization of an analog with all internal thiols blocked.

The thia zip cyclization was applied in the synthesis of circulin B **3** and CPT **4**. The unprotected peptides with or without purification were subjected to thia zip cyclization. The condition used for cyclization of the unprotected peptide via trans-thioesterification

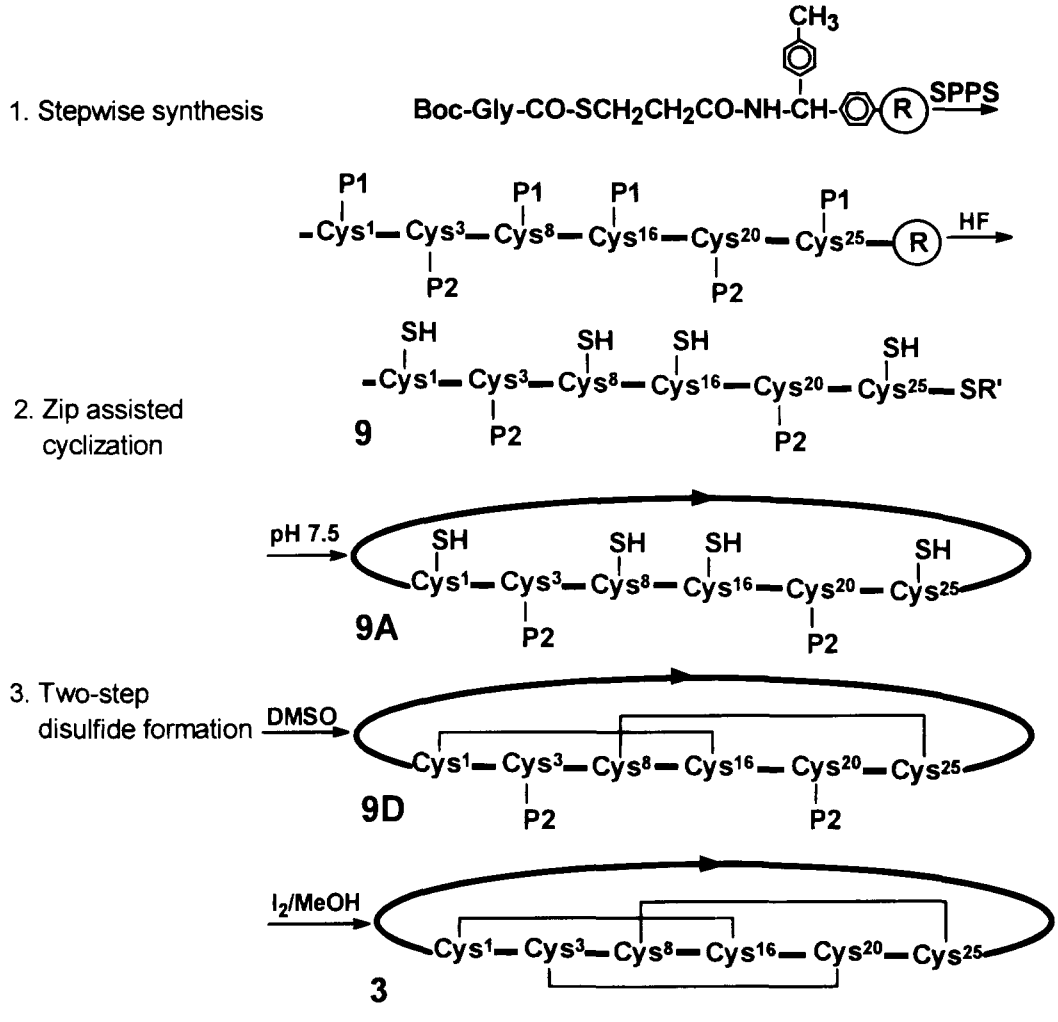

Fig. 3. A scheme for the synthesis of circulin B. Protected peptide is obtained by stepwise synthesis (step 1) and the cyclic peptide from the zip-assisted cyclization (step 2). The disulfide pairs are obtained from the two-step approach (step 3). Four protecting groups (P1 = MeBzl) were cleaved by HF and two disulfide bonds are formed by DMSO oxidation to give three disulfide isomers (see Fig. 4A,B). The desired isomer **9D** is treated with I_2 to remove the second set protecting groups (P2 = Acm) to form the third disulfide pair. R′ = $SCH_2CH_2CONH_2$; R = resin. Cysteine positions are based on circulin B (see Fig. 1).

was generally similar to that previously described by our laboratory for the thioester ligation of two unprotected peptide segments (Tam et al., 1995; Zhang & Tam, 1997). In the one-pot reaction, the crude peptides cleaved from the resin support by high HF were extracted by 8 M urea solution at pH 7.5 in a highly reductive environment containing a 5–10-fold excess of TCEP (Burns et al., 1991) to prevent disulfide formation. The urea solution was dialyzed to allow the dissociation from the peptide of small organic molecules and aromatic scavengers such as anisole and *p*-cresol. Sequentially lowering the urea concentrations (<2 M) not only allowed refolding, but also the occurrence of the thia zip cyclization. The progress of the end-to-end cyclization was monitored by HPLC and MS, and the whole process was complete within 10 to 20 h. The yield of the end-to-end cyclic peptides was about 70%.

