Synthesis of Cyclic Peptides through Direct Aminolysis of Peptide Thioesters Catalyzed by Imidazole in Aqueous Organic Solutions

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Received July 10, 2009

A promising method for the synthesis of cyclic peptides through the direct aminolysis of peptide thioesters is presented. The cyclization step was carried out in a mixture of acetonitrile and 1.5 M aqueous imidazole solution with no observable oligomers. Studies on the N- and C-terminal residues show that the choice of C-terminal residue has a more significant effect on the success rate of cyclization than the choice at the N-terminal residue.

Introduction

Cyclic peptides are important synthetic targets because of their potential usefulness as promising lead compounds in the drug discovery process.[1] Their constrained conformation may result in increased resistance to proteolysis and higher receptor-binding affinities compared to their linear analogues. Typically, head-to-tail amide-cyclized peptides are synthesized by cyclization of a linear precursor either in solution or anchored to a resin support.[2] Classical approaches for the synthesis of the cyclic peptides rely on the use of orthogonally protected linear precursors which are selectively activated and cyclized. The success rate of the macrocyclization step in these synthesis schemes is heavily dependent on the peptide coupling reagents.[3,4] Cyclization via these conventional carboxylate activation chemistries is often inefficient, providing a complex mixture of linear and cyclic molecules, as well as oligomers.[4]

Recently, the C-terminal thioesters have been used in the synthesis of cyclic peptides. The most common method for the synthesis of cyclic peptides using a C-terminal thioester is through native chemical ligation.[5] Though the chemical ligation approach has proven to be an extremely efficient method, it has been limited by the necessity for an N-terminal cysteine. Removable thiol-containing auxiliary strategies have been developed to extend the ligation site to non-cysteinyl residues including alanine,[6] glycine,[7] phenylalanine,[8] and valine.[9] A cysteine-free ligation method for the synthesis of proteins utilizing a intermolecular direct aminolysis of the peptide thioester was recently reported.[10] By the use of a mixed organic solvent with aqueous buffer, the ligation conjugation was extended to a wide range of amino acid residues at the N-termini without the use of auxiliaries. Silver ion-assisted lactamization of C-terminal thioester provides another ligation approach for making cyclic peptides through non-N-cysteine residue.[11] A silver ion coordination of the reactive ends of an unprotected peptide thioester permits the long-range acyl migration with an N-terminal amino functionality to form a cyclic peptide in aqueous solution. Conformational dependent cyclization of linear thioesters was also reported in the synthesis of the cyclic decapeptide antibiotic Tyrocidine A and its analogues from their fully deprotected linear thioester precursors on a solid support in aqueous ammonia solution.[12]

Imidazole catalysis of the hydrolysis and transfer of activated acyl groups has been studied since 1950s. Early studies on hydrolytic enzymes found that the imidazolyl group on histidine forms a portion of the catalytic site for many hydrolytic enzymes. From this observation, the ability of imidazole to catalyze the hydrolysis of thioesters, phenyl acetates, and et cetera has been investigated and demonstrated.[13] The mechanism of imidazole catalysis is thought to work through the direct attack of imidazole on the carbonyl group to form an acyl imidazole intermediate, which is subsequently hydrolyzed by water. Since the amino group is a stronger nucleophile than water, the application of imidazole to catalyze the cyclization of peptide thioesters in aqueous solution should be anticipated. Herein, we report the synthesis of cyclic peptides by the direct aminolysis of peptide thioesters in an imidazole aqueous organic solvent.

Results and Discussions

Synthesis of Peptide Thioesters Using "Volatilizable" Support. The linear peptide α-thioester was synthesized by a solid-phase approach using mercaptomethylphenyl functionalized silica gel as a 'volatilizable' support.[14] Starting from the mercaptomethylphenyl functionalized silica gel **1**, the linear peptide α-thioester was synthesized by the use of Boc-amino acids and a standard PyBOP/DIEA coupling approach (Scheme 1). After cleavage with anhydrous HF and evaporation of the anhydrous HF with a gaseous nitrogen stream, the unprotected peptide α-thioester **4** was obtained

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10.1021/cc900100z CCC: $40.75
Published on Web 10/06/2009

