Synthesis and Application of Unprotected Cyclic Peptides as Building Blocks for Peptide Dendrimers

Lianshan Zhang and James P. Tam*

Contribution from the Department of Microbiology and Immunology, Vanderbilt University, A5119 MCN, Nashville, TN 37232-2363

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Abstract: We describe an efficient regiospecific method for cyclization of unprotected peptide segments based on intramolecular transthioesterification of unprotected cysteinyl peptide thioesters under the control of ring−chain tautomeric equilibrium in aqueous buffered solutions at pH ranging from 5 to 7.5. The initial cyclization to form an intramolecular thioester under the ring−chain tautomeric equilibrium is reversible and could be performed in relatively high concentrations without observable oligomerization. This method overcomes the limitation of conventional cyclization methods that require high dilutions. The reaction becomes irreversible by a subsequent, spontaneous proximity-driven *S*- to *N*-acyl transfer to the adjacent N^{α}-amine of cysteine to form an end-to-end cyclic peptide. The cyclization is regioselective. No side reactions were observed with side-chain functionalities such as the N^{ϵ}-amine of lysine, thiol of internal cysteine, or imidazole of histidine. Since a free thiol group was introduced to the product after cyclization, these cyclic peptides were exploited as building blocks for synthesizing peptides with unusual architectures such as bicyclic peptides containing end-to-end backbones and disulfide bridges as well as cascade branched peptide dendrimers.

Introduction

The design and synthesis of novel artificial peptides and proteins with supramolecular characteristics represents an active field of research at the interface of chemistry and medical science.[1,2] Peptide dendrimers, which are a class of biomolecules with extensive uses in immunology and other biomedical applications, can be placed in this category.[3] They are composed of a defined number of copies of a bioactive peptide attached to a lysinyl core matrix. One example is cascade peptide dendrimers, known as multiple antigen peptides (MAPs), that usually contain four or eight copies of the peptide and have been used as immunogens,[4,5] diagnostic agents,[6] and artificial enzymes.[7] They have been recently employed in mimicking the pore structure of ionic channels[8] and in the development of antibiotics[9] and drug delivery vehicles.[10] One of the challenges in synthesizing peptide dendrimers is the construction of constituent molecular building blocks. A useful building block is an unprotected cyclic peptide containing a pendant sulfhydryl group that can be ligated to a branched core matrix via thiol chemistry[5a,b] to form a cyclic peptide dendrimer. The use of unprotected cyclic peptides for such an assembly has the advantages of good aqueous solubility and accessibility to analysis and purification by well-established methods.[5c]

Cyclic peptides have several advantages over their open-chain counterparts. Cyclization of bioactive peptides generally improves their biological properties, such as metabolic stability and receptor selectivity.[11,12] Cyclization imposes constraints that enhance conformational homogeneity and facilitate conformational analysis.[2,13] Consequently, stepwise structural rigidification through cyclization may provide insights into the biologically active conformation of linear peptides. Cyclic peptides have also been used to mimic the surface antigens of proteins for immunological studies.[14] Thus, a cyclic peptide dendrimer may have advantages observed in monomeric cyclic peptides.

A convenient method for the synthesis of a cyclic dendrimer is through a modular scheme in which unprotected cyclic peptides are assembled onto a dendritic core matrix through a pendant sulfhydryl group of the cysteinyl side chain.[5] Several approaches to the synthesis of cyclic peptides have been developed employing a variety of linkages in different forms.[15]

* To whom correspondence should be addressed. Phone: (615) 343-1465. Fax: (615) 343-1467.

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Nearly all are based on linear protected peptide precursors either in solution or anchored on a resin support.[15−17] Recently, we and others have developed methods for macrocyclization of unprotected peptide precursors.[18] One particularly useful form of macrocyclization method is to link unprotected peptides in an end-to-end fashion, forming a peptide bond as frequently occurs naturally.[19]

There are two major considerations in the macrocyclization of unprotected peptide segments to form end-to-end cyclic peptides. First and foremost is regioselectivity. The achievement of regioselectivity of amide bond formation without protecting groups has been demonstrated in the intermolecular coupling of linear peptides.[20−22] A common principle in obtaining regioselectivity of amide bond formation is entropic activation achieved by placing the N^{α}- and C^{α}-termini in close proximity to allow an intramolecular acyl shift, thus forming a peptide bond. Brenner et al.[23] advocated the template-driven amino acyl insertion approach while Kemp et al.[24] have developed a proximity-driven acyl transfer approach that results in highly effective molarity in amide bond formation. We have investigated other variations and refer to these amide bond approaches as orthogonal coupling methods.[25]