Two-step method to form disulfide bonds

Disulfide bond formation in disulfides is a step critical for the successful syntheses of peptides and proteins containing two or more disulfides. In peptides, the one-step disulfide forming reaction, in which all disulfide bonds are formed simultaneously, often proceeds in poor yield due to the formation of polymers or isomers with disulfide pairings different from those of the desired product. This is a particularly serious concern in the synthesis of circulins, which have a unique cystine-knot motif. Furthermore, our strategy preorganizes the structure as an end-to-end cyclic peptide, which would further place all cysteines in close proximity. Thus, it is anticipated that the formation of all 15 disulfide isomers would be possible, rendering the synthesis unsuccessful. Indeed, results obtained by a one-step disulfide formation of circulin by air oxidation on GSH/GSSG exchange yielded 2.5% of the correctly folded disulfide isomer (Table 1). For these reasons, we employed a two-step method developed by our laboratory (Yang et al., 1994).

In the two-step disulfide forming strategy (Fig. 3), a two-tiered protecting group scheme of 4-methylbenzyl (MeBzl) and acetamidomethyl (Acm) is used. In the first step, the benzyl-based protecting group on the side-chain functional group including the S-MeBzl is cleaved by HF acidolysis during the release of the

Table 1. *Yields of differently protected circulin B* **3** *and CPT* **4** *obtained after disulfide bond formation either by one-step or by two-step approach*

Compound	Cys[a] protecting group 1,4	2,5	3,6	Disulfide isomers obtained	Yield (mol%)
Circulin B **11**	MeB	MeB	MeB	>9	3
Circulin B **5**	MeB	Acm	MeB	3	32
Circulin B **10**	MeB	MeB	Acm	3	29
CPT	MeB	Acm	MeB	3	30

[a]Position of cysteine from N to C direction based on cysteine order in the sequence (see Fig. 1) to show their pairwise connection.

peptide from the resin supports. The Acm–thiol protecting group remains stable due to its resistance to acid. The second pair is then liberated by a method that will not interfere with the stability of the previously formed disulfide bonds by using an electrophilic agent such as I_2 under acidic conditions to prevent disulfide rearrangement (Kamber et al., 1980). Thus, the two-step method simplifies the synthetic scheme without the use of an elaborate three-tiered cysteine protecting group strategy. This method has an additional advantage in that the thiol-protected peptide is purified before oxidation by I_2. In our experiments with six cysteines, there are 15 possible arrangements of the disulfide bonds. However, the described two-step strategy can reduce the possible disulfide bonds from 15 to 3 in the first step. Accordingly, in all our experiments (Table 1) four of six Cys were selectively protected with MeBzl and two with Acm. Using the disulfide pairings of Kalata B1: Cys 1–4, 2–5, and 3–6 (Fig. 1), three separate syntheses were performed. In synthesis (1), Cys 2,5 of circulin B and CPT were protected by Acm and the other four by MeBzl. In synthesis (2) Cys 3,6 of circulin B was protected by Acm and the other four protected by MeBzl, and in the control synthesis (3) all six cysteines were protected by MeBzl as previously described.

In the disulfide formation of (Cys 2,5–Acm) circulin B, three two-disulfide isomers of cyclic peptide **9B, 9C**, and **9D** were isolated by HPLC after the oxidation by 15% DMSO (Tam et al., 1991) in 2 M urea solution (Fig. 4A). The distribution of these three disulfide isomers is statistical and ~30% yield was obtained for each isomer **9B–9D** (Table 1). Partial acid hydrolysis showed that the disulfide bond connectivity of peaks **9B**, **9C**, and **9D** were Cys 1–3, 4–6; 1–6, 3–4; and 1–4, 3–6 (Fig. 4B), respectively. Similar results were obtained in the two-step disulfide formation of (Cys 2,5–Acm) CPT and (Cys 3,6–Acm) circulin B with the correct isomer in ~30% yield. All nine isomers of two-disulfide peptide were isolated by RP-HPLC, and their disulfide connectivity were determined by partial acid hydrolysis. In the second step of the two-step approach oxidation by I_2/MeOH form the third pair of disulfide bond of the nine two-disulfide isomers to produce, as expected, the three-disulfide isomer in 75–85% yield to complete the synthesis of the end-to-end cyclic proteins.

Synthetic circulin B coeluted with the native circulin B in HPLC (Fig. 4C), while the other misformed disulfide isomers had different retention times and did not coelute with the native product. Furthermore, synthetic circulin B exhibited similar antimicrobial profiles as the native circulin B, but lower activity (twofold to fivefold) against common pathogens such as *E. coli*, and *Staphylococcus* compared to defensins. Taken together, these results show that the synthetic circulin B is chemical and biological, similar to the native circulin B and, thus, confirming its disulfide connectivity of Cys 1–4, 2–5, and 3–6, a cystine motif also found in kalata and CPT (Tam & Lu, 1997).

