Thia Zip Reaction for Synthesis of Large Cyclic Peptides: Mechanisms and Applications†

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Abstract: This paper describes the mechanism and application of an efficient thia zip cyclization that involves a series of intramolecular rearrangements in a cysteine-rich peptide for the synthesis of large end-to-end cyclic peptides. Key functional groups required in this reaction include an N^{α}-cysteine, a thioester, and at least one internal free thiol in a peptide. The zip reaction is initiated by intramolecular transthioesterification through an internal thiol with the thioester. A thiolactone is formed under ring−chain tautomeric equilibrium that favors ring formation in aqueous buffered solution at pH > 7. Successive ring expansions through thiol−thiolactone exchanges in the direction of the amino terminus lead finally to a large N^{α}-amino thiolactone which then undergoes a spontaneous and irreversible ring contraction through a sequence-independent S to N acyl isomerization to form an end-to-end lactam. The reversible thiolactone exchanges are sequence-dependent, and the rate-determining steps are shown by rate studies on model peptides. The assistance of internal thiols in reducing the ring sizes and hence the entropy of the thiolactone exchanges correlates well with cyclization rates. Zip-assisted end-to-end cyclizations forming 93- and 99-atom rings through a series of small thiolactone intermediates were 60−200-fold faster under strongly denaturing conditions such as 8 M urea than the corresponding unassisted lactamization. The thia zip reaction has been applied successfully to the synthesis of a 31-amino acid cyclic peptide, the naturally occurring cyclopsychotride that shows the antimicrobial activity. In addition, the thia zip reaction also enables the synthesis of an engineered cyclic 33-amino acid animal defensin by replacing the end-to-end disulfide with a lactam, which retains the antimicrobial activities of the native open-chain form.

Introduction

Ring enlargement is a common strategy in macrocyclic synthesis.[1−6] In particular, a plethora of methods have focused on ring expansions at the lactone or lactam carbonyl ring junction **1** by a tethered arm containing an oxa or aza nucleophile yielding a bicyclic intermediate **2** (Figure 1a). Subsequent heterolytic cleavage of the bridgehead bond in the bicyclic results in an expanded ring and a new carbonyl ring junction **3**. When the tethered arm contains multiple, interspatially dispersed nucleophiles **4**, ring enlargements can be repeated for several cycles to give the macrocyclic product **6**. This type of successive ring enlargement is known as the zip reaction. [5,6] The aza zip reaction has been particularly successful in the synthesis of polyaza lactams of up to 60 ring atoms.[6]

Thia zip-assisted ring enlargement (Figure 1b) to form macrocyclic peptides under mildly basic aqueous conditions appears to be possible in view of our recent work as well as others' on orthogonal cyclization strategies.[7−12] In one such strategy, an unprotected α-cysteinyl peptide thioester undergoes an end-to-end thiolactone formation and then a spontaneous ring contraction.[10−12] We envision that an unprotected peptide such as **8** obtained from a protected peptide by solid-phase synthesis and containing one or more internal cysteinyl thiols forms a

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† Abbreviations: Standard abbreviations are used for the amino acids and protecting groups [IUPAC-IUB Commission for Biochemical Nomenclature. *J. Biol. Chem.* **1985**, *260*, 14]. Other abbreviations are as follows: TFA, trifluoroacetic acid; HF, hydrofluoric acid; DMSO, dimethyl sulfoxide; DMF, dimethylformamide; DCM, dichloromethane; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DIC, *N*,*N*-diisopropylcarbodiimide; HOBt, *N*-hydroxybenzotriazole; DIEA, *N*,*N*-diisopropylethylamine; TCEP, tris(carboxyethyl)-phosphine; BOP, benzotrizol-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate; MBHA, 4-methyl benzhydrylamine; RP-HPLC, reverse-phase high performance liquid chromatography; MALDI/MS, matrix-assisted laser desorption ionization mass spectrometry.

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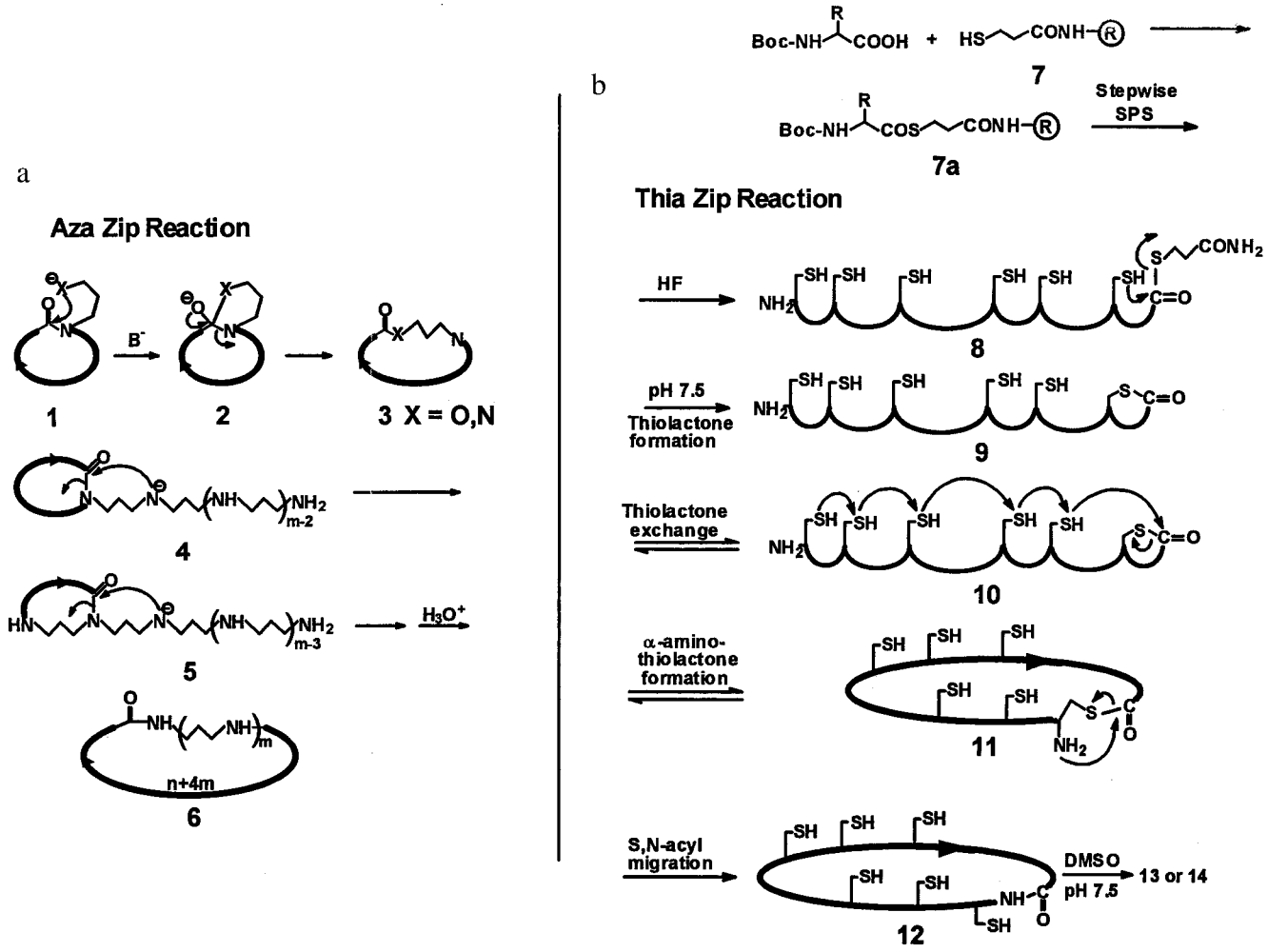

