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Optimal Oxidative Folding of the Novel Antimicrobial Cyclotide from *Hedyotis biflora* Requires High Alcohol Concentrations

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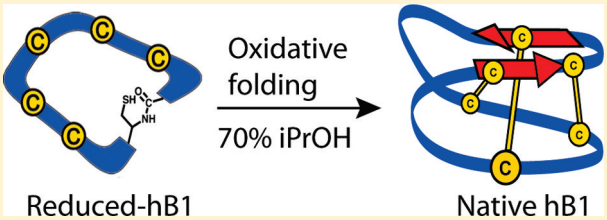
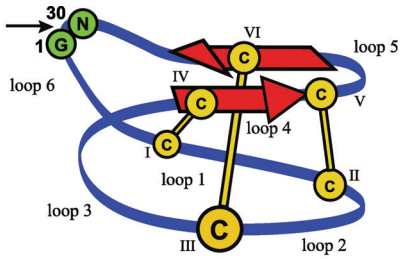
ABSTRACT: Hedyotide B1, a novel cyclotide isolated from the medicinal plant *Hedyotis biflora*, contains a cystine knot commonly found in toxins and plant defense peptides. The optimal oxidative folding of a cystine knot encased in the circular peptide backbone of a cyclotide poses a challenge. Here we report a systematic study of optimization of the oxidative folding of hedyotide B1, a 30-amino acid cyclic peptide with a net charge of +3. The linear precursor of hedyotide B1, synthesized as a thioester by solid phase synthesis, was cyclized quantitatively by a thia-zip cyclization to form the circular backbone and then subjected to oxidative folding in a thiol−disulfide redox system under 38 different conditions. Of the oxidative conditions examined, the nature of the organic cosolvent appeared to be critical, with the use of 70% 2-propanol affording the highest yield (48%). The disulfide connectivity of the folded hedyotide was identical to that of the native form as determined by partial acid hydrolysis. The use of such a high alcohol concentration suggests that a partial denaturation may be necessary for the oxidative folding of a cyclotide with the inverse orientation of hydrophobic side chains that are externalized to the solvent face to permit the formation of the interior cystine core in the circularized backbone. We also show that synthetic hedyotide B1 is an antimicrobial, exhibiting minimal inhibitory concentrations in the micromolar range against both Gram-positive and -negative bacteria.



The cyclotides make up a family of plant-derived cysteine-rich peptides (CRPs) with a circularized peptide backbone in which their N- and C-termini are joined by an amide bond. They consist of 28−37 amino acid residues and are organized in a cystine-knot scaffold (Figure 1).[1] Cystine knot, which has the characteristic Cys I−IV, II−V, III−VI disulfide connectivity, is commonly found in CRPs such as animal toxins and plant defense peptide families, including plant defensins, heveins, and knottins.[2−4] The combination of a backbone-cyclized and disulfide-knotted structure confers upon cyclotides exceptionally high stability against chemical or enzymatic degradation. These properties have made cyclotides an attractive scaffold for engineering of therapeutic peptides,[5,6] and a target for developing methods for their efficient synthesis and oxidative folding.



Cyclotides	Sequences	Net charge
Cys loop	6 I 1 II 2 III 3 IV 4 V 5 VI 6	
Hedyotide B1	G-TR-CGETCFV-LPC-WSAKFGCYCQKG-FCYRN	+3
Circulin A	G-IP-CGESC-VWIPC-ISAALGCSC-KNKVCYRN	+2
Circulin B	GVIP-CGESC-VWIPC-ISTLLGCSC-KNKVCYRN	+2
Cyclopsychotride	S-IP-CGESC-VFIPCTVTALLGCSC-KSKVCYKN	+2
Cycloviolacin O2	G-IP-CGESC-VWIPC-ISSAIGCSC-KSKVCYRN	+2
Kalata B1	G-LPVCGETC-VGGTC-NT--PGCTC-SWPVCTRN	0

Figure 1. Schematic structure and sequence comparison of various cyclotides. Amino acids between cysteines (Cys I−VI) form loops 1−6 and three disulfide bonds in a cystine-knot arrangement (Cys I−IV, II−V, III−VI).

The first cyclotide kalata B1 was discovered by Gran in the 1960s when he noticed that the African women in Congo drank a decoction from the plant *Oldenlandia affinis* to induce labor and to facilitate childbirth.[7] In the 1970s, he reported that one of the active ingredients was kalata B1.[8] For the next 20 years, only four cyclotides were discovered, largely attributed to institutional drug screening programs. In the past decade, the number of discoveries has greatly increased, and currently, ~150 cyclotide sequences have been identified. Cyclotides are most abundantly found in the families Rubiaceae and Violaceae.[9] They display a wide range of bioactivities that include membrane-active functions associated with either inhibitory or killing activity against cancer cell lines, HIV, bacteria, and fungi as well as insect growth.[10−15] In addition,

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cyclotides also exhibit hormonal functions that include neurotensin and uterotonic activity.[16,17]

Cyclotides are gene-encoded products, and their mature precursors undergo two key bioprocessing steps: disulfide formation of their linear precursor and backbone cyclization by a specific enzyme. Recent works suggest that protein disulfide isomerase (PDI) is likely involved in the disulfide formation and an asparaginyl endoproteinase, a thiol proteinase, in backbone cyclization.[18,19] However, in the chemical synthesis of a cyclotide, the sequence of these two steps is invariably reversed because of synthetic expedience. Because oxidative folding after backbone cyclization in a chemical synthesis is counter to the natural biosynthetic process, the optimal oxidative folding of a cystine knot restricted by the conformational flexibility of the cyclotide circular backbone has posed a synthetic challenge.

Cyclotide sequences are defined by six backbone loops [loops 1−6 (Figure 1)] among the six conserved Cys residues that form the cystine knot. On the basis of structure and sequence similarity, cyclotides can be divided into two major subfamilies, Möbius and bracelet.[20] Möbius cyclotides differ from the bracelet cyclotides in loop 5 by the presence of a *cis*-Pro bond that induces a twist in their circular backbone. In general, amino acid sequences of loops 1 and 4 in all cyclotides are highly conserved but are moderately conserved in the other four loops within the Möbius and bracelet subfamilies. Irrespective of their subfamilies, determinations by nuclear magnetic resonance and X-ray crystallography have shown that cyclotides share a common structural feature with the interior occupied by a cystine knot that displaces the bulky hydrophobic side chains to the exterior solvent face.[21] This is in contrast of the structural folds of globular proteins with bulky hydrophobic side chains shielded from the solvent face. The "inverse orientation of the hydrophobic side chain" of the cyclotide side chains contributes to the hydrophobic character of cyclotides as shown by their late retention in reverse phase high-performance liquid chromatography (RP-HPLC) and may account for their difficulty in oxidative folding.