Although the overall syntheses of circulin and CPT were efficient, particularly the end-to-end cyclization via the thia zip reaction, the lower than expected yields of obtaining the desired disulfide isomer could be attributed to the following. First, the energetic of forming these 15 disulfide isomers with a cystine knot are rather similar, as shown by the study on Kalata (Saether et al., 1995). The closed structure of these cystine-knot peptides may be an additional contributing factor. Second, oxidation-mediated DMSO is a slow and equilibrating process. Thus, under such a condition, the oxidation produces all possible nine products: three two-disulfide and six one-disulfide isomers. Figure 4 shows the oxidation pathway after 10 h with the three two-disulfide isomers as major peaks, together with several one-disulfide isomers. The formation of all of the three disulfide isomers usually would require >36 h. The long duration required for DMSO-mediated disulfide formation of closely packed cysteines is not unusual. The two-step oxidation of scratch peptide (Rao & Tam, 1996), a 12 amino acid peptide containing six cysteine residues, requires 96 h to reach final equilibrium to obtain the desired disulfide isomer. The yields of the DMSO-mediated oxidation can be improved by optimizing the pH and the DMSO concentration as well as recycling the incorrectly formed isomer by reduction and a next round of oxidation. However, changing the disulfide protecting group pairing as in (Cys 3.6–Acm) circulin B did not significantly affect the outcome of the disulfide formation (Table 1).

Partial acid hydrolysis for assignment of disulfide bonds

Partial acid hydrolysis was then used to obtain proteolyzed segments suitable for assignment of disulfide bonds in the synthetic products of highly constrained end-to-end cyclic circulin and CPT. More than 30 years ago, several laboratories had already firmly established that partial acid hydrolysis is not a random process in the cleavage of peptide bonds (Light, 1967; Schultz, 1967). However, problem of identifying overlapping peptides proved to be too laborious for practical use. With the advance of mass spectrometry, Zhou and Smith (1990) were able to achieve the successful assignment of the disulfide linkages in hen egg-white lysozyme by partial acid hydrolysis with 0.25 M oxalic acid and FABMS analysis. More importantly, they showed that disulfide bonds are stable under experimental conditions of partial acid hydrolysis with a dilute acid. This is an important point because most proteases have maximum activity in neutral and alkaline conditions, which are the also conditions under which a disulfide bond may undergo disulfide rearrangements (Wetlaufer, 1984; Smyth, 1967). Furthermore, we envision two additional advantages of the acid hydrolysis method. First, the extent of hydrolysis can be controlled by varying the conditions time, temperature, and/or pH. Second, the cleavage sites may be predicted by the chemical basis of anchimeric side-chain assistance as well as steric and electronic factors. These advantages could permit controlled acid catalyzed proteolysis to obtain the desired fragments.

Of all the factors contributing to the acid susceptibility of peptide bonds, anchimeric assistance is best understood. For example, the hydroxylic side chain of Ser and Thr can isomerize in acidic conditions through N,O-acyl transfer to an ester that then can be