Figure 1. Zip reaction through sequential ring expansion: (a) aza zip reaction **1**−**6** through amino insertion of a bridgehead lactam **2** and (b) proposed thia zip reaction **8**−**12** through thiolactones and thiolactone expansion **9**−**11.** The initial thiolactone is derived from transthioesterification between an internal thiol and the α-thioester **9** under the control of ring−chain tautomeric equilibrium. An S,N-acyl migration of the thiolactone **11** at the N-terminal cysteine trap the reversible process as an end-to-end cyclic peptide **12**. DMSO oxidation of the thiols to disulfides form the cyclic peptides **13** and **14**.

thiolactone such as **9** under a ring−chain tautomeric equilibrium.[13−14] It could then proceed through a series of small thiolactone intermediates, such as **10** and **11**, by successive ring expansions at the carbonyl ring junctions (Figure 1b) analogous to the aza zip reaction. These reversible equilibrations of thiol-thiolactone exchanges could result in the formation of an N^{α}-amino thiolactone linking the N and C termini **11**. However, unlike the aza zip reaction, this large N^{α}-amino thiolactone intermediate should readily undergo a ring contraction through an S to N acyl transfer that results in a spontaneous isomerization to give an end-to-end lactam **12**. The S to N acyl migration of an N^{α}-amino thiolactone to a lactam at the N^{α}-Cys residue is sequence-independent, irreversible, and provides the driving force for the proposed thia zip reaction.

The proposed thia zip cyclization potentially offers a facile approach and has been successfully exploited in the synthesis of cyclic large peptides or proteins with multiple disulfide bonds.[11] Such an approach, however, is significantly different from the conventional methods of peptide cyclization. In conventional methods, fully or partially protected peptide precursors and enthalpic cyclization are used either in solution or anchored on a resin support.[15−17] With these methods, N to C cyclization would be unfavorable for large peptides because of the entropy penalty involving a very large ring. In contrast, the thia zip cyclization using unprotected precursors would involve a series of intramolecular rearrangements of smaller rings ending with an irreversible S,N-acyl migration at the α-amino terminus for the ring closure to afford a large cyclic peptide. As a result, the thia zip-assisted cyclization which would undergo smaller, discrete cyclic intermediates is more entropically efficient than the corresponding one-step end-to-end cyclization. It is also a chemoselective cyclization process that distinguishes an α-amine from the ϵ-amines or other nucleophilic side chains in a peptide without any protecting group. Such a method, unencumbered by protecting groups or enthalpic

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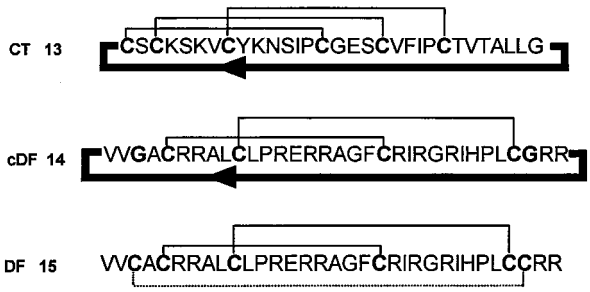

Figure 2. Amino acid sequences and disulfide pairings of cyclopsychotride (CT) **13**, the natural occurring α-defensin NP-1 (DF) **15,** and its engineered lactam cyclic defensin (cDF) **14**. Note, $Cys^{3,31}$ and the 1,6-disulfide pair of **15** shown as a dotted line is replaced by two Gly in **14**.

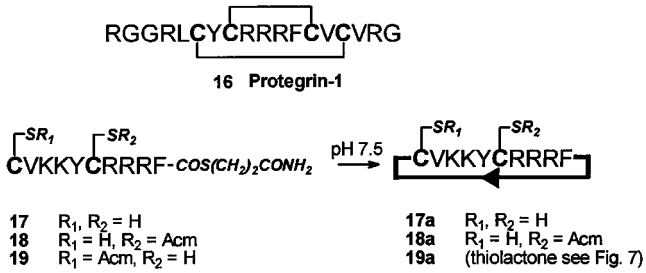

Figure 3. Amino acid sequences of the linear precursors and the lactam analogues of the protegrin (PG). Acm = acetamidomethyl.

activation by a coupling reagent, would greatly simplify synthetic schemes and enhance the efficiency of macrocyclization. Furthermore, thia zip cyclization has the additional advantage of avoiding oligomerization through ring−chain tautomerization because of the intrinsic high effective molarity for an intramolecular reaction.[9] Recently, we and others have successfully exploited this principle of ring−chain tautomerization in developing methods for the synthesis of cyclic peptides using unprotected peptide precursors.[9−12] Indeed, at appropriate concentrations these methods generally give high yields of cyclic peptides without observable oligomerization or the need for high dilution.

To test whether the proposed thia zip reaction would meet the challenge of macrocyclization of large peptides, we have undertaken the synthesis of cyclopsychotride **13** (CT), which is reported to be one of the largest naturally occurring cyclic peptides,[18a] and cyclic animal defensin **14** (cDF), which contains an engineered lactam replacing the native end-to-end disulfide bond of the rabbit α-defensin,[19] NP-1 **15** (Figure 2). CT contains 31 amino acids and belongs to the family of cystine−knot cyclic peptides with three intertwining cystine bonds and an end-to-end cyclic structure. This family, which includes circulins[18b] and kalata,[18c] has shown a diverse range of biological activities including antiviral activity against HIV-1. Although CT activates neurotensin receptors, as shown by receptor screening assays, it likely belongs to a family of plant antimicrobial defense agents collectively known as plant defensins based on their sequence similarity.[19,20] A facile method for the synthesis of the CT family of cyclic peptides can provide synthetic samples to verify this hypothesis.

Rabbit NP-1 **15** (referred to as linear DF in this paper), a member of the α-defensin family,[19] is also a broad spectrum antimicrobial peptide, but is produced by vertebrates as part of their innate defense mechanisms. It has an open-ended sequence with three disulfide bonds which are different from the disulfide pairing motif of CT **13** (Figure 2). Like many other disulfide-rich small proteins, including protease inhibitors and toxins,[21,22] linear DF **15** has a circular-permutated arrangement, owing to a disulfide linkage that places both termini at close proximity. These circular-permutated peptides or proteins are amenable to disulfide replacement with an end-to-end cyclic structure. As a model for preparing cyclic proteins, we therefore engineered the linear DF **15** into cyclic structure **14** by replacing the end-to-end disulfide with a lactam while retaining the two internal disulfide bonds. Despite differences in both sequences and disulfide pairing motifs, DF **15** and CT **13** probably share a triple β-strand structure based on their sequence homology with their respective members whose structures have been determined.[18a,19]

In this paper, we describe the development of the thia zip reaction for the cyclization of unprotected cysteinyl peptide thioesters and its validation for the synthesis of large end-to-end cyclic antimicrobial peptides such as CT **13** and cDF **14**. Furthermore, we show that the cDF **14** with a lactam replacement retains its antimicrobial activity, suggesting the general structural and functional relatedness of vertebrate and plant defensins.