Previously, we have synthesized both Möbius and bracelet cyclotides by the thia-zip cyclization and regioselective disulfide formation to confirm the disulfide-knotted arrangement.[12,22,23] Backbone cyclization of an end-to-end macrocyclic peptide such as cyclotide is energetically disfavored because of the great distance (more than 90 atoms apart for the 30-residue hedyotide B1) between the C- and N-termini during cyclization. Conventional methods such as on-resin or solution cyclization are relatively inefficient.[24,25] The thia-zip cyclization is the current method of choice. It is based on the intramolecuar ligation of unprotected linear peptide precursors containing an N-terminal cysteine and a C-terminal thioester leading to the irreversible formation of a circular amide backbone between the two termini with high regiospecificity.[26] This method has also been applied to the synthesis of cyclic protein as well as peptide engineering.[27,28]

Although the problem in backbone cyclization of cyclotides has been overcome by the thia-zip cyclization, a direct oxidative folding to form the native cystine-knot cyclotides remains empirical and poorly understood. Compared with the number of cyclotides being discovered, only a few cyclotides have been synthesized, including kalata B1, circulin A and B, cyclopsychotride,[12,22] cycloviolacin O1 and O2,[29,30] and trypsin inhibitors MCoT-I and MCoT-II.[31,32] Recently, several laboratories have reported cyclotide syntheses and approaches for the direct oxidative folding for the formation of the disulfide-knotted arrangement under a range of conditions. The Möbius cyclotides can be folded successfully in moderate yields. In contrast, the direct oxidative folding of the bracelet cyclotides that account for nearly 70% of all cyclotides discovered today has been difficult to achieve.

Although cyclotides share a high degree of sequence and structure similarity within subfamilies and across different plant species, the Möbius cyclotides, notably kalata B1, can be folded by using 50% 2-propanol as a cosolvent in moderate to acceptable yields.[33] In contrast, the oxidative folding of bracelet cyclotides under oxidative folding conditions similar to those of kalata B1 has been difficult to achieve. Recently, Aboye et al.[30] reported the first successful oxidative folding of a bracelet cyclotide, cycloviolacin O2 (CVO2), directly from its reduced backbone cyclized precursor in 35% dimethyl sulfoxide (DMSO) with a cocktail consisting of detergent, EDTA, and redox thiols (GSH/cystamine). They also found that 50% 2-propanol, which was a useful condition in the folding of Möbius cyclotide kalata B1, was ineffective in the oxidative folding of the bracelet cyclotide CVO2.[27,28]

Recently, we have successfully isolated from *Hedyotis biflora* a panel of cyclotides (unpublished data). The prototypic cyclotide of this panel is hedyotide B1 (GTRCGETCFVLPCWSAKFGCYCQKGFCYRN), a bracelet cyclotide. Interestingly, hedyotide B1 is unique among cyclotides. It contains the largest number of aromatic (six) and cationic (four) amino acids. Cyclotides generally have a net charge ranging from −4 to +3, and hedyotide B1 with a net charge of +3 is one of the most basic cyclotides discovered so far, suggesting that it may be contain antimicrobial activity consistent with the known characteristics of other cationic antimicrobial peptides.[34]

Here, we describe a total synthesis and oxidative folding of hedyotide B1. We examined 38 oxidative folding conditions to determine key parameters that influence disulfide formation in the folding process of hedyotide B1. Our results show that a key parameter is the use of denaturing conditions using a high concentration of 2-propanol to facilitate the formation of the cystine-knot structure of hedyotide B1. In addition, we show that hedyotide B1 exhibits antibacterial activities against both Gram-positive and Gram-negative bacteria.

EXPERIMENTAL PROCEDURES

Solid Phase Peptide Synthesis of Reduced Hedyotide B1. Hedyotide B1 was synthesized by Boc chemistry. All amino acids and reagents were provided by Peptide Institute, Inc. (Osaka, Japan). Other chemical and solvents were obtained from Nacalai Tesque (Kyoto, Japan). Mercaptopropyl-Arg-Arg-Arg-PAM resin (0.393 mmol/g), a thiol handle-functionalized resin, was used as the solid support. To increase the solubility of the thioester handle after its cleavage from the resin support, three arginines were coupled to the PAM resin before the coupling of mercaptopropionic acid (MPA) that served as a linker handle for converting the C-terminal acid into a thioester. Because the peptide must contain an N-terminal cysteine and a C-terminal thioester to effect a thia-zip cyclization, we selected Gly as the C-terminal amino acid for which the C-terminal carbon of Gly is less sterically hindered than other amino acids, thus improving the cyclization yield. On the basis of this, we chose the ligation site between Cys(IV)

and its adjacent Gly as the N- and C-terminal amino acid residues, respectively. Sequential coupling was performed with an automated ABI 433A peptide synthesizer mediated by an in situ neutralization coupling protocol with HCTU and HOCt. The following side chain-protected amino acids were used; Cys(MeBzl), Ser(Bzl), Thr(Bzl), Arg(Tos), Lys(ClZ), Tyr-(BrZ), Asn(Xan), and Trp(Hoc). After assembly of the sequence on the solid support, the peptide thioester was cleaved by a high-HF protocol [8:2 (v/v) HF:*p*-cresol] for 60 min at −5 °C. HF was then removed by vacuum and washed with ether to remove the organic scavengers. The ether-precipitated peptide was purified by preparative RP-HPLC with a gradient from 20 to 40% and a flow rate of 20 mL/min in 80 min on a YMC-ODS (250 mm × 30 mm × 5 μm) preparative column, and the products were characterized by electrospray ionization mass spectroscopy.

Cyclization of the Peptide Thioester. After the HPLC purification, the peptide thioester was cyclized in aqueous 0.1 M NaH_2PO_4 buffer (pH 7.5) containing 6 M GnHCl with a 100-fold excess of thiophenol at room temperature for 3 h. The reaction was monitored by HPLC and matrix-assisted laser desorption ionization time of flight (MALDI-TOF). After the completion of the reaction, DTT (30 equiv) was added with stirring for 10 min at room temperature. HCl (2 M) was added to adjust the reaction solution to pH 2, followed by ether extraction to remove thiophenol. The reaction mixture was then purified by preparative RP-HPLC, and the desired fractions containing the putative cyclized and reduced hedyotide B1 were dried by lyophilization.

Oxidative Folding of Hedyotide B1. Various folding conditions were investigated using different buffers, times, and temperatures as well as concentrations of the redox reagent GSH/GSSG, cosolvents, and detergents (Table 1). For each condition, 0.1 mg of peptide was dissolved in a total volume of 3 mL of solvent to a final concentration of 9.79 μM (Table 1). At 24 h intervals, samples were acidified with 2 M HCl to terminate the folding process and the product profiles analyzed by analytical RP-HPLC with a 10 to 60% buffer B gradient over 30 min. Hedyotide B1 purified from the plant was used as a standard to monitor the folding reaction in RP-HPLC. The Shimadzu HPLC program was used to calculate the area of the peaks in the HPLC profile.

Partial Acid Hydrolysis and Liquid Chromatography−Tandem Mass Spectroscopy (LC−MS/MS) Analysis of Cyclotide Connectivity. The disulfide bond connectivity of both synthetic and native hedyotide B1 was separately determined for comparison by partial acid hydrolysis as reported by Sze et al. in 2009.[35] The synthetic hedyotide B1 after oxidative folding was purified by semipreparative RP-HPLC and lyophilized. A final concentration of 0.1 mM for the hedyotide B1 solution was reached by dissolving 1 mg of peptide into 1 mL of HPLC-grade water. Two microliters of the solution was then mixed with 100 μL of 1 M HCl in a glass vial and the mixture deoxygenated with nitrogen for 2 min, and the glass vial was sealed. The partial acid hydrolysis was performed at 100 °C in a heating block. At 15 min intervals, samples were analyzed by MALDI-TOF to determine the extent of hydrolyzed products. An optimal time was determined to be 45 min. For LC−MS/MS analysis. the partially acid-hydrolyzed sample was mixed with 0.1% formic acid in MeCN and injected with an autosampler. Chromatographoc separation was conducted with a RP-HPLC system directly into a pico-frit nanospray tip (New Objective). The gradient was set at 200 nL/min and ramped from 5 to 35% MeCN over 40 min, increased to 80% MeCN at 45 min, and held for 3 min. The eluate was ionized by online nanospray at 1800 V into the LTQ-FT-Ultra instrument (ThermoFinnigan, Bremen, Germany). The MS scan was performed in the ICR cell at 100000 resolution. The LTQ was operated in a data-dependent mode by performing MS/MS scans.