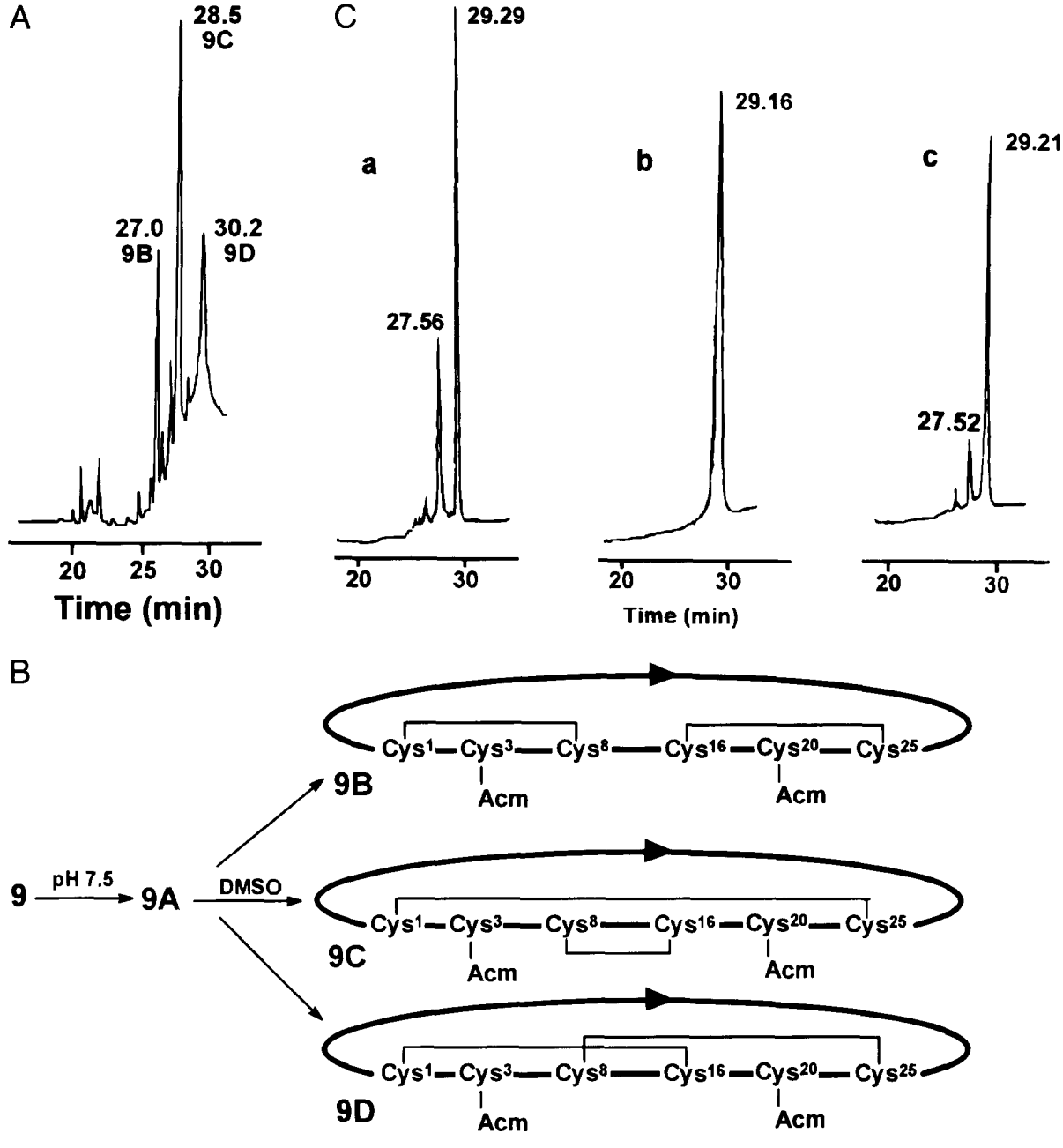

Fig. 4. A: HPLC profile of the three isomers of [$Cys^{3,20}$-Acm] circulin B obtained from crude products after HF and DMSO oxidation. Eluent A: 0.045% TFA/5% CH_3CN/H_2O; B: 0.04% TFA/60% CH_3CN/H_2O. Linear gradient from 0–85% B in 30 min at 1 mL/min. Detection at 225 nm. **B:** A two-step disulfide formation of [$Cys^{3,20}$-Acm] circulin B. Three isomers of disulfide bond [$Cys^{3,20}$-Acm] circulin B, **9B**, **9C**, and **9D**, were obtained by DMSO oxidation. Cysteine positions are based on circulin B (see Fig. 1). **C:** Comparison of synthetic and native circulin B: (a) native circulin B retention time (29.29 min) together with circulin A (27.56 min), (b) synthetic circulin B **3**, (c) co-elution of native and synthetic circulin B. HPLC profile on Vydac column (C_{18} 4.6 × 250 mm). Eluent A: 0.045% TFA/5% CH_3CN/H_2O; B: 0.04% TFA/60% CH_3CN/H_2O. Linear gradient from 0–85% B in 30 min at 1 mL/min. Detection at 225 nm.

readily hydrolyzed (Sakakibara et al., 1962). Anchimeric assistance of the carboxylic side chain of Asp and Glu through either a five- or six-member transition state accelerates hydrolysis at the carboxyl side. The amide side chains of Asn and Gln are also known to undergo aspartimide or glutarimide formation at the carboxyl side in protein degradation. The reaction pathway can also proceed through deamidation of Asn or Gln to Asp or Glu followed by their anchimeric assistance of the carboxylic side chains. In general, the five-member ring formation by Asp/Asn is about 100-fold faster than the corresponding reactions by Glu/Gln. In contrast, Ser and Thr offer anchimeric assistance through their β-hydroxylic groups to form N,O-acyl isomerization and hydrolysis at the amino side. Although the thiolytic side chain of cysteine can readily participate in N,S-acyl migration similar to

Ser-Thr leading to hydrolysis, the free thiol of Cys is usually tied up as a disulfide, which prevents its participation in the fragmentation pathway.

Steric factors and bond strains also contribute to the susceptibility of peptide bonds. Gly is favored in the hydrolytic pathway because it offers little steric hindrance. On the contrary, amino acids with sterically hindered branched side chains such as Ile are resistant to acid hydrolysis. The tertiary amide of proline produces bond strain and is more acid susceptible than the corresponding secondary amide bonds. Asp–Pro is one of the most acid susceptible bonds and widely used for partial acid cleavage reactions. Thus, based on these chemical precedents, acid susceptible bonds will include sites containing Asp, Glu, Ser, Thr, Asn, Gln, Gly, and Pro residues. A combination of two or three such amino acids should increase their acid susceptibility, as found in the Asp–Pro sequence, whose cleavage rate is often several hundred-fold higher than other peptide bonds. Based on this rationale, we refer to these "hot spots" as the primary cleavage sites, with those containing isolated Asp, Glu, Ser, Thr, Asn, Gln, Gly as the susceptible cleavage sites. In acid fragmentation, three such primary cleavage sites are predicted in circulin and two in CPT (Table 2). The confirmation of the proposed primary cleavage sites can be realized by isolation of the high molecular, single-nicked products.