Results

Peptide Thioester Synthesis. All linear peptides were prepared as thioesters by stepwise solid-phase synthesis[23] using Boc chemistry. The first amino acid was directly esterified onto the resin **7**[24] containing a cleavable thioproprionyl linker attached to MBHA resin using BOP, which converted the C-terminal Boc-amino acid to a thioester **7a** (Figure 1b). Sequential coupling was mediated by BOP or DCC/HOBt protocol. After assembly of the sequence on the solid support, peptide thioesters were cleaved from the resin, purified by reverse phase HPLC (RP-HPLC), and characterized by MALDI-MS. Thus, all peptides used in this study contained an N-terminal cysteine and an α-thioester with a 3′-propionic carboxamide such as **8**, but were generally free of side-chain protecting groups. In several peptides, selected internal thiols were protected with acetamidomethyl (Acm)[25] to study thiol participation in the thia zip reaction.

Cyclization Conditions. Cyclizations (Figure 1b) of the linear unprotected precursors were performed at pH 7.5 in phosphate buffer[9] containing a 3−5-fold excess of TCEP, a water-soluble

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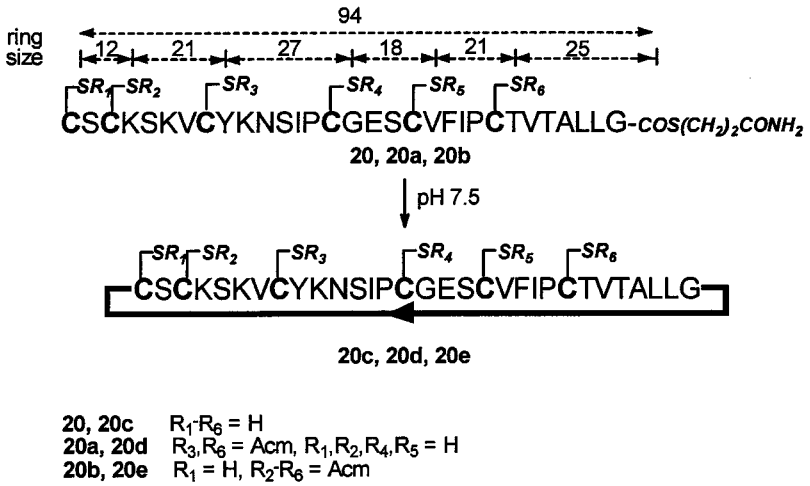

Figure 4. Amino acid sequences of the linear precursors and the lactam products of cyclopsychotride (CT).

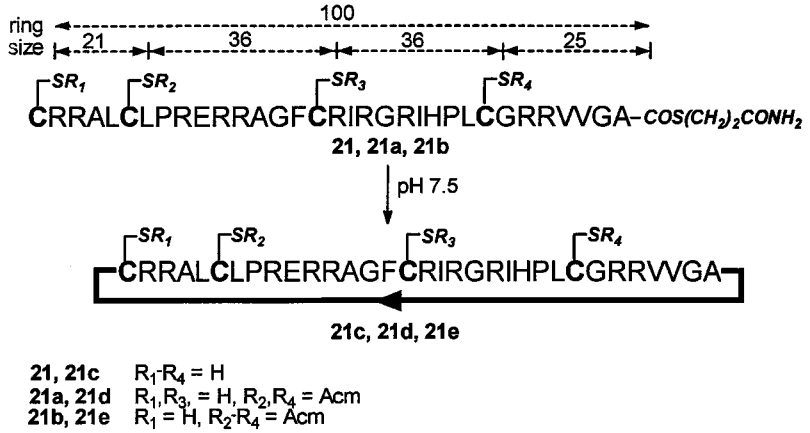

Figure 5. Amino acid sequences of the linear precursors and the lactam products of α-defensin (DF). The ring size required for thiolactone formation or exchange is shown at the top of the figure.

tris(carboxyethyl)phosphine[26] and a 2-fold excess of 3-thiopropionic acid. The role of the small thiol is to minimize the formation of diacyl byproduct. TCEP was added to prevent disulfide formation.[27] Previous examination of the pH dependence of cyclization has determined[9] that the pH range of 7−8 is optimal for cyclization with minimal hydrolysis of the thioester.

Three groups of unprotected peptide thioesters **17**−**21** containing 10, 31, and 33 amino acid (aa) residues were used to test the thia zip reaction in aqueous solutions at pH 7.5. The first group **17**−**19** with a disulfide bond consisted of 10 aa's to form a 30-member ring lactam (Figure 3). These are analogues of the antimicrobial peptide protegrin (PG) **16**.[28] By using purified unprotected peptide precursors, the cyclization of PG analogues **17**−**19** with their sulfhydryls proceeded efficiently in yields >95%. As reported previously by our laboratory,[9] the cyclization is chemoselective for the N^{α}-amine (referred to as an orthogonal process for its ability to distinguish between α- and ϵ-amines). Side-chain amines of lysine are unaffected. Peptides **17**−**19** contain multiple arginines and lysines in their sequences, and end-group analysis by Sanger's reagent[29] failed to detect products due to incorrect acyl-transfer reactions.

The second and third groups consisted of the 31-residue CT analogues **20**, **20a**, and **20b** (Figure 4) and the 33-residue DF analogues **21** and **21a** (Figure 5). Cyclizations of these large peptides to their respective cyclic forms **20c**,**d** and **21c**,**d** were equally facile and complete in 6 h. The cyclization could be run in moderate concentrations at 0.1−1 mM levels without affecting the yield. Although peptide oligomerization is often the case in cyclizations using protected peptide segments, it was not detected in thia zip cyclization by RP-HPLC. In all three cases, more than 90% of cyclic products were recovered as the isolated yield after RP-HPLC purification. Thus, in the model studies, the cyclization appeared to be concentration-independent and conformed with the expected equilibrium behavior of ring−chain tautomerism.