The extracted ion chromatogram was integrated over the MS scan with Qual Browser version 2.0 with a bandwidth of 0.05 Da of the precursor ion m/z. The relative abundances of the partial acid hydrolysis products were measured by chromatographic peak areas of the precursor. A home-written program (available for download at http://proteomics.sbs.ntu.sg/cyclotide_SS) was used for subsequent data analysis and

Table 1. Conditions and Yields of Oxidative Folding Experiments for Hedyotide B1

run[a]	temp (°C)	concn (μM)	cosolvent	additive	yield (%)
1	4	500	–	–	<5[b]
2	4	100	–	–	<5[b]
3	4	100	–	2 M GnHCl	<5[b]
4	4	100	–	4 M GnHCl	<5[b]
5	4	50	–	–	<5
6	25	10	–	–	<5
7	4	10	–	–	<5
8	4	10	–	2 M NaH_2PO_4	<5
9	25	10	–	2 M GnHCl	<5
10	25	10	–	4 M GnHCl	<5
11	4	10	–	2 M GnHCl	<5
12	4	10	–	4 M GnHCl	<5
13	25	10	–	–	<5
14	25	10	–	–	<5
15	4	10	20% EG	–	<5
16	25	10	20% EG	–	<5
17	4	10	20% MeCN	–	7
18	25	10	20% MeCN	–	8
19	4	10	20% 2-PrOH	–	12
20	25	10	20% 2-PrOH	–	14
21	25	10	20% DMSO	–	<5
22	25	10	20% DMSO	–	<5
23	25	10	20% glycerol	–	<5
24	25	10	20% glycerol	–	<5
25	25	10	50% MeCN	–	12
26	25	10	50% MeCN	–	19
27	25	10	50% 2-PrOH	–	28
28	25	10	50% 2-PrOH	6% Tween 20	7
29	25	10	50% 2-PrOH	6% Tween 40	9
30	25	10	50% 2-PrOH	6% Tween 60	7
31	25	10	70% 2-PrOH	–	40
32	25	10	70% 2-PrOH	–	48
33	25	10	70% 2-PrOH	–	23
34	25	10	70% 2-PrOH	–	6
35	25	10	70% 2-BuOH	–	9
36	25	10	70% 2-PrOH	6% Briji	37
37	25	10	80% 2-PrOH	–	48
38	25	10	90% 2-PrOH	–	<5[b]

[a]All reactions were performed at pH 7.5 except for runs 32 and 36−38 (pH 8.5), run 33 (pH 9.5), and run 34 (pH 10.5). [b]Precipitation occurred.

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disulfide bond determination. The program calculated all possible combinations of disulfide pairing and singly or doubly charged ions. The experimental ion masses were matched to the tabulated ion masses. When the peak list matched more than 20% of the theoretical CAD b and y ions within 0.8 Da mass errors, the spectra were manually inspected to confirm the assignment.

Antimicrobial Assay. Six bacterial strains were tested: Gram-positive *Micrococcus luteus*, *Staphylococcus aureus*, *Bacillus cereus*, and *Bacillus megaterium* and Gram-negative *Pseudomonas aeruginosa* and *Escherichia coli*. Antimicrobial assays were performed according to Lehrer's two-stage radial diffusion. In brief, the radial diffusion assay requires two different layers of agrose gel.[36] The underlay gel was prepared by mixing a constant amount of bacteria with minimal nutrient provided to the bacteria. Then equal size holes (2.5 mm diameter) were punched in the underlay gel, and each hole was filled with an aliquot (3 μL) of hedyotide B1 at different concentrations. The peptide and the underlay gel with bacteria were incubated at 37 °C for 3 h. The underlay gel was covered with a nutrient-rich overlay gel, and the bacteria were allowed to grow overnight. The minimal inhibitory concentration (MIC) was calculated by measuring the diameter of the clear zone against different peptide concentrations.

RESULTS

Synthesis and Cyclization. The solid phase synthesis of hedyotide B1 was performed on a HF-cleavable benzhydrylamine resin attached to a thiol handle that afforded a thioester at the conclusion of the synthesis (Figure 2). Hedyotide B1 contains a Trp that is often protected by an N^i-formyl group to minimize alkylation side reactions during HF cleavage. In this synthesis, we used the N^i-cyclohexyloxycarbonyl (Hoc) group instead of the N^i-formyl group as the protecting group for the indole side chain of tryptophan. The Hoc group is base-resistant and readily cleaved by HF.[37]

After the synthesis had been completed on the resin support, hedyotide B1 was treated with HF to remove all protecting groups. The resulting peptide thioester was purified by HPLC and then subjected to the thia-zip cyclization in an aqueous buffer (pH 7.5) containing 6 M GnHCl with a 100-fold excess of thiophenol at room temperature for 3 h. The reaction was monitored by HPLC and MALDI-TOF. The thia-zip cyclization initiated a series of nucleophilic attacks by the cysteinyl thiols toward the C-terminal thioester, leading to the formation of thiolactone and ring expansion. The final step involved an irreversible S-to-N acyl shift via a five-membered ring thiolactone, resulting in a cyclic backbone. HPLC monitoring showed that the cyclization proceeded quantitatively and was completed within 2.5 h, and importantly without detectable side products due to oligomerization. Such a high cyclization efficiency of an unprotected peptide thioester is likely attributed to the regioselectivity of the thia-zip mechanism.[23]

Figure 2 shows the HPLC profiles of the purified and S-reduced peptide thioesters, the S-reduced cyclized peptide, and the native hedyotide B1, with each eluting at a different retention time (15.2, 17.6, and 19.1 min, respectively). Their distinctive elution times permitted convenient monitoring by HPLC in the optimization of the oxidative folding process.