Scheme 1. Synthesis of Peptide Thioester through Volatilizable Support

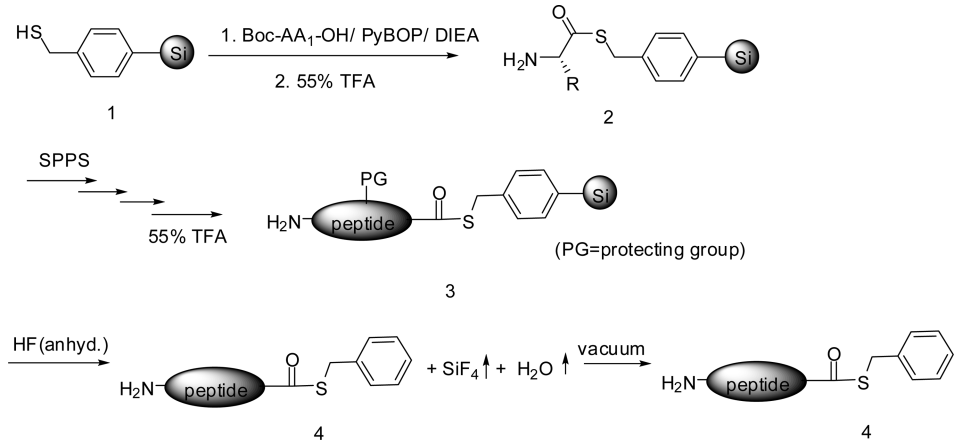


Table 1. Peptide-Thioesters and Monomeric Cyclic Peptides

	linear			cyclic			
sequence	product	purity (%)[a]	yield (%)[b]	product	yield (%)[c]	MW (calcd)	MW (found)[e]
DLTFG	**4a**	88	91	**5a**	23	533.25	534.26
DYFRG	**4b**	70	70	**5b**	46	638.28	639.32
GYGGAL	**4c**	77	80	**5c**	43[d]	518.25	519.24
DLGYRG	**4d**	76	77	**5d**	23[d]	661.32	662.34
GFDNYLG	**4e**	74	65	**5e**	24[d]	766.33	767.34
GGFSYTFA	**4f**	67	40	**5f**	15[d]	830.36	853.50
DYGLYNTFA	**4g**	55	71	**5g**	20[d]	1044.46	1045.47
DYGGFANYLA	**4h**	60	80	**5h**	27[d]	1071.47	1072.52
GDYGGFSLYKA	**4i**	73	46	**5i**	25[d]	1158.53	1159.54

[a] Purity (in percent) is determined by the peak area of HPLC at 214 nm. [b] Yield (in percent) is based on the weight of the crude products and are relative to the substitution of the resin. [c] Yield is based on isolated product after HPLC purification and the substitution of the resin. [d] 4−10 days. [e] Determined by ESI-HRMS.

in excellent yield and purity following lyophilization (Table 1, **4a**−**i**). The crude unprotected peptide thioesters were directly used in the cyclization.

Cyclization through Direct Aminolysis in Imidazole Aqueous Organic Solution. The cyclization was performed in various organic solvents mixed with aqueous imidazole solutions. Hydrolysis of the peptide thioester is a competitive side reaction to the cyclization. H-Glu-Ala-Phe-Tyr-Gly-SBzl was used as the model peptide for optimizing the head-to-tail cyclization. DMF, NMP and acetonitrile were chosen as the organic solvents. The peptide thioesters were completely soluble in all three organic solvents mixed with aqueous imidazole solution. DMF and NMP both gave a broad UV peak at 214 nm which sometimes overlapped with the UV absorption of the cyclic peptide in our experimental range. Acetonitrile was chosen as the organic solvent for its preferred UV absorptions characteristic when monitoring the reaction by LC-MS. Moreover, acetonitrile is more readily removed after cyclization, thus facilitating the purification of the desired cyclic products.

The cyclization was performed at a concentration of 1 mmol/L in a mixed solvent. Varying ratios of acetonitrile and imidazole solution were examined. The cyclization was monitored by LC-MS after quenching the reaction with 15% TFA in water (Figure 1). It was found that the simple thioester hydrolysis was maximally suppressed with a mixture of acetonitrile and 1.5 M aqueous imidazole solution with a volume ratio of 7:1 (Scheme 2). After reaction for 72 h at 37 °C, the model linear peptide thioester was converted to its expected cyclic product without observable oligomers. On the basis of these results, all experiments, unless noted otherwise, were conducted under these conditions.