Mechanistic Study. The thia zip reaction can be divided into two general types of reactions based on the nucleophiles involved (Figure 1b): (1) side-chain thiols as nucleophiles in a series of sequence-dependent transthioesterifications **8**→**11** consisting of thiolactone formation and exchanges which ultimately result in the formation of the N^{α}-amino thiolactone **11**, an end-to-end intermediate in the penultimate step of the zip-assisted pathway, and (2) N^{α}-amine as nucleophile in the final step of the sequence-independent S,N-acyl isomerization of the N^{α}-amino thiolactone to the end-to-end lactam **12** which

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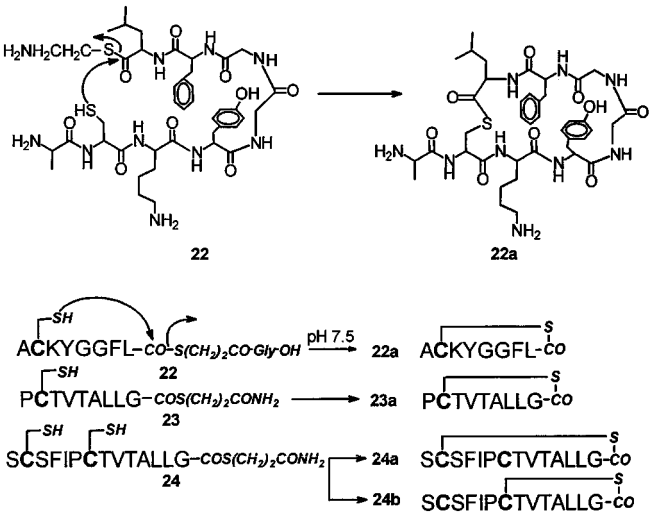

Figure 6. Amino acid sequences and their thiolactones obtained after treatment with pH 7.5 in aqueous conditions.

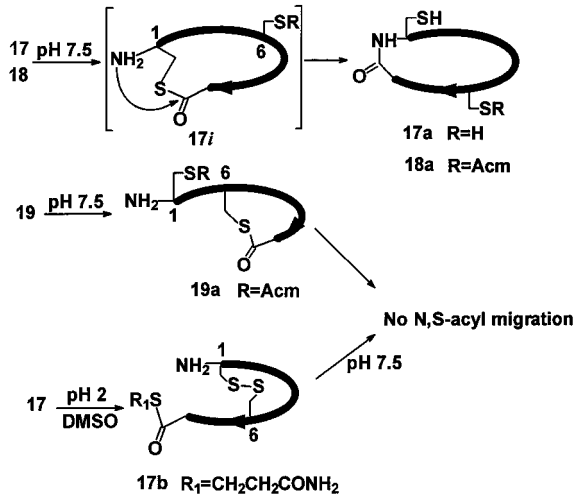

Figure 7. Model study to show the requirement of N^{α}-amino thiolactone formation. The amino thiolactone intermediate **17i** is not observed and undergoes a facile S,N-acyl migration to form lactam **17a** or **17b**. Position of Cys in the PG analogues **17**−**19** is numbered as 1 and 6.

involves the invariant α-cysteine at the N-terminal. Both types of reactions were studied.

1. Formation of Thiolactone Intermediates. Thiolactone formation represents the first step in the proposed thia zip reaction. Since the N^{α}-amino thiolactone intermediates in the thia zip reaction are prone to isomerization through an S,N-acyl migration to lactams, we prepared Xaa-Cys (Xaa=Ala, Ser or Pro) peptide thioesters **22**−**24** (Figure 6) whose N^{α}-amines were extended by an amino acid to obtain stable thiolactone intermediates for characterization.

Three types of thiolactone precursors were prepared. The first model ACKYGGFL **22** derived from Leu-enkephalin contains a GlyGly dipeptide sequence and represents a flexible peptide. The second model PCTVTALLG **23** derived from the COOH-terminal nine residues of CT **20** is hydrophobic and represents a peptide with a high propensity to form an ordered structure. Indeed, this sequence is part of the β-strand structure found in kalata.[18c] The third model SCSFIPCTVALLG **24** derived from the COOH-terminal 13 residues of CT represents an ordered peptide containing two Cys, which permit the formation of two thiolactones from the truncated analogue CT **20**. The thiolactone formation of **22** at pH 7.5 was fast, irreversible, and took <2 min. Similarly, **23** and **24** with ordered peptide structures required <10 min while peptide **24** with two Cys afforded thiolactones **24a** and **24b** in about equal molar ratio. Furthermore, these thiolactones with free α-amine atoms were found to be relatively stable in aqueous solutions at pH 7.5 for 6 h without significant hydrolysis or ring contraction via an S to N shift to give end-to-end cyclic peptides.

Table 1. Correlation of Rates and Sequence Dependence of Thiol−Thiolactone Exchange for the Thia Zip Reaction Using the Peptide Thioester, Cys-Val-X-Y-Thr-Cys-Arg-Arg-Phe-$OCH_2CH_2SCONH_2$

	analogues X−Y	rate (cyclic peptide)	
		k_1 (min^{-1})	$t_{1/2}$ (min^{-1})
Lys-Lys	**17**	0.21	3.3
D-Lys-Lys	**17a**	0.24	3.1
Lys-D-Lys	**17b**	0.29	2.5
D-Lys-D-Lys	**17c**	0.3	2.4
D-Lys-Leu	**17d**	0.13	5.9
Leu-Lys	**17e**	0.09	7.7
Leu-Leu	**17f**	0.004	161
Val-Leu	**17g**	0.005	141

Previously, we have shown that peptide thioester CKYG-GFL-X (X= $SCH_2CH_2CONH_2$), in which the N-terminal Ala of **22** is eliminated to explore Cys at the N-termini, cyclizes as an end-to-end lactam in <10 min, and the thiolactone intermediate cannot be observed.[9] However, the presence of an additional noncysteinyl amino acid at the amino terminus, such as those found in **22**−**24**, significantly retards the S,N-acyl isomerization because of the cis bond requirement at the X−Cys peptide bond (X = N-terminal amino acid). Other evidence supporting the stability of non-N^{α}-amino thiolactones will be discussed later (see Figure 7). Experimental results showing that **22a**−**24b** were stable thiolactone intermediates included the change of retention time in RP-HPLC, the correct molecular weight in MALDI-MS, and their susceptibility to hydroxylamine at pH 9. The corresponding hydroxylamine derivatives were isolated and further confirmed by MALDI-MS.

2. Sequence-Dependent Thiolactone Exchanges as the Rate-Determining Step. The second and intermediate steps in the proposed thia zip reaction are thiolactone exchanges. To show that the exchange is a sequence-dependent, rate-limiting step leading to the formation of the N^{α}-amino thiolactone, we studied the sequence correlation and cyclization rates in a series of protegrin (PG) analogues. PGs are double β-strand antiparallel antimicrobial peptides and share striking sequence homology with α-DF.[28] Eight PG analogues, **17a**−**g** (Table 1), with two amino acid substitutions at positions 3 and 4 centered between Cys 1 and Cys 6, were designed to alter their conformation in the thiolactone exchange to arrive at the N^{α}-amino thiolactone. However, the initial irreversible thiolactone formation is not affected in these analogues because the sequences between Cys 6 and the C^{α}-thioester remain unchanged. Substitution with D-amino acids **17a**−**d** promotes disorder and flexibility that can assist thiolactone exchange. In contrast, substitution with two hydrophobic amino acids in **17f** and **17g** gives a stretch of six consecutive hydrophobic amino acids and may favor formation of ordered structures that would slow the thiolactone exchange reaction. Cyclization followed the first-order rate equation, and the rate constants K_1 ranged from 0.30 to 0.004 min^{-1}, a >80-fold differences. Peptides **17**−**17e** with hydrophilic or D-amino acid residues cyclized with $t_{1/2}$ within 7.7 min while peptides **17f** and **17g** with hydrophobic amino acid residues proceeded slowly with $t_{1/2}$ > 2.4 h. These results confirm the conformation-dependent nature of the zip-assisted cyclization, since faster rates