We used oxalic acid for partial acid hydrolysis to test our samples of nine isomers containing two-disulfide bonds with one pair of cysteines protected by Acm. These disulfide isomers were obtained after the first step and prior to the second step in the two-step method to form disulfide bonds. Each of the two-disulfide isomers (Fig. 4A) was purified and subjected to hydrolysis by 0.25 M oxalic acid solution at 110 °C for 5 h. Fragments of disulfide-containing peptides obtained by RP-HPLC fractions of the hydrolysates were analyzed by MALDI/MS. In general, many one-disulfide fragments were obtained for each isomer to facilitate the determination of disulfide connectivity. Part of the representative data obtained from one of the disulfide isomer, **9D** of (Cys 2,5–Acm) circulin B, is shown in Table 3. In this particular run, 15 fragments could be assigned to 1–4 disulfide linkages and 13 fragments to 3–6 disulfide linkages. Thus, **9D** was unambiguously assigned disulfide pairings of 1–4 and 3–6. Similarly, **9B** and **9C** were assigned to contain Cys 1–3, 4–6, and 1–6, 3–4, respectively (Fig. 4B).

Table 2. *Predicted and observed proteolytic fragments by 0.25 M oxalic acid hydrolysis of circulin B and CPT*

Predicted primary cleavage sites	Sequence[a]	Mass	
		Calculated	Determined
[Cys^{3,20}-Acm]Circulin B[b]		3,428.2	3,429.3
N11-G12	ΔN11-G12	3,275.1	3,277.2
	ΔN11G12-S27	3,206.0	3,204.4
	ΔN11G12-S27T28	3,104.9	3,104.3
G17-E18	ΔG17-E18	3,260.1	3,261.0
	ΔG17E18-S27	3,190.9	3,191.0
E18-S19	ΔE18-S19, S27-G31	2,776.4	2,778.1
	ΔG17E18S19-S27-L30	2,776.4	2,778.1
S27-T28	ΔS27	3,359.2	3,359.9
	ΔS27-T28	3,258.1	3,259.0
[Cys^{8,24}-Acm]CPT[b]		3,375.1	3,376.4
N11-S12	ΔS12	3,306.1	3,308.3
	ΔK10-S12	3,064.1	3,065.0
G16-E17	ΔG16-E17, K10-P14	2,686.1	2,688.2
E17-S18	ΔG16-S18, K4-I13	1,916.8	1,918.4

[a]Excised amino acids or peptides from the sequence.
[b]Cysteine position based on sequence shown in Figure 1.

Two general conclusions can be drawn from the hydrolysis of nine samples consisting of different disulfide isomers of circulin peptides. First, we have found that fragmentation follows the predicted the primary cleavage sites (Table 2). The arrows in Figure 5A indicate the primary and susceptible sites at which cleavage occurred to give the one-disulfide bond containing peptides listed in Table 3. Second, the constraints of these cyclic peptides permit isolation of high molecular fragments. Thus, the fragmentation pattern can be reconstructed to aid the primary and susceptible cleavage sites and to provide confidence in identifying both of one- and two-disulfide bonded fragments. For example, initial cleavage at one of these primary cleavage site Asn^{11}, Gly^{12} of **9D** produces single-nicked products with the loss of either Asn^{11}, Gly^{12}, or both (Fig. 5B). However, single-nicked **9D** (Fig. 5B) at the other two primary cleavage sites were also observed confirming the propensity of fragmentation at these sites. Further fragmentation of **9D** at Ser^{27}, Thr^{28}, and/or Gly^{17} Glu^{18} Ser^{19} produced 13 double-nicked and 12 triple-nicked **9D**. These two-disulfide–linked peptides were isolated and confirmed by MS. Subsequent fragments at the susceptible sites, particularly at Asn^{5}, produced 28 quadruple-nicked one-disulfide–paired segments useful to establish the disulfide connectivity of **9D**. Similar pathways of proteolytic fragmentation of the other eight disulfide isomer were found, i.e., first through the primary cleavage sites and then the susceptible sites. It should be point out even through the proteolytic fragmentation follow closely through the described pathways, secondary degradation of both amino and carboxylic amino acid residues of the nicked fragments also occur simultaneously and contributed to the heterogeneity in these observed peptide fragments.

Our results on acid-catalyzed fragmentation patterns are in general agreement with those obtained by others. In the partial hydrolysis of the four-disulfide, 49-amino acid Echistatin, Bauer et al. (1993) reported 28 one- or two-disulfide–linked segments in which 54 of the 60 amino terminal residues are Cys (Cys), Glu, Thr, Gly, or Pro, while 44 of the carboxyl terminal residues are derived from cleavage of X-Glu, Ser, Thr, Asp, Asn, Gly, or Pro bonds. Similarly, by acid hydrolysis of the four-disulfide, 129 amino acid residue–hen egg–white lysozyme, Zhou and Smith (1990) have identified 46 single disulfide-linked segments with predominant fragmentation occurring at these sites.