Oxidative Folding. Immediately after the HPLC purification of the thia-zip cyclized product, the fully S-reduced

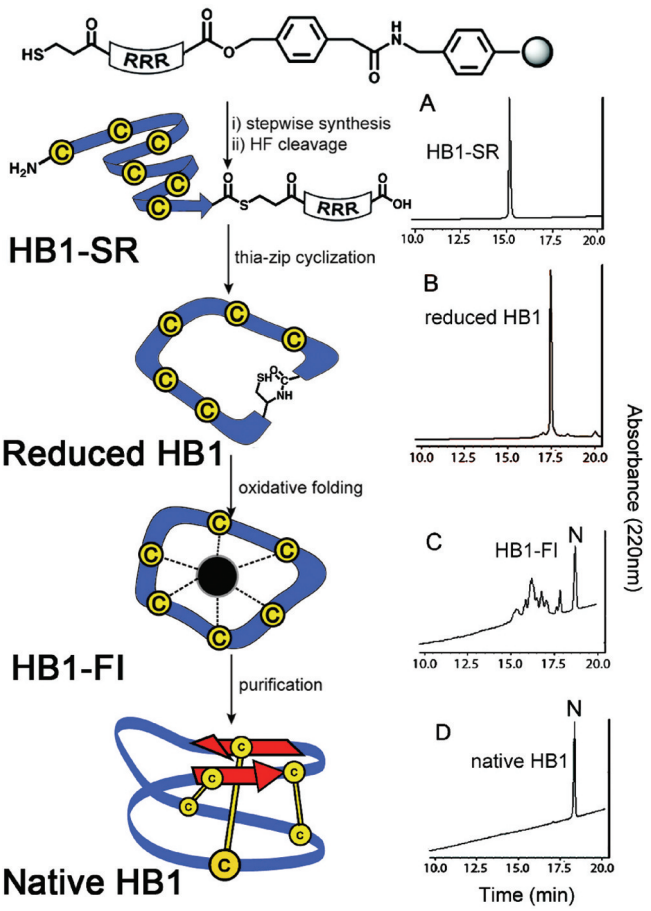

Figure 2. Synthetic scheme and HPLC profiles of (A) hedyotide B1 thioester (HB1-SR) (linear peptide thioester eluted at 15.2 min), (B) hedyotide B1 thioester after cyclization of the S-reduced hedyotide B1 (17.6 min), (C) the hedyotide B1 folding intermediate (HB1-FI) during the oxidative folding process, and (D) purified hedyotide B1 with native disulfide connectivity.

synthetic hedyotide B1 was subjected to the oxidative folding under 38 different conditions (Table 1). We followed the general experimental framework in the use of redox agents, buffers, and additives previously established by many laboratories in the oxidative folding of cysteine-rich peptides (CRPs) that included conotoxin,[38−40] defensins,[41,42] scorpion toxin, and cyclotides.[29,30,33,43]

For the redox reagent, we used a mixture of reduced and oxidized glutathione (GSH/GSSG), a typical choice for the thiol−disulfide exchange reaction in many successful oxidative folding reactions of small disulfide-rich proteins. For all experiments, we used the redox agent GSH/GSSG at a 10:1 molar ratio. In the first six runs (runs 1−6), we tested a suitable peptide concentration for the prevention of precipitation. At a peptide concentration of 10−50 μM, no precipitation was observed, whereas at 100 μM, even in the presence of 4 M GnHCl, precipitation occurred. In all subsequent runs, the peptide concentrations were reduced to 10 μM and the folding experiments monitored by HPLC at 24 h intervals.

From runs 1−20 in our study, only runs 17−20 exhibited reasonable yields of the native folded product (Figure 3). All four runs contained an organic cosolvent. Thus, we eliminated parameters such as denaturant or ethylene glycol that failed to improve the folding yield. There was also no significant

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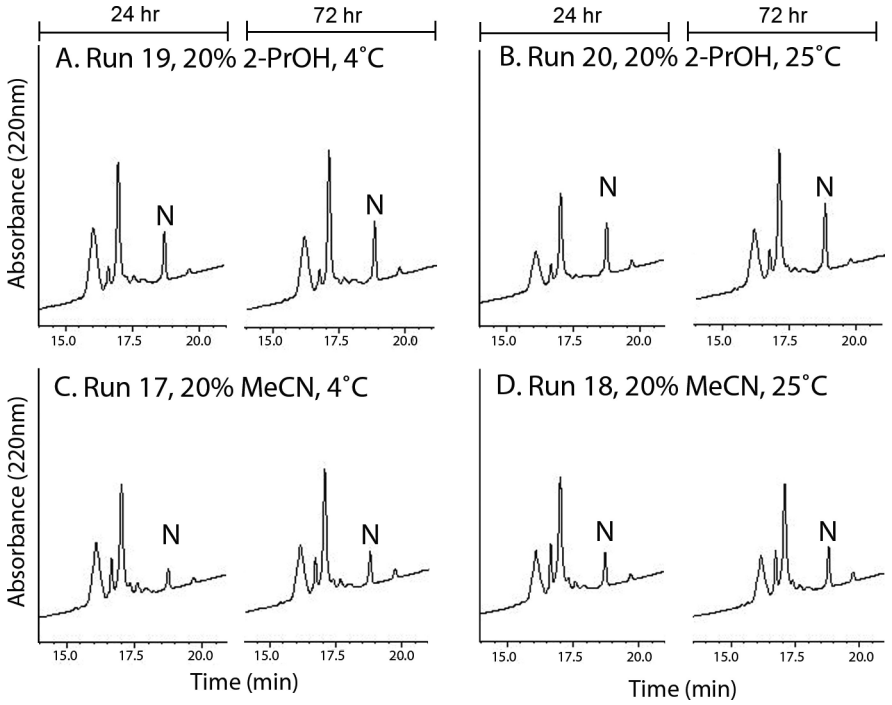

Figure 3. HPLC profiles highlighting the effects of cosolvent in the oxidative folding of synthetic hedyotide B1. N denotes the native form of hedyotide B1 eluting at 18.7 min: (A) 20% iPrOH at 4 °C (run 19), (B) 20% iPrOH at room temperature (run 20), (C) 20% MeCN at 4 °C (run 17), and (D) 20% MeCN at room temperature (run 18). The left panels show the reaction performing for 24 h and the right panels that performed for 72 h.

difference in yield whether the reaction was performed at 4 °C or room temperature. The oxidative folding process was slow and required 72 h for completion. Prolonging the folding reaction beyond 72 h showed no improvement in yield. All the non-native products could be separated by HPLC, S-reduced again, and then subjected to the same oxidative folding process to give similar HPLC profiles, indicating that oxidative folding involves a steady state of native, misfolded, and kinetic-trapped products.

The second set of runs [runs 21−31 (Table 1)] tested the nature and concentration of organic cosolvents that included acetonitrile (MeCN), 2-propanol (iPrOH), and DMSO. In 50 and 70% acetonitrile, the yields obtained after 72 h were 12 and 19%, respectively. DMSO instead of air oxidation has been used effectively for oxidative folding of cysteinyl-containing peptides.[44] The cosolvent DMSO, a strong aprotic polar solvent that would minimize protein aggregation, did not appear to be compatible with our test conditions with GSH/GSSG as the redox reagent. Among the three cosolvents, 2-propanol was shown to be the best cosolvent for the oxidative folding of hedyotide B1, affording yields ranging from 28 to 48% (Figure 4). We also tested the effect of pH on the oxidative folding using the 2-propanol system (runs 32−34). In 70% 2-propanol and at pH 7.5, the yield obtained after 72 h was 40% but increased to 48% at pH 8.5. At pH 9.5 and 10.5, the yield decreased to 23 and 6%, respectively, suggesting that pH 8.5 was optimal for the oxidative folding of hedyotide B1 under the proposed conditions using the 2-propanol cosolvent system.