Table 2. Unassisted and Zip-Assisted Cyclization of CT and DF under Nondenaturing (Aqueous) and Denaturing (8 M Urea) Conditions

peptide		internal free thiol groups	ring atoms: thiolactone intermediate	ring atoms: lactam product	$t_{1/2}$ for cyclization (h): aqueous	$t_{1/2}$ for cyclization (h): 8 M urea
CT	**20**	5	12−27	93 (**20c**)	0.3	0.6
CT-2Acm	**20a**	3	18−40	93 (**20d**)	0.9	2.6
CT-5Acm	**20b**	0	94	93 (**20e**)	2.0	120.0
DF	**21**	3	21−36	99 (**21c**)	0.3	2.6
DF-2Acm	**21a**	1	51−55	99 (**21d**)	n.d.[a]	3.4
DF-3Acm	**21b**	0	100	99 (**21e**)	2.7	186.0

[a] n.d. = not determined.

were observed in flexible PG peptides and slower rates in rigid, and ordered analogues.

3. Correlation of Rates with Ring Sizes of Thiolactone Intermediates. To show the involvement of thiols in the thia zip reaction, linear precursors CT **20**, DF **21** and their respective analogues with various numbers of intervening internal thiols (Figures 4 and 5) were used for rate studies in end-to-end cyclizations containing 93 and 99 ring atoms, respectively. Linear CT **20** contains five internal free thiols that can undergo thiol−thiolactone exchanges to provide small thiolactone intermediates ranging from 12 to 27 ring atoms. Similarly, linear DF **21** contains three internal thiols that can form thiolactones of 21−35 ring atoms. Linear CT-2Acm **20a** and DF-2Acm **21a**, both containing two Acm groups but three and one internal free thiols in their respective sequences, would allow exchange reactions to occur with the thiolactone intermediates of 15−55 ring atoms. Finally, linear CT-5Acm **20b** and DF-3Acm **21b** with all their intervening thiols blocked permit only direct N-to-C-terminal thiolactone formation to yield the 94- or 100-member ring of N^{α}-amino thiolactone in one step.

Cyclizations of linear CT **20**−**20b** and DF **21**−**21b** were performed at pH 7.5 under two conditions: (1) physiological condition in 0.2 M phosphate buffer alone and (2) denaturing condition in 8 M urea solution. Under the physiological condition, the zip-assisted cyclizations of linear CT **20** and DF **21** were exceedingly rapid with $t_{1/2}$ of 0.3 h^{-1} (Table 2). The corresponding unassisted cyclizations of CT-5Acm **20b** and DF-3Acm **21b**, both without any internal thiols, were 7 and 10 times slower. However, the half-lives of 2.0 and 2.7 h^{-1} are still fast and useful for synthesis, considering their respective 93 and 99 atom ring sizes. Because linear CT **20**, DF **21**, and their analogues have circularly permutated arrangements that place their N and C termini at close proximity, their cyclizations should be favorable. However, even with the aid of such favorable conformations, the thiolactone formation and exchanges undergone by small thiolactone intermediates significantly enhance the cyclization process.

To limit conformational assistance, linear CT **20**, DF **21,** and their analogues were cyclized under denaturing conditions. As shown in Table 2, zip-assisted cyclization of linear CT **20** and CT-2 Acm **20a** required 0.6 and 2.6 h for a 50% conversion to the corresponding lactams **20c** and **20d**, respectively. In contrast, the unassisted cyclization of CT-5 Acm **20b** was 200 times slower than CT **20,** requiring 120 h. Similarly, while zip-assisted cyclization of DF **21** required 2.6 h for a 50% conversion, the corresponding cyclization of DF-3 Acm **21b** required 186 h under strongly denaturing conditions.

4. S,N-Acyl Isomerization of the N^{α}-Amino Thiolactone. The final step in the proposed reaction is the irreversible and fast S,N-acyl isomerization to form the lactam. In previous studies,[9,27] the S,N-acyl isomerization of the N^{α}-amino thiolactone via a five-member transition state at pH > 7 was too fast to be observed in either segment ligation or intramolecular cyclization Thiolactone intermediates have been isolated and characterized in a strained ring system of cyclic pentapeptides at pH 3.[9] However, attempts to trap the thiolactone intermediate of the decapeptide **18** at pH 3 were unsuccessful. We then turned our attention to substantiate the requirement of a thiol at the N^{α}-Cys to form the amino thiolactone that leads to sequence-independent, five-member ring S,N-acyl isomerization.

PG analogue peptides **17**−**19** containing two Cys were again used as models (Figure 7). The N^{α}-Cys thiol of these analogues was blocked in different ways to prevent formation of an N^{α}-Cys amino thiolactone. When the N^{α}-Cys thiol of **19** was blocked by the Acm group to yield **19a**, or when **17** was converted at pH 2 by DMSO to its disulfide form **17b** under acidic conditions, no cyclization was observed in either peptide **19a** or **17b** in 20 h at pH 7.5. The requirement of the N^{α}-Cys thiol in N^{α}-amino thiolactone formation and the subsequent isomerization step were thus confirmed. In the control experiment, however, blocking the internal Cys 6 thiol in PG **18** with the Acm group did not significantly inhibit cyclization, which was completed in 1 h to afford **18a**. Although the analogue **17b** lacked the N^{α}-Cys thiol, the N^{α}-amine was held in relatively close proximity to the thioester as a 22-member ring through the Cys 1−6 disulfide bond. Similarly, **19** rapidly converted to its thiolactone **19a** at pH 7.5, but no lactamization of **19a** through a 20-member thiolactone was observed after 12 h. As shown previously, N^{α}-amino thiolactones **22a** and **23a** that could have isomerized through an eight-member ring to form lactams were found to be stable at pH 7.5. Taken together, these experiments show the general lack of reactivity of thioesters or thiolactones to amines at pH 7.5 under aqueous conditions and also that sequence-independent S,N-acyl isomerization in the zip reaction occurs via an entropically favored five-member ring transition state. Furthermore, our results eliminate the alternative pathways of the zip reaction, which involves a lactamization via the non-N^{α}-amino thiolactone or a direct N-to-C lactamization via N^{α}-thioester.

5. Disulfide Formation to Complete the Synthesis of CT and cDF and Their Characterizations. To complete the synthesis of CT **13** and cDF **14** after zip-assisted cyclization, two different routes were used for their disulfide formation. Since cDF **14** contains only two disulfide pairs it was obtained essentially in a same-pot reaction by direct oxidation of the four thiols of cDF **20c** in 10% DMSO[30] after the zip-assisted cyclization. A major disulfide isomer was obtained in 87% yield. The overall yield was 38% based on the crude product after HF cleavage followed by HPLC. Disulfide connectivity was confirmed by comparing this purified product with the cDF **20d** obtained through the unambiguously selective sulfur−sulfur bonds formed between two disulfide pairs by differentially protected DF-2Acm **20a**. This bond formation took place under sequential oxidation by 10% DMSO and I_2 in MeOH to afford cDF **14**. Synthetic cDF **14** retained antimicrobial activity similar to the native defensin in assays involving three different microbes. However, it also exhibited an antimicrobial profile that was distinct from the native form. For example, under physiological conditions containing a high salt concentration (100 mM NaCl) α-defensin NP-1 was inactive against *Escherichia coli* while cDF **14** was found to be active, which bodes well for its therapeutic usefulness.