Effect of C-Terminal Amino Acid. The effect of the C-terminal residue on the cyclization with direct aminolysis of thioester was tested. Analogue peptides H-Ala-Tyr-Gly-Lys-Xxx-SBzl with different amino acids at the C-terminal were examined. Ala, Asn, Asp, Arg, Cys, Gln, Gly, His, Leu, Lys, Met (O), Phe, Ser, Thr, Trp, Tyr, and Val were all tested as the C-terminal residues (Table 2). Cyclization with Ala, Asp, Cys, Gly, Lys, Met (O), and Ser as the C-terminal residue was completed within 72 h with more than 80% of desired cyclic product observed in each case. Peptide thioesters having Arg, Asn, Gln, His, Leu, and Trp at the C-termini were completely reacted within 72 h but yielded more hydrolysis byproducts. Cyclization with Phe and Tyr as the C-terminal residue needed longer reaction times and yielded a higher percentage of hydrolysis byproducts. It was found that the starting peptide α-thioesters were almost unchanged after a 15 day reaction when either Thr or Val was used as the C-terminal residue. This result is in accord with the suggested mechanism of imidazole catalysis. A β-branched C-terminal residue inhibits the nucleophilic attack by imidazole on the carboxyl carbonyl group of the peptide thioester.

Effect of N-Terminal Amino acid. The effect of the N-terminal residue on cyclization was also tested. Analogue peptide α-thioesters H-Xxx-Tyr-Gly-Lys-Gly-SBzl having different amino acids at the N-terminal residue were used in this experiment. Ala, Asn, Asp, Cys, Glu, Gln, Gly, His, Leu, Met (O), Phe, Ser, Tyr, Trp and Val were used as the

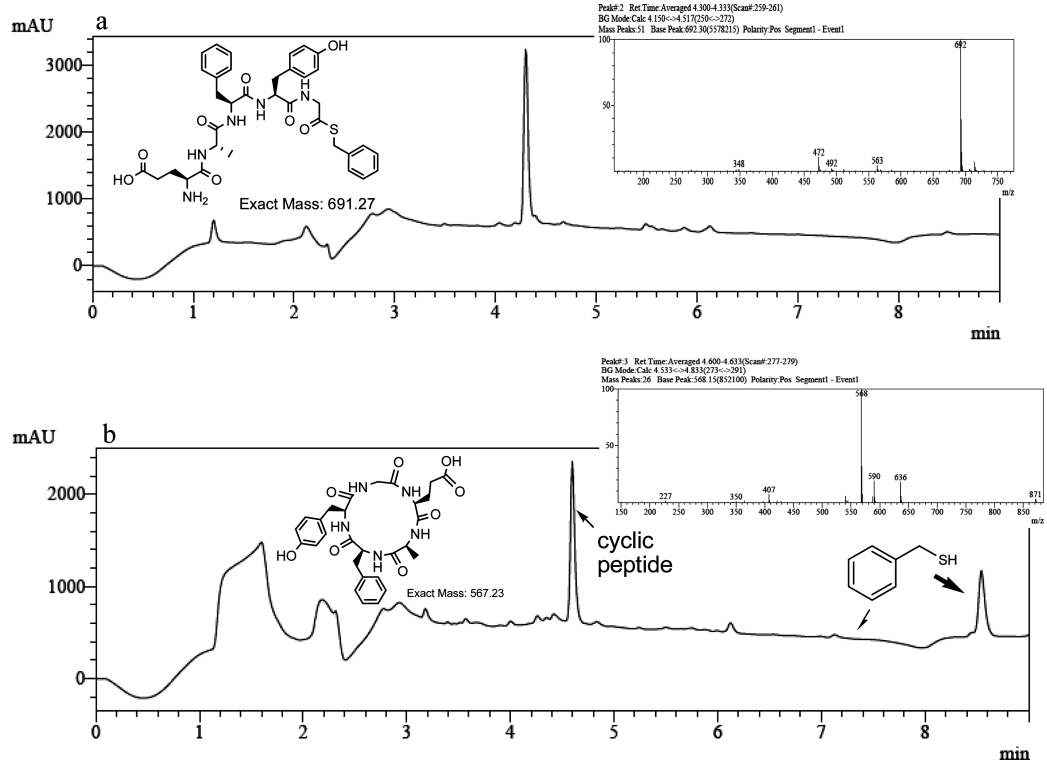
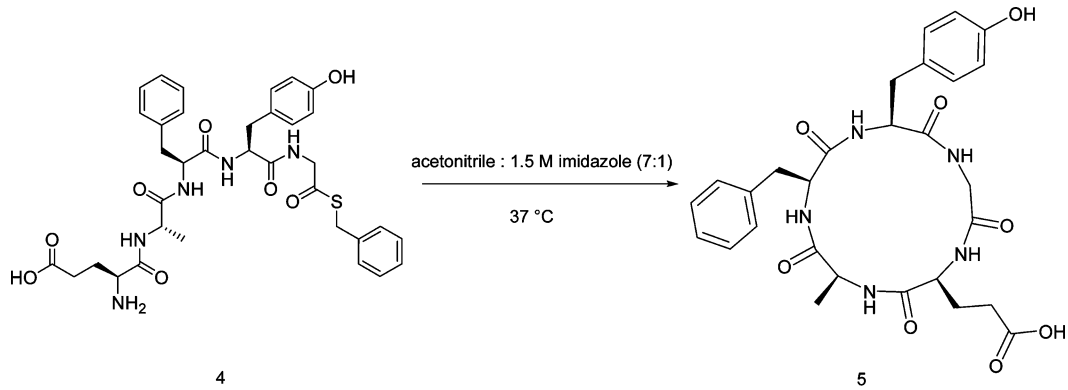