To confirm that 70% 2-propanol was sufficient to afford a high yield for the oxidative folding of hedyotide B1, other cosolvents such as 2-butanol (run 35) and additives such as detergents, Brij 35, were tested (run 36). The use of a

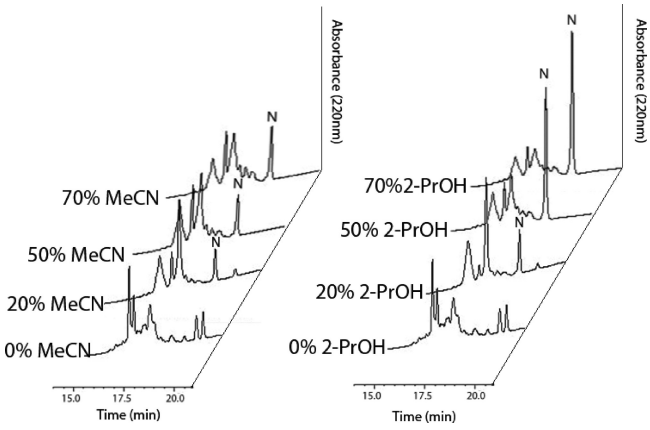

Figure 4. HPLC profiles showing the effects of different organic cosolvents on the oxidative folding of synthetic hedyotide B1. N denotes the native form of hedyotide B1.

hydrophobic organic solvent and detergents might favor the folding of the hydrophobic peptides (Table 1); however, none of the conditions described above showed improvement over the use of 70% 2-propanol.

Partial Acid Hydrolysis and LC−MS/MS Analysis of Cyclotide Connectivity. To confirm the disulfide connectivity of the folded synthetic hedyotide B1, we used partial acid hydrolysis of the intact sample[22] and de novo MS/MS determination by the method reported by Sze et al.[35] HPLC-purified synthetic hedyotide B1 after oxidative folding was treated with 1 M HCl and subjected to partial acid hydrolysis at 100 °C. At 15 min intervals, samples were analyzed by MALDI-TOF to determine the extent of acid hydrolysis for useful fragmentations of hedyotide B1. Table 2 shows some of the

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Table 2. Selected SS-Linked Fragments Obtained from Partial Acid Hydrolysis and Matched by the Computer Program for Determining the Disulfide Bond Connectivity of Hedyotide B1

fragment	molecular weight	assigned fragment	connectivity
1	1163	FVLPC + FCYR	III–VI
2	1635	VLPCWSAKF + FCYR	III–VI
3	1289	NGTRCGET + KFGC	I–IV
4	1144	ETCFVL + CEKG	II–V
5	1112	TCFVLP + CEKG	II–V

single-SS-linked fragments obtained for MS/MS determination after a 45 min treatment. Consistent with our previous results, many of the peptide bond fragmentations occurred at sequences containing Gly, Ser, Asn/Asp, or Gln/Glu, residues that either are acid-susceptible or can offer anchimeric assistance in the fragmentation of peptide bonds (Figure 5).[22]

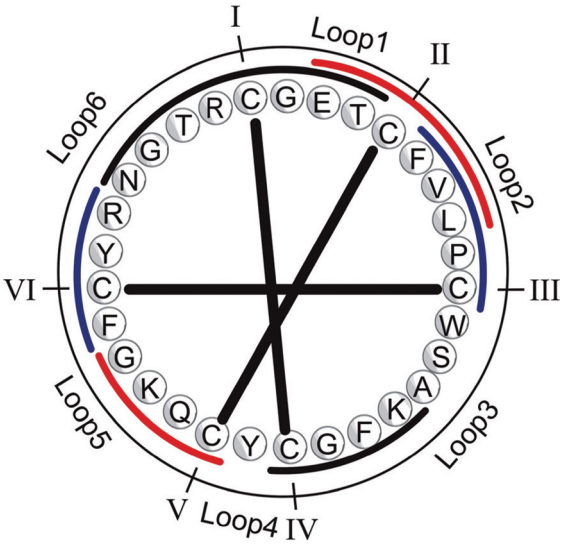

Figure 5. Disulfide connectivity of hedyotide B1 as determined by partial acid hydrolysis. The three pairs of colored lines represent some of the SS-linked fragments obtained from partial acid hydrolysis.

The partial acid-hydrolyzed fragments were analyzed by a home-written program (available for downloading) for disulfide bond determination. The program calculated all possible combinations of disulfide pairing and singly or doubly charged ions. The experimental ion masses were matched to the tabulated ion masses. When the peak list matched more than 20% of the theoretical CAD b and y ions within 0.8 Da mass errors, the spectra were manually inspected to confirm the assignment. For comparison, the SS connectivity of hedyotide B1 isolated from a natural source was also analyzed in parallel. Both synthetic and naturally occurring hedyotide B1 yielded similar results, confirming the presence of a cystine-knot motif.

Antimicrobial Assays. The antimicrobial activity of antimicrobial peptides such as defensins and protegrins, RTD-1, and tachyplesins has been known to correlate with their number of net charges. Furthermore, charge–activity relationships have been demonstrated by cationic peptide dendrimers, showing an increased antimicrobial activity increases with cationic charges.[45] In contrast to the highly cationic defensins, cyclotides generally carry few cationic amino acids and possess a net charge below +3. Hedyotide B1 is one of the few exceptions and carries a net charge of +3, sharing similar charge properties with circulin A, circulin B, and cyclopyschotide and suggesting its antibacterial potential (Figure 1). Indeed, we showed that hedyotide B1 was active against both Gram-positive and Gram-negative bacteria like cyclopsychotride and circulin B. It inhibited *E. coli*, *B. megaterium*, *P. aeruginosa*, and *B. cereus* with MICs of 1.98, 1.0, 4.4, and 2.7 μM, respectively. It was moderately active against *S. aureus* with a MIC of 39 μM but was inactive against *M. luteus* (Table 3).

Table 3. Antimicrobial Activities of Hedyotide B1 (hB1), Circulin A (cirA), Circulin B (cirB), and Cyclopsychotide (CPT) against a Panel of Tested Bacteria[a]

bacteria	hB1	CirA[b]	CirB[b]	CPT[b]
S. aureus	37.0	0.2	13.5	39.0
E. coli	2.0	>500	0.4	1.6
P. aeruginosa	4.4	>500	25.5	13.5
M. luteus	>100	>500	>500	48.0
B. cereus	2.7	NA[c]	NA[c]	NA[c]
B. megaterium	1.0	NA[c]	NA[c]	NA[c]

[a]The data are expressed as minimal inhibitory concentrations (micromolar). [b]Data from ref 12. [c]Not available.

DISCUSSION

In this study, we have examined experimentally several parameters for the successful oxidative folding of a bracelet cyclotide containing a cystine knot. Our findings are likely relevant to the oxidative folding of other cyclotides and cystine-knot plant defense peptides. These parameters can be classified into three groups for the oxidative folding cyclotides and CRPs: (1) prevention of aggregation, (2) maintaining a proper redox potential, and (3) use of a structure-enhancing solvent system.

Prevention of Aggregation. Experimentally, a practical consideration in the oxidative folding of cyclotides containing multiple thiols is preventing polymer formation, leading to precipitation. Polymer formation is favored because CRPs such as cyclotides are usually rich in β strands and hydrophobic patches that enhance aggregation. Aggregation and intermolecular cross-linking by the thiols form precipitates during oxidative folding that are often "dead-end" products that are irreversibly denatured. Cumulative works on amyloid formation and structure determination by Eisenberg and co-workers have shown aggregation can occur with short β strands in protein.[46] The first five runs of our experiments were designed to test the minimal peptide concentration in oxidative folding without aggregation leading to precipitation. When oxidative folding was performed at 50–100 μM, copious precipitation occurred in the presence of 2 or 4 M GnHCl and could not be reversed by reducing agents (Table 1, runs 1–5). Consequently, we compromised on folding conditions using a peptide concentration of 10 μM to prevent aggregation and precipitation. Under such conditions, precipitation is minimized, but the folding yields are generally low, ranging from 5 to 28%. Additives such as denaturant GnHCl (runs 11–14) and detergents (runs 28–30 and 36) were included in the testing conditions. We also tested additives coupled with organic cosolvents such as low-alkyl chain alcohols (runs 19, 20, 27, and 31), ethylene glycol (runs 15 and 16), acetonitrile (runs 17, 18,

25, and 26), DMSO (runs 21 and 22), and glycerol (runs 23 and 24). Interestingly, the combination of the detergent Tween and an organic cosolvent (runs 28−30) was incompatible under our test conditions, resulting in yields of <10%. However, the use of ≥20% concentrations of MeCN or iPrOH as a cosolvent afforded yields ranging from 10 to 48%. In general, the overall folding yield improved with the use with a more hydrophobic organic cosolvent at a higher concentration.