In conclusion, our results demonstrated orthogonal ligation through a thia zip reaction provides a simple and direct method for synthesis of cyclic peptides with multiple disulfide bonds. In addition, the combination of partial acid hydrolysis and MALDI/MS is a valuable method for locating disulfide bonds in these highly constrained cyclic peptides. These methods are likely to be useful for the synthesis and confirmation of cyclic proteins that have not been generally explored.

Table 3. *Assignment of disulfide-containing peptides found in partial acid hydrolysis of [Cys^{3,20}-Acm] circulin B (**9D**)*

No.	Sequence of observed fragments	Mass Calculated	Mass Determined	C-C linkage
1	LLGC(Acm)K GCPI	1,180.4	1,182.9	1,4
2	C(Acm)KNK GCPI	1,238.4	1,241.4	1,4
3	C(Acm)KNK SEGCPIV	1,325.4	1,326.1	1,4
4	C(Acm)KN(-H_2O) SEGCPIVGN	1,391.5	1,391.8	1,4
5	C(Acm)KN EGCPIVGN(-H_2O)	1,391.5	1,391.8	1,4
6	TLLGC(Acm)KN SEGCPIV	1,400.5	1,400.1	1,4
7	C(Acm)KN EGCPIVGN	1,409.5	1,410.7	1,4
8	C(Acm)KN EGCPIVGNR	1,565.7	1,565.0	1,4
9	C(Acm)KN(-H_2O) SEGCPIVGNR	1,634.8	1,636.6	1,4
10	LLGC(Acm)KN EGCPIVGN	1,692.9	1,694.5	1,4
11	TLLGC(Acm)KN(-H_2O) SEGCPIV	1,692.9	1,691.3	1,4
12	LLGC(Acm)KNK EGCPIVGN	1,821.1	1,821.5	1,4
13	LLGC(Acm)KN(-H_2O) EGCPIVGNR	1,977.3	1,978.9	1,4
14	LLGC(Acm)KNK SEGCPIVGNR	2,064.3	2,065.8	1,4
15	C(Acm)KNK PIFVC(Acm)SEGCPIV	2,084.3	2,086.5	1,4
16	NKVCYR IC	1,013.2	1,015.4	3,6
17	NKVCYR TSIC	1,045.2	1,047.3	3,6
18	KVCYR TSIC	1,087.3	1,086.9	3,6
19	NKVCYR SCI	1,100.3	1,102.6	3,6
20	KVCYR TSICP	1,184.4	1,186.4	3,6
21	NKVCYRNG SIC	1,271.5	1,271.6	3,6
22	NKVCYRNGVIP SIC	1,580.9	1,580.9	3,6
23	KVCYR TSICPIFVC(Acm)	1,718.1	1,719.3	3,6
24	KVCYR TSICPIFVC(Acm)S	1,805.1	1,804.0	3,6
25	KVCYR TSICPIFVC(Acm)SE	1,934.2	1,934.9	3,6
26	NKVCYR TSICPIFVC(Acm)SE	2,162.4	2,162.6	3,6
27	KVCYR TSICPIFVC(Acm)SE	2,219.5	2,218.7	3,6
28	NKVCYRN TSICPIFVC(Acm)SEG	2,219.5	2,218.7	3,6

Materials and methods

Peptide synthesis and general procedure

Solid-phase peptide syntheses were performed manually or on an ABI 430 synthesizer. All thioester peptides were synthesized on Boc–amino acid–SCH_2CH_2CO–MBHA resin that was prepared according to a modified procedure described by Hojo and Aimoto (1991). All amino acids were protected with Boc group. The side-chain protections were as follows: Arg(Tos), Asp(OcHex), Cys(4-MeBzl), Cys(Acm), Glu(OBzl), Lys(ClZ), Ser(Bzl), and Thr(Bzl). One cycle of the synthesis using 15–20 mL solvent per gram of resin consisted of the following two operations: (a) a 20-min deprotection with 50% trifluoroacetic acid/CH_2Cl_2, and (b) coupling with 4 equiv. each of Boc–amino acid and BOP (Castro et al., 1975) in the presence of 12 equiv. DIEA in DMF for 45–60 min. In manual synthesis, coupling reactions were monitored by the Kaiser ninhydrin test (Kaiser et al., 1970) to determine the extent of completion, and double coupling was used with 2 equiv. of Boc–amino acid when necessary. In synthesis by the ABI 430 synthesizer, a double coupling with DCC/HOBt protocol was employed. The thioester peptide was cleaved from the resin by high HF (HF/anisole, 9:1, v/v). After HF removed, the resulting peptides were washed with diethyl ether to remove the organic scavenge. The peptide was extracted with 8 M urea, pH 7.5, containing 100 mg of TCEP to prevent polymeric disulfide formation. The peptide solution was dialyzed (MW cutoff 1,000 from Spectrum Medical Industries, Inc., Houston, Texas) to allow the dissociation of small organic molecules and aromatic scavengers. Sequentially lowering urea concentrations to 2 M allowed cyclization that was monitored by analytical C_{18} reversed-phase HPLC and MS. The dialyzed solution was then diluted to 1 M urea by water for the first-step folding after completed cyclization. Analytical HPLC was run on a Shimadzu system with a C_{18} Vydac column (250 × 4.6 mm) at a flow rate of 1 mL/min with a linear gradient of 0–85%B (60% acetonitrile in H_2O/0.04% TFA) in buffer A (5% acetonitrile in H_2O/0.045% TFA) for 30 min with UV detection at 225 nm. MALDI/MS was performed on a Kompact MALD III instrument. Samples were dissolved in 2 μL of a 1:1 mixture of H_2O–CH_3CN containing α-cyano-4-hydroxycinnamic acid. Measurements were made in the linear mode.