Figure 1. LC-MS profiles during the cyclization: (a) LC-MS of starting peptide thioester H-Glu-Ala-Phe-Tyr-Gly-SBzl and (b) after 72 h at 37 °C cyclization.

Scheme 2. Cyclization of Peptide Thioester H-Glu-Ala-Phe-Tyr-Gly-SBzl



[a] Yield is based on the peak area of the cyclic products and linear peptide thioester at 214 nm. [b] Yield is based on isolated product after HPLC purification and the substitution of the resin.

Table 2. Effect of C-Terminal Residues on Cyclization

residue		A	D	C[b]	G	K	M	S	R	N	H	Q	L	W	Y	F	V	T
ratio of product (%)[a]	cyclic	90	91	90	88	95	90	90	36	42	46	45	46	23	25	34		
	hydrolysis	10	9	10	12	5	10	10	64	58	54	55	54	77	56	46	9	10
ratio of linear precursor (%)[a]															19	20	91	90

[a] Ratio (in %) is based on LC-MS detection at 214 nm after cyclization at 37 °C for 72 h. [b] Side chain group of cysteine was oxidized to $-S-S-CH_2Ph$ under the reaction condition.

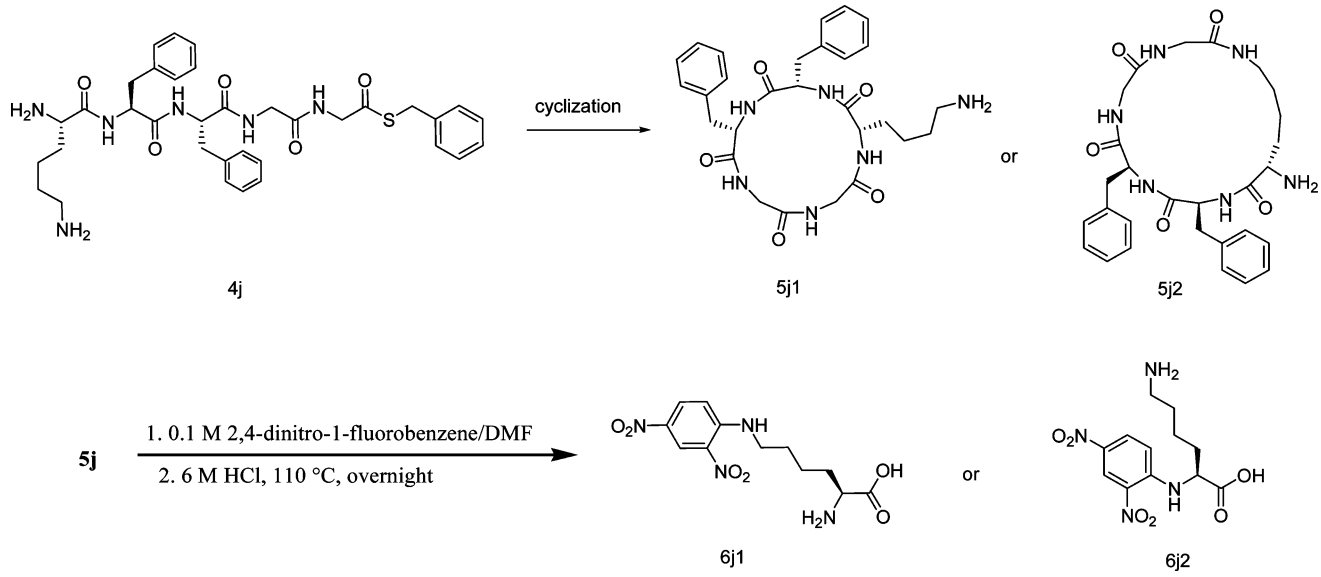
Table 3. Effect of N-terminal Residues on Cyclization