Two other factors commonly used for oxidative folding are worthy of discussion, although they are ineffective under our test conditions. We have tested the effects of concentration of both buffer solutions at 0.1 and 2 M because the presence of a high salt concentration may help to suppress electrostatic repulsion between the highly charged residues in the peptide and, in turn, may help to provide a transition from random to a folded structure[47] (runs 8 and 10). This method has been useful in the oxidative folding of highly charged peptides such as defensins to prevent intermolecular aggregation. In ω-conotoxin, an increase in the yield of the correctly folded peptide has been reported by addition of anions such as SO_4^{2-} and Cl^-.[38] Apparently, the hydrophobic factor dominates the charge factor in the oxidative folding of hedyotide B1.

Maintaining a Proper Kinetic and Redox Potential. Successful oxidative folding depends on structural factors, including proximity and accessibility.[48,49] Proximity drives the formation of kinetic products that often pair two neighboring thiols as disulfides. In the case of cyclotides, the kinetic product that is also the major misfolded product is the "ladder" type with Cys I−II, III−IV, V−VI disulfide connectivity. These kinetic misfolded cyclotides have been observed by Gunaskera et al. in the synthesis of different cyclotide analogues using 50% 2-propanol as a cosolvent[33] and by Aboye et al.[30] in the synthesis of bracelet cyclotide CVO2.

Second, it is necessary for the reactive thiols to come sufficiently close to form the native disulfide bonds. However, proximity may not be a determining factor in a "closed" system such as cyclotides whose cyclic backbone constrains cysteinyl thiols to equal proximity. In this case, backbone propensity, loop entropy, and enthalpy factors become crucial determinants. Although it is difficult to prevent the formation of the kinetic product such as the misfolded ladder cyclotide in the folding reaction, this type of reaction can be reversed by properly maintaining the redox potential of the system. In our study, the addition of DMSO disrupts such a redox potential. DMSO, a mild oxidant, alters the GSH:GSSG ratio and the redox system to an oxidizing environment, leading to an irreversible accumulation of "kinetically misfolded" products. For example, the addition of 20 and 30% DMSO (runs 21 and 22, respectively, in Table 1) reduced the yield of the correctly folded hedyotide significantly. Recently, Aboye et al.[30] have nicely circumvented the kinetically misfolded product problem in the DMSO-mediated folding of bracelet cyclotide CVO2 by adding GSH/cystamine at regular intervals during the folding reaction to restore the proper redox potential in their DMSO-mediated folding scheme.

The enthalpic factor (folding at 4 or 25 °C) may also affect oxidative folding, but there is no substantial evidence of its contribution to the yield under our experimental conditions. Another factor is the pH that affects the thiol−disulfide exchange reactions. The pK_a of the cysteinyl thiol is 8.5, at which 50% of its thiol is in the reactive thiolate for forming and reshuffling of disulfide bonds. Accordingly, oxidative folding reactions performed with buffers at pH values ranging from 7.8 to 8.7 have been reported. However, the pK_a of a cysteinyl thiol is also known to vary in the presence of an organic cosolvent, which was included in our folding conditions. In 70% 2-propanol, the folding of hedyotide B1 appears to be optimal at pH 8.5 (run 32). Decreasing the pH to 7.8 or increasing the pH to 9.5 and 10.5 resulted in a lower folding yield (runs 31, 33, and 34).

Entropy, Inverse Configuration, and Use of a Structure-Enhancing Solvent for Oxidative Folding. Oxidative folding of a "closed-end" peptide such as cyclotides faces an unusual problem. Within cyclotides, it is sterically crowded during the thiol−disulfide exchange reactions in the folding process upon moving from the kinetic to the native product. At the completion of the process, cyclotide adopts an "inverse configuration" with the cystine knot occupying the interior and displacing the bulky hydrophobic side chains to the exterior. Thus, the folded native cyclotide is generally more hydrophobic than their unfolded intermediates as shown by RP-HPLC profiles (Figure 4). Experimentally, the use of a high concentration of 2-propanol in the folding process appears to provide a solution for overcoming such a problem. 2-Propanol solvates the hydrophobic side chains of the cyclotides and leads to their externalization to the surface, thus providing accessibility for the thiol−disulfide exchange reactions inside the peptide core.[50] It may also promote the solubility of different disulfide intermediates, which form during the oxidative folding process. Intermediates are reactive species to which the disulfide bonds are usually exposed, and the bonds are in contact with the surrounding redox reagents.[51] We have also tested the effects of 80 and 90% 2-propanol under the same system (runs 37 and 38). No improvement in yield was observed with 80% 2-propanol at pH 8.5. In contrast, the yield decreased to 12% at 90% 2-propanol because of the insolubility of GSH and GSSG as well as the peptide leading to precipitation. Thus, the use of 70% 2-propanol is optimal under our experimental conditions that employ GSH/GSSG as a redox system. Once the correct disulfide bonds are formed, the stable tertiary structure with the buried disulfide bonds as the interior core is somewhat protected and less accessible to redox reagents, hence permitting an accumulation of the native product.

Goldenberg et al.[39] have demonstrated in buffered aqueous solutions that the success of oxidative folding of conotoxins that are open chain cystine CRPs correlates inversely with the peptide hydrophobic composition. Accordingly, the use of denaturants such as urea and GnHCl facilitates the oxidative folding process of hydrophobic but not hydrophilic conotoxins. Thus, denaturants minimize noncovalent interactions between hydrophobic residues to facilitate the folding process. However, the denaturing properties of 70% 2-propanol and 8 M urea in the oxidative folding process are different. Our data show decreased folding yields with denaturing agents such as urea or GnHCl, suggesting that noncovalent interactions between residues are one of the determinants in the oxidative folding process, but insufficient to be the only determinant in a successful folding of CRPs. It is likely that 70−80% 2-propanol not only weakens the peptide noncovalent interactions but also enhances the "inverse configuration" of hedyotide B1, which is expected to be similar to the native peptide where the side chains are externalized to the surface. Previously, Daly et al.[33] have shown that 50% 2-propanol is sufficient for the oxidative

folding of kalata B1, a Möbius cyclotide with only one aromatic amino acid residue and its hydrophobic residues dispersed in loops 5 and 6. In contrast, hedyotide B1 is a bracelet type with six aromatic residues and contains hydrophobic residues as a nearly continuous patch in loops 2 and 3. Indeed, our HPLC analysis shows that hedyotide B1 is more hydrophobic than kalata B1. Thus, the use of a higher alcohol concentration is logical, and indeed successful, for folding a highly hydrophobic cyclotide such as hedyotide B1. The use of an alcohol cosolvent has also found success in the folding of other CRPs. For example, α-conotoxins such as α-ImI, α-GI, and α-SI containing ∼40% hydrophobic residues in their sequences have been folded successfully in 20−40% ethanol. In contrast, the highly hydrophilic ω-conotoxins (MVIIC) containing ∼10% hydrophobic amino acids can be folded in high-salt solutions (to suppress charge residues) rather than in solutions containing an organic cosolvent.[38,39,42]