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Table 3. Antimicrobial Activities (MIC) of NP-1 **15**, Circularized NP-1 **14,** and Synthetic CT **13**[a]

		defensins		circulin
strain		NP-1 **15**	cDF **14**	CT **13**
E. coli	H-salt	>500	16.1	>500
	L-salt	1.5	1.6	1.5
C. albicans	H-salt	>500	>500	>500
	L-salt	5.4	19.0	>500
P. acruginosa	H-salt	5.9	6.0	50.2
	L-salt	2.6	4.8	13.5

[a] Antimicrobial activity was performed in radial diffusion assay underlaid with 1% agarose and 10 mM NaP buffer with (H-salt) or without (L-salt) 100 mM NaCl. MIC stands for minimum inhibitory concentration. Structures are shown in Figure 4.

Linear **20** CT contains six cysteinyl thiols, and therefore a direct same-pot oxidation of the cyclized CT **20c** by DMSO after the zip reaction can result in the formation of all possible 15 disulfide isomers. In HPLC there were eight peaks representing three three-disulfide and five two-disulfide isomers while the desired disulfide isomer **13** was obtained in only 2% yield. The difficulty in forming the correct disulfide bonds in CT **20c** can be attributed to its unique cystine knot motif[22] in which all three disulfides are clustered together in close proximity with one disulfide threading through the other two. That these disulfide bonds are configured in a closed end-to-end structure may also exacerbate the situation. Thus, to limit the number of disulfides, a two-step disulfide formation[31] using the cyclized CT-2Acm **20d** was chosen to improve the yield of the target compound. Such a strategy has been used successfully for confirming the disulfide pairings of both circulin B and CT.[11] Treatment of the cyclic CT-2Acm **20d** containing four cysteinyl thiols in 10% DMSO could yield three possible disulfide isomers. In fact, all three were obtained in this first step in about equal molar ratio.

The disulfide connectivity of each isomer after separation by HPLC was determined by partial acid hydrolysis in 0.25 M oxalic acid[11a] (Supporting Information). Because these cyclic peptides are highly constrained with end-to-end cyclic structures and two disulfide bonds, identification of their disulfide connectivities requires multiple cleavage sites. The use of the conventional approach by enzymatic digestions, therefore, was unsuccessful. In contrast, acid-catalyzed fragmentation of small amino acids such as Gly, Ser, Asn, Asp, and Glu was more convenient in yielding useful discrete disulfide-linked segments which could be unambiguously identified by mass spectrometric analysis after HPLC separation. Although the desired isomer was found in about 32% yield, the yield could be improved by recycling the misformed disulfide. In the second step, the Acm group was reduced, and the disulfide was formed using I_2 at pH 4 to yield CT. The overall yield starting from the thia zip cyclization and without the recyclizing process was 15%. Synthetic CT coeluted with the native CT in HPLC while the two other misformed disulfide isomers had different retention times.[18b] Furthermore, synthetic CT exhibited antimicrobial activity (Table 3) similar to the analogues plant defensin such as circulins.

Discussion

Without conformational assistance, cyclization of very large peptides is generally expected to be slow because of the vast number of atoms between the two ends. Our results support this conclusion. Indeed, under denaturing conditions to limit conformational assistance, end-to-end cyclizations of CT (31 amino acids) and DF (33 amino acids) through a one-step N- to C-thiolactone formation and then a spontaneous ring contraction via an S,N-acyl migration were exceedingly slow. The rate-limiting step is the N^{α}-amino thiolactone formation. The half-lives for forming these two 94- and 100-member N^{α}-amino thiolactones were more than 120 h. In contrast, under the same conditions, cyclization of these peptides with two or more free internal thiols by the zip-assisted mechanism with discrete small thiolactone intermediates of 12−35 ring atoms proceeds >100 times faster. However, even the help of one internal thiol can confer a beneficial effect on N^{α}-amino thiolactone formation. For example, **21a** containing one internal free thiol, cyclized about 60-fold faster than the corresponding DF-3Acm **21b**, which lacks internal thiol. These results therefore suggest that the presence of internal thiol facilitates the thiol−thiolactone exchange and assists ring enlargement in the thia zip reaction.

The efficiency of the thia zip reaction stems from two types of reactions: the reversible S-acylation through intramolecular transthioesterifications and the irreversible S,N-acyl migration of the amino thiolactone. Similar to the sulfur−sulfur bond exchange reactions in disulfide bond formation, the reversible S-acylations are aided by ring−chain tautomerism in limiting competing oligomerization. Ring−chain tautomerism is made possible by the weakly activated C^{α}-thioester that exchanges with a free thiol to form a thiolactone. However, in our study, the initial step of thiolactone formation shown in model peptides **18**−**19** and **22**−**24** is strongly favored over the open-chain thioester and appears to be irreversible. Since the final step of the S,N-acyl migration is essentially spontaneous, the rate-determining step is the intramolecular exchanges of free thiols with the thiolactone. The resulting cyclic intermediates thus arrive at the N^{α}-amino thiolactone, which terminates the equilibrium through the spontaneous S,N-acyl transfer at pH 7.5. The slow step of thiolactone exchanges **10**→**11** as proposed in Figure 1b is supported by studying a series of model peptides **17a**−**g**. The thia zip reaction also provides an explanation for the slow kinetics often observed in segment ligation of large peptides containing multiple cysteines.[27] Capture of the thioester by an internal cysteine of an N-terminal peptide segment with multiple internal cysteines would have to undergo the proposed thia zip reaction to the N-terminal cysteine to drive the reaction to completion.

Intermolecular S-acylations through transthioesterification are facile and reversible, but generally have limited synthetic utility. However, when the S-acylations are coupled with the S,N-acyl transfer reactions, the combinations provide a new and exciting chemistry for the formation of a peptide bond between two unprotected peptide segments[27] and cyclization of unprotected peptides with high efficiency and regiospecificity. The high regiospecificity is largely due to the thiols, which are stronger nucleophiles than amines for the S-acylation reaction at nearly neutral pH. Thus, there are no significant side reactions in the peptides containing two side-chain functionalities such as the amine of lysine or guanidine of arginine. The S-acyl intermediate at the α-amino terminus promptly undergoes a proximity-driven S to N acyl transfer to complete the intramolecular orthogonal formation of a stable peptide bond in an unprotected peptide.