residue		A	D	E	C[b]	G	H	M	S	Y	V	Q	L	F	N	W
ratio of product (%)[a]	cyclic	80	82	95	90	90	90	80	90	80	90	90	66	41	67	10
	hydrolysis	20	18	5	10	10	10	20	10	20	10	10	34	59	33	90

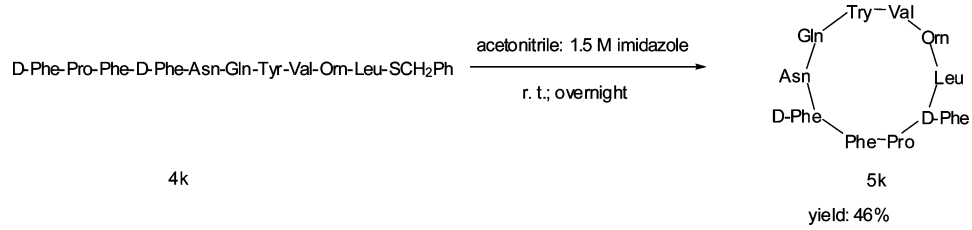
[a] Ratio (in %) is based on LC-MS detection after cyclization at 37 °C for 72 h. [b] Side chain group of cysteine was oxidized to $-S-S-CH_2Ph$ under the reaction condition.

N-terminal residues (Table 3). Native ligation with an N-terminal cysteine residue was used as a control to evaluate the ligation efficiency of other N-terminal residues. Ligation/cycliczation with N-cysteine under above condition yielded more than 90% of the desired cyclic product. It was found that the cyclization was complete within 72 h, and only a

Scheme 3. Regioselectivity of N-Terminal Lysine on Cyclization



Scheme 4. Synthesis of Tyrocidine A



small amount of hydrolysis byproduct (<10%) was formed when Ala, Asp, Glu, Gln, Gly, His, Met (O), Ser, Tyr, and Val were used at the N-terminal. The results showed these N-terminal residues had the similar reactivity as N-cysteine when they were used in the direct aminolysis of benzyl thioester. Prolonged cyclization time was needed and the amount of hydrolysis byproducts was found to increase when Asn, Leu, Phe, and Trp were used as N-terminal residues.

Regioselectivity. The effect on cyclization when lysine is incorporated into a linear thioester was also tested. N-terminal lysine and internal lysine were used to determine the regioselectivity of lysine on the cyclization. A pentapeptide thioester (**4j**) with lysine as the N-terminal residue was cyclized (Scheme 3). There are two possible cyclic products, **5j1** for N^{α}-amino cyclization (15-membered ring) and **5j2** for N^{ε}-amino cyclization (19-membered ring). In this case, only one cyclic product was observed. To verify whether the cyclization was an α- or ε- cyclization, N^{ε}-(dinitrophenyl)-lysine (**6j1**) and N^{α}-(dinitrophenyl)-lysine (**6j2**) were synthesized in solution and were used as controls. The cyclic product **5j** was purified and treated with 2,4-dinitro-1-fluorobenzene (Sanger's reagent) and hydrolyzed with 6 M HCl at 110 °C overnight. RP-HPLC analysis showed that only N^{α}-(dinitrophenyl)-lysine was detected, indicating that cyclization took place through the N^{ε}-amino group (Figure shown in Supporting Information).