Oxidation to form disulfide bond (first step)

Disulfide formation was achieved by adding 15% DMSO to the peptide urea solution (1 M) and was complete in 10 h, as monitored by HPLC and MS. All three isomers were generally obtained. The isomer peptides were purified on a preparative C_{18} Vydac reversed-phase (RP) HPLC column (250 × 22 mm), with a linear gradient of H_2O containing 0.1% TFA and 60% acetonitrile in H_2O containing 0.1% TFA at a flow rate of 20 mL/min.

Concomitant removal of the S-acetamido group and formation of the third disulfide bond (second step)

A DMSO/urea peptide solution was adjusted pH to 4 by acetic acid. The solution was bubbled with nitrogen for 10 min before dropwise addition of iodine/methanol until a brown color persisted. The reaction, maintained in nitrogen atmosphere and in a darkened vessel, was completed in 45 min, as monitored by HPLC. The solution was cooled in an ice bath, and excess iodine was

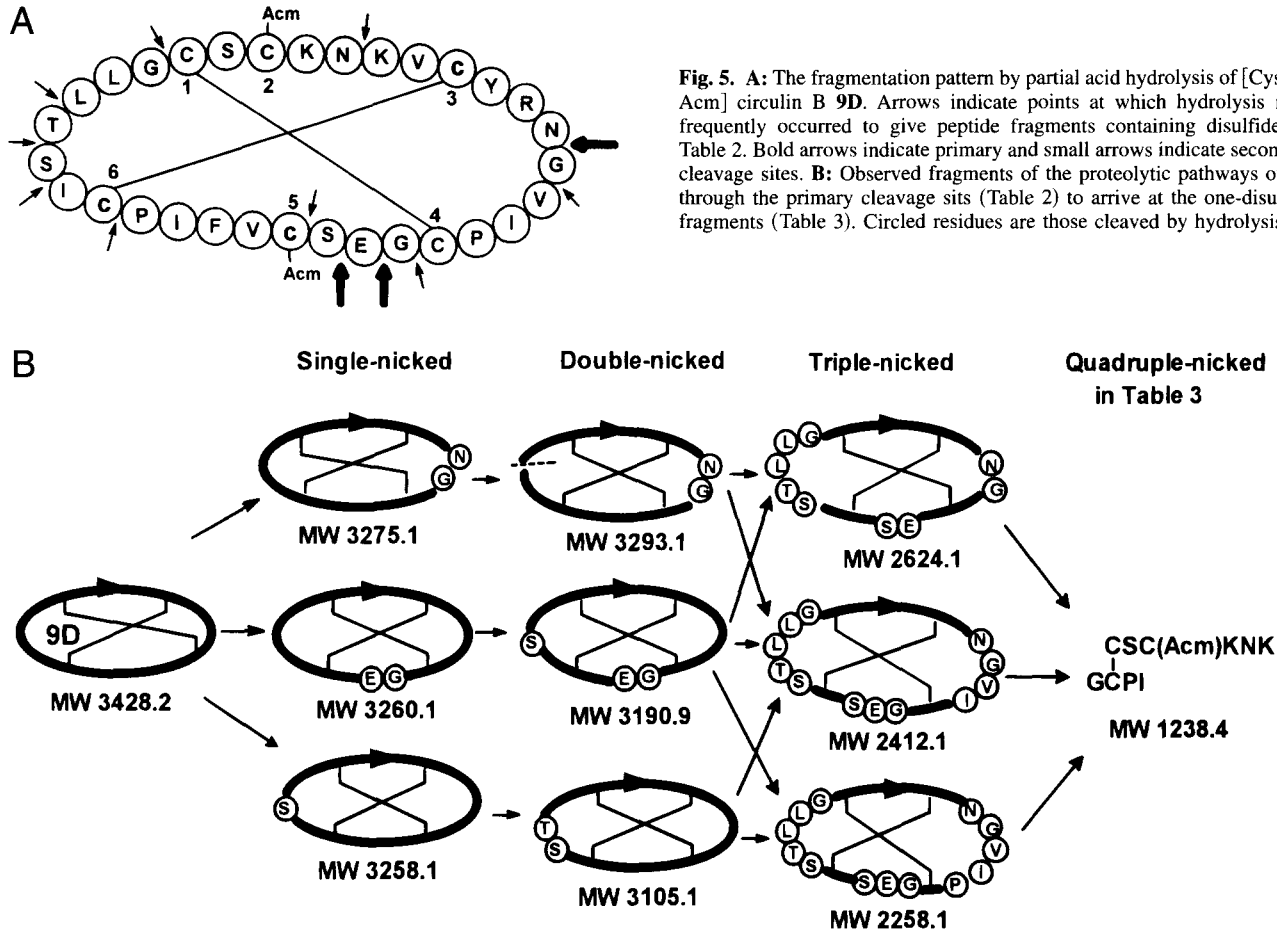

Fig. 5. A: The fragmentation pattern by partial acid hydrolysis of [$Cys^{3,20}$-Acm] circulin B **9D**. Arrows indicate points at which hydrolysis most frequently occurred to give peptide fragments containing disulfides in Table 2. Bold arrows indicate primary and small arrows indicate secondary cleavage sites. **B:** Observed fragments of the proteolytic pathways of **9D** through the primary cleavage sits (Table 2) to arrive at the one-disulfide fragments (Table 3). Circled residues are those cleaved by hydrolysis.

quenched by ascorbic acid. The peptide was purified on preparative HPLC in the same manner as described above.

Synthesis of circulin B

Circulin B was synthesized by ABI 430 on Boc-Gly-SCH_2CH_2CO-MBHA resin (500 mg, 0.26 mmol/g). The unprotected peptide thioester (MW cal. 3,538.17, found $M+H^+$ 3,538.9) was cleaved from the resin support (250 mg) by high HF. Peptide was extracted into 8 M urea (150 mL) and then dialyzed against 8 M urea (2,000 mL) to allow cyclization in a descending concentration of 8 to 2 M urea solution. The whole dialysis process with concomitant thia zip cyclization was complete within 20 h. A single major peak of cyclic peptide with four reduced thiols and Cys 2,5 (Acm) was found (MW cal. 3,432, found $M+H^+$ 3,432.2) in RP-HPLC. Disulfide formation was achieved by adding 15% DMSO to the peptide solution and was complete in 10 h (MW cal. 3,428.17, found $M+H^+$ 3,429.5), yielding three disulfide isomers (Fig. 4A) with the