Although this work has established the requirements of an N^{α}-Cys at the N-terminus, a thioester and one or more internal thiols for the thia zip reaction, which leads to fast and irreversible thiolactone formation with thiol-thiolactone exchanges as the rate-determining steps and N,S-acyl isomerization as the spontaneous final step, we have not been able to establish

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the exact pathways of the thiol−thiolactone exchanges. We do not know whether the thia zip reaction proceeds through the discrete steps involving all thiols, as shown in Figure 1, or other pathways involving only a few thiols. Product profile analyses of CT **20** and cDF **21** show formation of multiple thiolactone intermediates that coalesce over time to the end-to-end cyclic peptides. Similar to thiol−thiol exchanges in the disulfide function of proteins, it is likely to be highly sequence dependent as well as dependent on the specific experimental conditions. Thus, the exact pathway for the thia zip reaction probably varies from one peptide or protein to another. However, we do know that under strongly denaturing conditions that remove the effects of conformation, internal thiols clearly play a role in increasing the rate cyclization resulting from the slow step of thiol−thiolactone exchanges.

In contrast to the known aza zip reactions that are performed in highly basic and nonaqueous conditions,[5,6] our results show that the thia zip reaction for macrocyclization of cysteinyl-rich peptides can be achieved with mildly buffered aqueous solutions. Furthermore, with the use of unprotected peptide segments, this method provides a facile approach for the synthesis of large cyclics without deprotection or activation steps.[11] We have shown that this strategy can be used successfully for the synthesis of cyclopsychotride **13**, a naturally occurring cyclic peptide which we show in this study to be an antimicrobial peptide. Thus far, cyclic proteins have not been found in nature. However, many proteins have circular permutated arrangements imposed by structural architectures such as the α,β-barrel design found in triose phosphate isomerase or by the end-to-end disulfide bridge in pancreatic trypsin inhibitor. In a step toward the synthesis of cyclic proteins, we have engineered the synthesis of an animal α-defensin by replacing the end-to-end disulfide bridge with lactam. Furthermore, we have shown that this type of replacement strategy retains the antimicrobial activities of the open-chain native animal α-defensin. A similar strategy has also been applied successfully to engineer the synthesis of the disulfides which belong to the family of β-sheet antimicrobial peptides including protegrin and tachyplesins.[28]

In recent years, orthogonal synthetic schemes have been developed to simplify the synthesis of peptides,[7−12,27,32,34] dendrimers,[33a,b] and carbohydrates[35] by eliminating the use of protecting groups. However, the orthogonal ligation concept[33−36] has coevolved independently with the newly discovered biosynthetic process of intein splicing,[33−37] in which two peptide segments are spliced through a catalytic intein and ligated in the final step by an uncatalyzed S,N-acyl migration. Thia zip cyclization is based on orthogonal ligation principles that in turn depend on entropic activation through proximity-driven intramolecular transfer reactions. Thus, the thia zip reaction provides a useful demonstration of the principle of entropic activation through proximity rearrangements to form large end-to-end cyclic peptides.

Experimental Section

Peptide Synthesis and General Cyclization Procedure after HF Cleavage. 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 which was prepared according to a modified procedure described by Hojo and Aimoto.[24] N^{α}-amino groups of all amino acids were protected with Boc group. The side-chain protections were as follows: Arg (Tos), Asn (Xan), Cys (4-MeBzl), Cys (Acm), Glu (OBzl), His (Dnp), Lys (ClZ), Ser (Bzl), Thr (Bzl), and Tyr (BrBzl). In manual synthesis, one cycle of the synthesis using 15−20 mL of solvent per gram of resin consisted of the following two operations: (i) a 20-min deprotection with 50% trifluoroacetic acid/CH_2Cl_2, and (ii) coupling with 4 equiv each of Boc−amino acid and BOP in the presence of 12 equiv of DIEA in DMF for 45−60 min. Coupling reactions were monitored by the Kaiser ninhydrin test 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 the HF was removed, the resulting peptides were washed with diethyl ether to remove the organic scavengers. For circulin and defensin analogues, **13**−**15**, **20**, and **21**, the peptide was extracted with 8 M urea, pH 7.5, containing 100 mg of TCEP to prevent intermolecular disulfide formation. The peptide solution was dialyzed (MW cut off 1000 from Spectrum Medical Industries, Inc.) to allow the dissociation of small organic molecules and aromatic scavengers. Gradually lowering of the urea concentration to 2 M allowed cyclization, which was monitored by analytical C_{18} reversed-phase HPLC and MS. After completion of cyclization, the dialyzed solution was then diluted to 1 M urea with aqueous buffer for the first step in disulfide oxidation by DMSO. Analytical HPLC was run on a Shimadzu system with a C_{18} Vydac column (250 × 4.6 mm) at 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 MALDI 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. For protegrin analogues, **17**−**19** and **22**−**24**, peptides were extracted to 50% acetic acid after cleavage and diluted solution (<5% acetic acid) was lyophilized.

General Procedure for Two-Step Oxidation To Form Disulfide Bond. a. DMSO Oxidation. Disulfide formation of the free sulfhydryls was achieved by adding 15% DMSO to the peptide urea solution (1 M) and was generally complete in 10 h as monitored by HPLC and MS. In the case of CT **20c** all three isomers were obtained. In CT **20d**, DF **21c,** or **21d**, DMSO oxidation yielded only one disulfide isomer. The isomeric disulfide peptides were purified on a preparative C_{18} 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.

b. Concomitant Removal of the *S*-Acetamidomethyl Group and Formation of the Second or Third Disulfide Bond. 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 quenched by ascorbic acid. The peptide was purified on preparative HPLC in the same manner as described above.

Thiolactone Formation. The purified peptide thioester **22** (0.1 mM, MW 1003.7, R_t = 20.7 min) was dissolved in a buffer (150 μL, 50% CH_3CN/H_2O pH 7.5 buffer = 1:2, v/v) containing 3 equiv of TCEP. After 30 min, a new peak was observed. Peptide isolated from HPLC was analyzed by MS (**22a** MW calcd 840.1, found 840.9; R_t = 21.9 min) and was treated with hydroxylamine to form a hydroxylamine

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peptide (MW calcd 872.1, found 872.5; R_t = 19.2 min). Similar treatments of **23** (MW 960.5; R_t = 21.2 min) and **24** (MW 1497.7; R_t = 27.4 min) yielded thiolactone **23a** (MW calcd 855.5; found, 855.7; R_t = 21.6 min), **24a**, and **24b** (calcd 1393.7, found 1393.5; R_t = 26.3 min; and calcd 1393.7, found 1394.2; R_t = 29.0 min), respectively.

Thia Zip Cyclization of CT 20, 20a, and 20b and DF 21, 21a, and 21b under Physiological Conditions. In physiological condition, the peptide thioester (10 μM) was dissolved in 0.2 M phosphate/0.1 M citric acid buffer (pH 7.6) containing 3 equiv of TCEP. The reaction was monitored by HPLC until completion. In denaturing condition, the peptide thioesters were dissolved in 8 M urea containing 0.1 M Tris-HCl at pH 7.6, 10 equiv each of TCEP and 3-mercaptopropionic acid were added to prevent undesirable byproduct formation. The 8 M urea solution was sequentially dialyzed in a descending solution of 8 to 2 M. The thiol−thioester exchange and the cyclization reactions were monitored by HPLC at frequent intervals by using small aliquots of the reaction. The identity of the peaks appeared in the HPLC profiles were confirmed by MALDI-MS and treatment with hydroxylamine.