Cyclization of the internal lysine peptide thioesters were investigated using a pentapeptide and a heptapeptide, Glu-Lys-Phe-Tyr-Gly-SCH_2Ph and Glu-Phe-Ala-Lys-Gly-Tyr-Gly-SCH_2Ph, respectively. Both peptides yielded two major peaks with identical molecular weights according to LC-MS detection, indicating both end-to-end (15- and 21-membered ring, respectively) and side-to-end (16-membered ring) cyclic products were formed during cyclization. The results revealed that the acylation preferred the N^{ε}-amino group of N-terminal lysine and had no preference when lysine was an internal residue. This is caused by the dual effect of ring size and the nucleophilicity of the amino group.

Effect of Ring Size. To determine the dependence of ring size on cyclization efficiency, peptide thioesters ranging in length from 5- to 11-amino acid residues with favored C- and N-terminal residues and random sequences were synthesized (Table 1, **5a**−**i**). It was found that the cyclization rate was related to the ring size and the peptide sequence. As expected, peptides with 5- and 6-amino acid residues were cyclized within 72 h. It took longer reaction times, usually 4−10 days, to complete the cyclization for 7- to 11- amino acid residues. No dimers or oligomers were found in any of these cyclic peptide syntheses. After purification, the desired isolated cyclic products were obtained in satisfactory yields, ranging from 15−46%.

Application to the Synthesis of Tyrocidine A and Effect of Conformation on Cyclization. Tyrocidine A is an antibiotic cyclic decapeptide produced by the bacteria *Bacillus brevis*. When imidazole aqueous organic solution was applied to the synthesis of tyrocidine A, the cyclization was complete after reacting at room temperature overnight with more than 90% of the desired product (Scheme 4). The reaction worked more efficiently than our randomly selected peptide thioester **4a**−**i**. Tyrocidine A has a confirmed β-turn

Table 4. Cyclization of Peptide Thioesters Having Same Sequence but Residue Stereoisomers

	product ratio[a]		
linear sequence	cyclic (%)	dimer (%)	hydrolysis (%)
L-L-L-L-L	95	0	5
L-L-L-D-L	5	21	74
L-D-L-D-L	78	2	20

[a] Product ratio (in percent) were determined by the peak area of HPLC at 214 nm. No linear precursory was detected in all three cases.

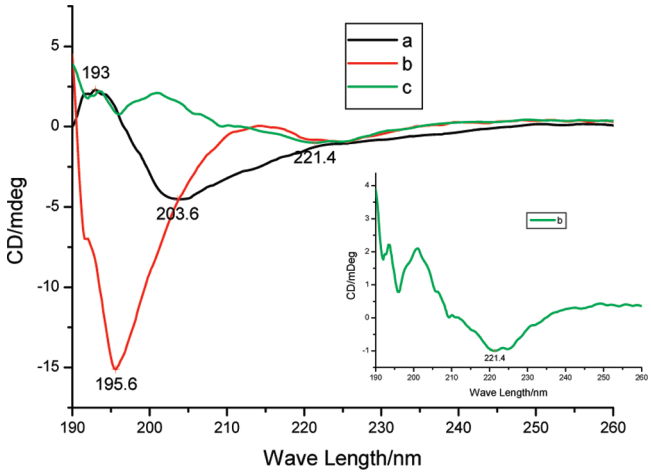


Figure 2. Circular dichroism (CD) of peptide α-thioesters: (a) H-Asp-Leu-Thr-Phe-Gly-SBzl, (b) H-Asp-Leu-Thr-D-Phe-Gly-SBzl, and (c) H-Asp-D-Leu-Thr-D-Phe-Gly-SBzl.