correctly folded isomer obtained in 30% yield. The third disulfide bond was formed by adjusting the pH to 4 and treatment with I_2/MeOH under nitrogen to remove the Acm protecting groups of Cys 2,5. The peptide was purified by preparative RP-HPLC and characterized by MS giving the expected molecular weight (MW cal. 3,283.96, found $M+H^+$ 3,285.8) with an overall yield of 5% based on HPLC of the crude peptide. The synthetic material was identical to the naturally isolated circulin B both chemically and biochemically (data not shown). Furthermore, synthetic and naturally isolated circulin B eluted as a single peak under several RP-HPLC conditions (Fig. 4C).

Synthesis of Cyclopsychotride

A procedure similar to that described above for the synthesis of circulin B was used for synthesis of CPT. The unprotected peptide thioester: (MW cal. 3,485.07; found $M+H^+$ 3,486.07), cyclic peptide with four reduce thiols and two Acm: (MW cal. 3,379.07; found $M+H^+$ 3,380); CPT (MW cal. 3,230.89; found $M+H^+$ 3,232.2).

The partial acid hydrolysis

The peptide (120 μg) in 0.25 M oxalic acid (200 μL) was hydrolyzed at 100 °C for 5 h in a glass tube, which had been evacuated and sealed. The hydrolysate was fractionated by RP-HPLC with a linear gradient from 0–90% B for 60 min, where buffer A (5% CH_3CN in H_2O/0.045% TFA) buffer B (60% CH_3CN in H_2O/0.04% TFA), C_{18} Vydac column (250 × 4.6 mm) at flow rate of 1 mL/min, UV detection at 225 nm. All chromatographic fractions were dried in a vacuum centrifuge and dissolved in buffer B (HPLC) (1 μL), and analyzed by MALDI/MS.

Synthesis of CPT by one-step disulfide bond formation

Synthesis was carried out on Boc-Gly-SCH_2CH_2CO-MBHA-resin (500 mg) by ABI 430 synthesizer. The unprotected peptide thioester (MW 3,341.96, found 3,342.2) was obtained after high HF cleavage (200 mg resin). Cyclization was carried out by the same procedure as described for circulin B to give the cyclic peptide (MW 3,236, found 3,236.7) at >70% yield in only 30 min. However, oxidation by GSSG and GSH gave more than nine disulfide isomers (retention time in HPLC from 22.6 to 30.8 min showed the same molecular weight). About 1% of the desired isomer has same retention time as the native CPT (30.1 min). This mixture of disulfide isomer solution was recycled by reduction in TCEP, and then oxidized by 15% DMSO at pH 7 to give six disulfide isomers and the desired CPT in 2% yield.

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