Kinetic Study. Kinetic analysis of **17** and **17a**−**g** was accomplished by RP-HPLC to determine the reaction progress. The title compounds were cyclized in a physiological condition of pH 7.6 in aqueous buffer. In addition, CT **20** and **20a**,**b** and DF **21** and **21a**,**b** were also performed in a denaturing 8 M urea condition. Aliquots (10 μL) were withdrawn at various time points, and CF_3CO_2H (5 μL of 10% solution) was added to quench the reaction. The progress of the cyclization was analyzed immediately by RP-HPLC. First-order plots [not shown] of ln [C]/[C_0] versus time (where [C] is the concentration of the linear thioester precursor and [C_0] is the amount of the linear thioester precursor at time 0) were used to calculate the rate and half-life of the cyclization reaction.

Synthesis of Cyclopsychotride 13. The purified peptide thioester containing the assembled protected peptide with $Cys^{3,6}$ (Acm) **20a** (MW calcd 3485.7, found 3485.7; R_t = 22.0 min) was obtained after cleavage from the resin support by HF (HF:anisole = 9:1, v/v) and purified on preparative HPLC as described in the general procedure. The crude peptide thioester was generally required strong denaturant for solubility during purification. For the one-pot cyclization, the crude peptide (50−80 mg) was extracted into 8 M urea solution (150 mL), at pH 7.6 in a highly reductive environment containing 5−10-fold excess of TCEP and then dialyzed against 8 M urea (2000 mL) to allow cyclization in a descending concentration of 8 to 2 M urea. The whole dialysis process with concomitant thia zip cyclization was completed within 20 h. A single major peak of cyclic peptide with four reduced thiols and $Cys^{3,6}$ (Acm) **20d** was observed in RP-HPLC profile (30−85% B/30 min) (MW calcd 3379.1, found 3380.0; R_t = 23.8 min). Disulfide formation was achieved by adding 15% DMSO to the peptide solution and was complete in 10 h, yielding three disulfide isomers of **20d** (MW calcd 3376.0, found 3376.0; R_t = 19.1, 21.3, 28.3 min) with the correctly folded isomer obtained in 32% 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 3,6. The peptide **13** was purified by preparative RP-HPLC and characterized by MS, giving the expected molecular weight (MW calcd 3230.9, found 3232.2) with an overall yield of 5% based on HPLC of the crude peptide. The synthetic material was identical to the naturally isolated CT both chemically and biochemically. Furthermore, synthetic and naturally isolated CT eluted as a single peak under several RP-HPLC conditions.[18b]

Synthesis of Cyclic Defensin 14. With Boc-Leu-thiopropionyl-MBHA resin **7a** as the starting material, the peptide thioester was assembled using a standard DCC/HOBt coupling protocol in a ABI 430A synthesizer. After chain elongation, the resin was treated with thiophenol/DMF (1:9 v/v) solution to remove Dnp protecting group from His. The resin was treated with high HF procedure as described in the general procedure and after purification yielded a purified peptide thioester (MW calcd 4034.9, found 4034.0). For the one-pot cyclization, the crude peptide thioester was extracted into 150 mL of 8 M urea after HF. To the urea solution was added 10 equiv of TCEP and 50 equiv of thiopropionic acid. The solution was dialyzed in 2000 mL of a descending gradient of 8 M to 2 M urea/0.1 M tris at pH 7.5 in 24 h to complete the thia zip cyclization. In the synthesis of cDF**14** through the two-step oxidation method, **21d** (MW calcd 3929.8, found 3929.0) was treated with DMSO (10% in volume). The oxidation at pH 7.0 was monitored by HPLC and was completed after 24 h to give cyclic peptide with Cys 1,3 disulfide and Cys 2,4 (Acm) (MW calcd 3927.8, found 3927.0). The solution was then adjusted to pH 3.0 by HOAc and treated with I_2/MeOH under nitrogen to form the second pair of disulfide bond. The total yield of cyclodefensin **14** (MW calcd 3783.6, found 3784.5) was 34.2% based on the HPLC area.

Characterization. Peptides were hydrolyzed with 6 N HCl at 110 °C for 24 h and amino acid analysis was performed to confirm the composition of the desired peptides. Analytical HPLC for all peptides was performed on a Vydac column (250 × 4.6 mm) with a linear gradient of 0−85% buffer B at a flow rate of 1 mL/min (buffer A, 0.045% TFA in 5% CH_3CN in H_2O; buffer B, 0.04% TFA in 60% CH_3CN in H_2O). All synthetic peptides were characterized by MALDI-MS.

Partial Acid Hydrolysis.[11a] Each of the peptide disulfide isomers CT **20d** with $Cys^{3,6}$ (Acm) (120 μg) was hydrolyzed in 0.25M oxalic acid (200 μL) at 100 °C for 5 h. The hydrolysate was fractionated by RP-HPLC with a linear gradient from 0 to 90% buffer B over 60 min using a 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. In general, many one-disulfide fragments for each isomer were obtained to facilitate the determination of disulfide connectivity. Data are shown in Supporting Information. For example in a typical run for the 1,4- and 2,5-disulfide isomer, 11 one-disulfide fragment such as VFIPC(Acm)TVTALLGC-(CGES) (MW calcd 1801, found 1801) and ALLGCS-(SIPC) (MW calcd 892, found 892) could be assigned to 1−4 disulfide linkage and nine another one-disulfide fragments to 2−5 linkage such as SC-(SCK)-VFIPC(Acm)TVTA (MW calcd 1475.6, found 1475.1). Similar results were obtained for the other disulfide isomers to persist the determination of their disulfide isomer as 1,2-disulfide linkage, CS(CKS) (MW calcd 524.6, found 523.3) and 4,5 KNSIPC(CVFI)GE (MW calcd 1325.5, found 1326.6) or as 1,5-disulfide linkage GC(CVFIPC(Acm)TVT) (MW calcd 1229.5, found 1230.5) and 2,4 C(IPC)KS (MW calcd 665.8, found 667.3).

Antimicrobial Assay. *E. coli* ML35, *Candida albicans*, and *Pseudomonas aeruginosa* were utilized in a radial diffusion assay described by Lehrer et al.[38] Bacteria were grown to mid-log phase in trypticase soy broth (TSB), diluted into 10 mL of warm (43 °C) 1% agarose containing 3 mg of TSB, buffered with 10 mM sodium phosphate, pH 7.4. Five milliliters of each peptide solution was pipeted into wells formed with a 4-mm cork borer and allowed to incubate at 37 °C for 3−4 h. Plates were then overlaid with 10 mL of sterile 1% agar containing 2 × TSB. Following incubation for 18−24 h, the diameter of the clear zone surrounding each well was measured using a magnified transilluminator.

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Supporting Information Available: Assignment of disulfide-containing peptides found in partial acid hydolysis of isomers [Cys 3,6-Acm]cyclopsychotride (CT). This material is available free of charge via the Internet at http://pubs.acs.org.

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