and β-pleated sheet conformation.[16] We hypothesize that the β-turn structure facilitates the cyclization. To test the effect of the conformation of the linear peptide on cyclization, peptides having the same sequence but residue stereoisomers were synthesized. Model pentapeptide thioesters H-Asp-Leu-Thr-Phe-Gly-SBzl, H-Asp-Leu-Thr-*D*-Phe-Gly-SBzl, and H-Asp-*D*-Leu-Thr-*D*-Phe-Gly-SBzl were synthesized and then cyclized under the general cyclization condition. After 72 h reaction, no linear precursory were detected by LC-MS in all three cases. The results of cyclization of these three peptides were highly sequence dependent (Table 4). Cyclization of peptide thioester made up of all L-amino acids gave 95% of the desired cyclic product and only 5% of the hydrolysis byproduct. Under the same conditions, cyclization of the peptide thioester with LLLDL-amino acid yielded 74% of the hydrolysis product, 5% of the desired cyclic product and 21% of the cyclic dimer. The peptide thioester with LDLDL-amino acid yielded 78% of the cyclic monomer, 2% of the cyclic dimer and 20% of the hydrolysis product. Circular dichroism (CD) study of these purified linear peptide thioesters in aqueous methanol showed that the conformations changed with D-amino acid replacement of residues (Figure 2). The peptide thioester having all-L-residues gave a positive peak at approximately 193 nm, and a negative peak at approximately 204 nm, suggesting the β-turn structure of the linear peptide thioester.[17] The CD spectra of peptide thioester having LLLDL-sequence gave a strong negative peak around 196 nm, suggesting a random coil structure. The CD spectra of LDLDL-peptide thioester was more complicated and had the characteristics of both an α-helical and random coil conformation. Comparison of the CD spectra with the results of cyclization indicates that the cyclization efficiency is correlated to the conformation of linear peptide thioester. Molecular dynamics simulations and quantum mechanics calculations were also applied to these three peptide thioesters.[18] The results are consistent with the cyclization tendencies.

In summary, we present here a general method for the synthesis of cyclic peptides by the intramolecular aminolysis of peptide thioesters. The cyclization was performed in an aqueous acetonitrile solution with imidazole as the catalyst and buffer agent. Studies on the N- and C-terminal residues show the choice of C-terminal residue has a more significant effect on the success rate of cyclization than the choice at the N-terminus. The use of sterically hindered, β-branched residues at the C-termini will greatly decrease cyclization. Regioselectivity studies show the ε-amino group has preference over the α-amino group for cyclization when lysine is at the N-terminus, but no preference when lysine is internally incorporated in the sequence.

Experimental Section

Solid-Phase Synthesis of Peptide α-Thioesters. All peptide α-thioesters were prepared by SPPS using mercaptomethyl phenyl silica gel as volatilizable support.[14] Parallel solid-phase synthesis of peptide α-thioesters were carried out using the "teabag" approach.[15] Stepwise peptide synthesis was carried out using a standard PyBOP/ DIEA coupling protocol. After peptide chain elongation, the resin bound peptide was treated with anhydrous HF for 2 h at 0 °C. Following evaporation of the anhydrous HF and volatile silica gel decomposition products with a gaseous nitrogen stream, the unprotected peptide α-thioester was obtained. The purity and yield data are summarized in Table 3.

General Procedure for the Cyclization of Cyclic Peptides. Linear peptide α-thioesters were dissolved at a concentration of 1 mM in a mixture of acetonitrile and 1.5 M aqueous imidazole solution with a volume ratio of 7:1. The reaction was allowed to proceed at 37 °C for 72 h. An aliquot of 0.1 mL was withdrawn and quenched with 15% TFA in water. The mixture was analyzed by LC-MS. For synthesis of cyclic peptides with larger than 5-amino acids rings, the actual reaction time depends on the rate of cyclization. When no remaining linear peptide α-thioester was detected by LC-MS, the reaction was quenched with 15% TFA in water. The solvents were then removed by lyophilization. The cyclic peptide was isolated by preparative HPLC. The molecular weight of each cyclic peptide was analyzed by HRMS-ESI.

Effect of C-Terminal Residues on Cyclization. A small analogue peptide thioester library H-Ala-Tyr-Gly-Lys-Xxx-SBzl with different amino acids at the C-termini was synthesized using the above procedure. Parallel cyclization was carried out at a concentration of 1 mM at 37 °C for 72 h. After quenched the reaction, the mixture was analyzed by LC-MS.

Effect of N-Terminal Residues on Cyclization. A small analogue peptide thioester library H-Xxx-Tyr-Gly-Lys-Gly-SBzl with different amino acids as the N-terminal residues was synthesized. Parallel cyclization was carried out at a

concentration of 1 mM at 37 °C for 72 h. After quenched the reaction, the mixture was analyzed by LC-MS.