Previously, our laboratory has shown a positive correlation between the number of cationic charges in cyclotides and antibacterial activities against Gram-negative bacteria.[12] Consistent with the previous results, hedyotide B1 that contains the largest number of cationic charged residues among all cyclotides is more active against *P. aeruginosa*, a Gram-negative bacteria, than the less cationically charged cyclotides such as circulin A and B as well as cyclopsychotide. Hedydotide B1 is relatively inactive against *S. aureus* and *M. luteus*, which are Gram-positive bacteria. Because cyclotides are plant defense peptides, we also tested hedyotide B1 against two different bacteria found in soil, *B. cereus* and *B. megaterium*. The low MICs of hedyotide B1 against these soil bacteria suggest that cyclotides likely evolved to target soil bacteria rather than human pathogens.

■ CONCLUSIONS

The total synthesis of hedyotide B1 has been successfully performed with a 48% yield in oxidative folding using 70% 2-propanol. The native SS connectivity was confirmed by partial acid hydrolysis. Without the use of an organic cosolvent, the oxidative folding yield substantially decreased to <20%, suggesting the useful role of a high concentration of 2-propanol in the formation of disulfide bonds of the interior cystine core in the oxidative folding of cyclotides that display an unusual inverse configuration with hydrophobic side chains externalized to the solvent face. Hedyotide B1 is an antimicrobial and displays moderate to mild activity against *E. coli*, *B. megaterium*, *P. aeruginosa*, and *S. aureus*.

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■ REFERENCES

(1) Saether, O., Craik, D. J., Campbell, I. D., Sletten, K., Juul, J., and Norman, D. G. (1995) Elucidation of the primary and three-dimensional structure of the uterotonic polypeptide kalata B1. *Biochemistry 34*, 4147−4158.

(2) Daly, N. L., and Craik, D. J. (2011) Bioactive cystine knot proteins. *Curr. Opin. Chem. Biol. 15*, 1−7.

(3) García-Olmedo, F., Molina, A., Alamillo, J. M., and Rodríguez-Palenzuéla, P. (1998) Plant defense peptides. *Biopolymers 47*, 479−491.

(4) Barbault, F., Landon, C., Guenneugues, M., Meyer, J. P., Schott, V., Dimarcq, J. L., and Vovelle, F. (2003) Solution structure of Alo-3: A new knottin-type antifungal peptide from the insect *Acrocinus longimanus*. *Biochemistry 42*, 14434−14442.

(5) Gunasekera, S., Foley, F. M., Clark, R. J., Sando, L., Fabri, L. J., Craik, D. J., and Daly, N. L. (2008) Engineering stabilized vascular endothelial growth factor-A antagonists: Synthesis, structural characterization, and bioactivity of grafted analogues of cyclotides. *J. Med. Chem. 51*, 7697−7704.

(6) Henriques, S., and Craik, D. (2009) Cyclotides as templates in drug design. *Drug Discovery Today 15*, 57−63.

(7) Gran, L. (1973) On the isolation of tetramethylputrescine from *Oldenlandia affinis*. *Lloydia 36*, 209−210.

(8) Gran, L. (1973) On the effect of a polypeptide isolated from Kalata-Kalata (*Oldenlandia affinis* DC) on the oestrogen dominated uterus. *Pharmacol. Toxicol. 33*, 400−408.

(9) Craik, D. J. (2009) Circling the enemy: Cyclic proteins in plant defence. *Trends Plant Sci. 14*, 328−335.

(10) Wang, C. K., Colgrave, M. L., Gustafson, K. R., Ireland, D. C., Göransson, U., and Craik, D. J. (2008) Anti-HIV cyclotides from the Chinese medicinal herb *Viola yedoensis*. *J. Nat. Prod. 71*, 47−52.

(11) Lindholm, P., Göransson, U., Johansson, S., Claeson, P., Gullbo, J., Larsson, R., Bohlin, L., and Backlund, A. (2002) Cyclotides: A novel type of cytotoxic agents. *Mol. Cancer Ther. 1*, 365−369.

(12) Tam, J. P., Lu, Y. A., Yang, J. L., and Chiu, K. W. (1999) An unusual structural motif of antimicrobial peptides containing end-to-end macrocycle and cystine-knot disulfides. *Proc. Natl. Acad. Sci. U.S.A. 96*, 8913−8918.

(13) Ireland, D., Wang, C., and Wilson, J. (2008) Cyclotides as natural anti-HIV agents. *Biopolymers 90*, 51−60.

(14) Svangård, E., Burman, R., Gunasekera, S., Lövborg, H., Gullbo, J., and Göransson, U. (2007) Mechanism of action of cytotoxic cyclotides: Cycloviolacin O2 disrupts lipid membranes. *J. Nat. Prod. 70*, 643−647.

(15) Gustafson, K., Sowder, R., Henderson, L., Parsons, I., Kashman, Y., Cardellina, J. H. II, McMahon, J. B., Buckheit, R. W. Jr., Pannell, L. K., and Boyd, M. R. (1994) Circulins A and B. Novel human immunodeficiency virus (HIV)-inhibitory macrocyclic peptides from the tropical tree *Chassalia parvifolia*. *J. Am. Chem. Soc. 116*, 9337−9338.

(16) Witherup, K. M., Bogusky, M. J., Anderson, P. S., Ramjit, H., Ransom, R. W., Wood, T., and Sardana, M. (1994) Cyclopsychotride A, a biologically active, 31-residue cyclic peptide isolated from *Psychotria longipes*. *J. Nat. Prod. 57*, 1619−1625.

(17) Gran, L., Sandberg, F., and Sletten, K. (2000) *Oldenlandia affinis* (R&S) DC. A plant containing uteroactive peptides used in African traditional medicine. *J. Ethnopharmacol. 70*, 197−203.

(18) Gruber, C. W., Cemazar, M., Clark, R. J., Horibe, T., Renda, R. F., Anderson, M. A., and Craik, D. J. (2007) A novel plant protein-disulfide isomerase involved in the oxidative folding of cystine knot defense proteins. *J. Biol. Chem. 282*, 20435−20446.

(19) Saska, I., Gillon, A. D., Hatsugai, N., Dietzgen, R. G., Hara-Nishimura, I., Anderson, M. A., and Craik, D. J. (2007) An asparaginyl endopeptidase mediates in vivo protein backbone cyclization. *J. Biol. Chem. 282*, 29721−29728.

(20) Rosengren, K. J., Daly, N. L., Plan, M. R., Waine, C., and Craik, D. J. (2003) Twists, knots, and rings in proteins. Structural definition of the cyclotide framework. *J. Biol. Chem. 278*, 8606−8616.

(21) Wang, C. K., Hu, S. H., Martin, J. L., Sjögren, T., Hajdu, J., Bohlin, L., Claeson, P., Göransson, U., Rosengren, K. J., Tang, J., Tan,

N. H., and Craik, D. J. (2009) Combined X-ray and NMR analysis of the stability of the cyclotide cystine knot fold that underpins its insecticidal activity and potential use as a drug scaffold. *J. Biol. Chem. 284*, 10672−10683.