Synthesis of Tyrocidine A. Linear peptide thioester was synthesized having the sequence of D-Phe-Pro-Phe-D-Phe-Asn-Gln-Tyr-Val-Orn-Leu-SCH_2Ph (**4k**). Cyclization was carried out in the same solvent mentioned above at a concentration of 1 mM. The reaction was performed at room temperature (20 °C) overnight. Then the solvents were removed and the resulting residue was purified by preparative HPLC to afford the product **5k**: yield 46%; MS calcd 1269.65 ($C_{66}H_{87}N_{13}O_{13}$); ESI-HRMS *m*/*z* found 1270.50 $[M + H]^+$. The results are presented in the Supporting Information.

Conformational Study of Peptide α-Thioester. The peptide α-thioesters were purified by preparative HPLC. The samples were dissolved in a mixture of 0.1 M phosphate buffer saline (pH 7.2) and methanol (1:1 v/v) at a concentration of 1 mg/mL. Circular Dichroism (CD) spectra was recorded at the range of 190−260 nm with the bandwidth of 1.00 nm and scanning speed of 50 nm/min. The average of five scans was recorded.

Determination of Regioselectivity of Cyclization Reactions. (1). Synthesis of the Linear Peptide Thioesters and Their Cyclic Products. Peptide thioesters of Lys-Phe-Phe-Gly-Gly-SCH_2Ph (**4j**), Glu-Lys-Phe-Tyr-Gly-SCH_2Ph and Glu-Phe-Ala-Lys-Gly-Tyr-Gly-SCH_2Ph were synthesized using the above method. Cyclization reaction was performed at a concentration of 1 mM at 37 °C for 72 h. After quenching the reaction and analyzing the reaction mixture by LC-MS, the solvents were removed and the cyclic product of **5j** was purified by HPLC: yield 32%; MS calcd 566.29 ($C_{29}H_{38}N_6O_6$); ESI-HRMS *m*/*z* found 567.20 $[M + H]^+$.

(2). Preparation of N^{α}-(Dinitrophenyl)-lysine Standards. Fmoc-Lys(Boc)-OH (47.5 mg) was dissolved in 2 mL of 20% piperidine in DMF for 20 min to remove the Fmoc protection group. After removal of the solvent in vacuo, 17 μL of DIEA in 1 mL of DCM was added. To the solution was added 2,4-dinitro-1-fluorobenzene (13 μL). The mixture was reacted at room temperature for 10 min. After removal of the solvent in vacuo, the residue was treated with 55% TFA in DCM for 30 min. The product was extracted with 25% acetonitrile in water and purified by preparative HPLC and confirmed by LC-MS (found 313.1, calculated 313.1 for $[M + H]^+$).

(3). Preparation of N^{ε}-(Dinitrophenyl)-lysine Standards. Fmoc-Lys(Boc)-OH (47.5 mg) was dissolved in 55% TFA in DCM for 30 min to remove the Boc protection group. After removal of the solvent in vacuo, 34 μL of DIEA in 1 mL of DCM was added. To the solution was added 2, 4-dinitro-1-fluorobenzene (13 μL). The mixture was reacted at room temperature for 10 min. After removal of the solvent in vacuo, the residue was treated with 2 mL of 20% piperidine in DMF for 10 min. The product was extracted with 25% acetonitrile in water with 10% TFA and purified by preparative HPLC and confirmed by ESI-MS (found 313.1, calculated for $[M + H]^+$ 313.1).

(4). Determination of N^{α}-(Dinitrophenyl)-lysine and N^{ε}-(Dinitrophenyl)-lysine to Verify the Coupling Site of N-Terminal Lysine. Purified cyclic peptide **5j** (2 mg) was dissolved in 1 mL DMF and then treated with 17 μL of DIEA and 13 μL of 2, 4-dinitro-1-fluorobenzene at room temperature for 10 min. After removal of the solvent in vacuum, the sample was hydrolyzed with 6 M HCl at 110 °C overnight. LC-MS was used to analyze the hydrolysate alone, hydrolysate with N^{α}-(dinitrophenyl)-lysine and N^{ε}-(dinitrophenyl)-lysine to detect the presence of the digestion product. The results are presented in the Supporting Information.

Acknowledgment. This work was supported by the National Science Foundation (RAH CHE 0455072) and the State of Florida, Executive Officer of the Governor's Office of Tourism, Trade and Economic Development, 1P41GM-079590, 1P41GM081261, and U54HG03916-MLSCN.

Supporting Information Available. Experimental details, NMR data of selected product **4a**, copies of HRMS for cyclic peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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