(22) Tam, J. P., and Lu, Y. A. (1998) A biomimetic strategy in the synthesis and fragmentation of cyclic protein. *Protein Sci. 7*, 1583−1592.

(23) Tam, J. P., Lu, Y., and Yu, Q. (1999) Thia Zip Reaction for Synthesis of Large Cyclic Peptides: Mechanisms and Applications. *J. Am. Chem. Soc. 121*, 4316−4324.

(24) Mcmurray, J. (1991) Solid phase synthesis of a cyclic peptide using fmoc chemistry. *Tetrahedron Lett. 32*, 7679−7682.

(25) Sager, C. (1999) Influence of cis-trans isomerisation on pentapeptide cyclisation. *Tetrahedron Lett. 40*, 7987−7991.

(26) Liu, C. F., and Tam, J. P. (1994) Peptide segment ligation strategy without use of protecting groups. *Proc. Natl. Acad. Sci. U.S.A. 91*, 6584−6588.

(27) Camarero, J. A., Fushman, D., Cowburn, D., and Muir, T. W. (2001) Peptide chemical ligation inside living cells: In vivo generation of a circular protein domain. *Bioorg. Med. Chem. 9*, 2479−2484.

(28) Clark, R. J., Jensen, J., Nevin, S. T., Callaghan, B. P., Adams, D. J., and Craik, D. J. (2010) The Engineering of an Orally Active Conotoxin for the Treatment of Neuropathic Pain. *Angew. Chem., Int. Ed. 49*, 6545−6548.

(29) Gunasekera, S., Daly, N. L., Clark, R. J., and Craik, D. J. (2009) Dissecting the oxidative folding of circular cystine knot miniproteins. *Antioxid. Redox Signaling 11*, 971−980.

(30) Leta Aboye, T., Clark, R. J., Craik, D. J., and Göransson, U. (2008) Ultra-stable peptide scaffolds for protein engineering-synthesis and folding of the circular cystine knotted cyclotide cycloviolacin O2. *ChemBioChem 9*, 103−113.

(31) Thongyoo, P., and Roqué-Rosell, N. (2008) Chemical and biomimetic total syntheses of natural and engineered MCoTI cyclotides. *Org. Biomol. Chem. 6*, 1462−1470.

(32) Thongyoo, P., Tate, E. W., and Leatherbarrow, R. J. (2006) Total synthesis of the macrocyclic cysteine knot microprotein MCoTI-II. *Chem. Commun.,*, 2848−2850.

(33) Daly, N. L., and Craik, D. J. (2000) Acyclic permutants of naturally occurring cyclic proteins. Characterization of cystine knot and β-sheet formation in the macrocyclic polypeptide kalata B1. *J. Biol. Chem. 275*, 19068−19075.

(34) Tam, J. P., Lu, Y.-A., and Yang, J.-L. (2002) Correlations of cationic charges with salt sensitivity and microbial specificity of cystine-stabilized β-strand antimicrobial peptides. *J. Biol. Chem. 277*, 50450−50456.

(35) Sze, S. K., Wang, W., Meng, W., Yuan, R., Guo, T., Zhu, Y., and Tam, J. P. (2009) Elucidating the structure of cyclotides by partial acid hydrolysis and LC-MS/MS analysis. *Anal. Chem. 81*, 1079−1088.

(36) Lehrer, R.I., Rosenman, M., Harwig, S. S., Jackson, R., and Eisenhauer, P. (1991) Ultrasensitive assays for endogenous antimicrobial polypeptides. *J. Immunol. Methods 137*, 167−173.

(37) Nishiuchi, Y., Nishio, H., Inui, T., and Kimura, T. (1996) Nin-Cyclohexyloxycarbonyl group as a new protecting group for tryptophan. *Tetrahedron Lett. 37*, 7529−7532.

(38) Kubo, S., Chino, N., Kimura, T., and Sakakibara, S. (1996) Oxidative folding of ω-conotoxin MVIIC: Effects of temperature and salt. *Biopolymers 38*, 733−744.

(39) Price-Carter, M., Gray, W. R., and Goldenberg, D. P. (1996) Folding of ω-conotoxins. 1. Efficient disulfide-coupled folding of mature sequences in vitro. *Biochemistry 35*, 15537−15546.

(40) DeLa, C., Whitby, F., Buczek, O., and Bulaj, G. (2003) Detergent-assisted oxidative folding of δ-conotoxins. *J. Pept. Res. 61*, 202−212.

(41) Wu, Z., Hoover, D. M., Yang, D., Boulegue, C., Santamaria, F., Oppenheim, J. J., Lubkowski, J., and Lu, W. (2003) Engineering disulfide bridges to dissect antimicrobial and chemotactic activities of human β-defensin 3. *Proc. Natl. Acad. Sci. U.S.A. 100*, 8880−8885.

(42) Kubo, S., Tanimura, K., Nishio, H., Chino, N., Teshima, T., Kimura, T., and Nishiuchi, Y. (2008) Optimization of the Oxidative Folding Reaction and Disulfide Structure Determination of Human α-Defensin 1, 2, 3 and 5. *Int. J. Pept. Res. Ther. 14*, 341−349.

(43) Altamirano, M. M., Garcia, C., Possani, L. D., and Fersht, A. R. (1999) Oxidative refolding chromatography: Folding of the scorpion toxin Cn5. *Nat. Biotechnol. 17*, 187−191.

(44) Tam, J., Wu, C., Liu, W., and Zhang, J. (1991) Disulfide bond formation in peptides by dimethyl sulfoxide. Scope and applications. *J. Am. Chem. Soc. 113*, 6657−6662.

(45) Tam, J. P., Lu, Y.-A., and Yang, J.-L. (2002) Antimicrobial dendrimeric peptides. *Eur. J. Biochem. 269*, 923−932.

(46) Sawaya, M. R., Sambashivan, S., Nelson, R., Ivanova, M. I., Sievers, S. A., Apostol, M. I., Thompson, M. J., Balbirnie, M., Wiltzius, J. J., McFarlane, H. T., Madsen, A. Ø., Riekel, C., and Eisenberg, D. (2007) Atomic structures of amyloid cross-β spines reveal varied steric zippers. *Nature 447*, 453−457.

(47) Korolev, N., Berezhnoy, N. V., Eom, K. D., Tam, J. P., and Nordenskiold, L. (2009) A universal description for the experimental behavior of salt-(in)dependent oligocation-induced DNA condensation. *Nucleic Acids Res. 37*, 7137−7150.

(48) Wedemeyer, W. J., Welker, E., Narayan, M., and Scheraga, H. A. (2000) Disulfide bonds and protein folding. *Biochemistry 39*, 4207−4216.

(49) Narayan, M., Welker, E., and Wedemeyer, W. (2000) Oxidative folding of proteins. *Acc. Chem. Res. 33*, 805−812.

(50) Shimizu, S., and Shimizu, K. (1999) Alcohol denaturation: Thermodynamic theory of peptide unit solvation. *J. Am. Chem. Soc. 121*, 2387−2394.

(51) Welker, E., Narayan, M., and Wedemeyer, W. (2001) Structural determinants of oxidative folding in proteins. *Proc. Natl. Acad. Sci. U.S.A. 98*, 2313−2316.