Orthogonal coupling is similar in concept to chemoselective ligation based on thiol or aldehyde chemistries,[26−29] but the resulting product contains an amide bond similar to that obtained in an amino acid coupling reaction. In end-to-end macrocyclization, the coupling reaction must be specific between an N^{α}-amine and a weakly activated C^{α}-acyl moiety and proceeds independently of the N^{ϵ}-amine of the side-chain amines and other side-chain functional groups. For our purpose of obtaining a cyclic peptide containing a thiol moiety which can then be used for attachment to a functionalized scaffolding, this could be achieved by orthogonal coupling via intramolecular transthioesterification of a linear peptide precursor containing an N-terminal cysteine and a C-terminal thioester. The thioester intermediate would subsequently undergo a proximity-driven *S*- to *N*-acyl transfer to give an end-to-end cyclic peptide with a peptide backbone and would introduce a thiol moiety that is necessary for the further assembly of peptide dendrimers. The feasibility of intermolecular coupling of two unprotected peptide segments was proposed 40 years ago by Wieland et al.[30] and was recently demonstrated in larger peptide segments by Dawson et al.[21] and our laboratory.[22] However, the feasibility and regioselectivity of intramolecular transthioesterification for preparing cyclic peptides have not been determined.

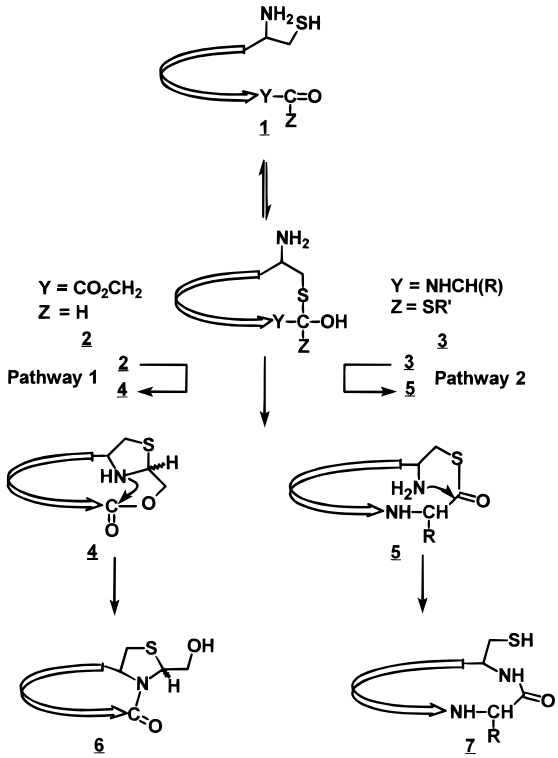

Figure 1. Cyclization of N-terminal cysteinyl peptides through transthioesterification under the control of ring−chain tautomeric equilibrium.

The second consideration in the macrocyclization of unprotected peptide segments is the avoidance of oligomerization.[31] Since both ends of a cysteinyl linear thioester peptide precursor are reactive moieties, oligomerization would result in low yields and would require that the macrocyclization be performed in high dilution. Recently, we have found that we could overcome this limitation by exploiting the equilibrium of ring−chain tautomerization deriving from the weakly activated nature of the C^{α}-acyl moiety used for orthogonal coupling. In ring−chain tautomerization (Figure 1), an open-chain system (**1**) containing

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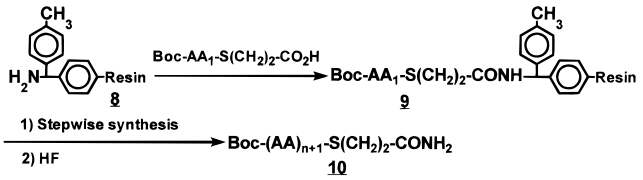

Figure 2. Preparation of peptide thioesters from stepwise solid-phase peptide synthesis.

an N^{α}-amine at one end and a polar multiple bond at the other end is transformed into a cyclic intermediate (**2** or **3**) through reversible intramolecular addition of the thiol group to the polar multiple bond.[32] A water, thiol, or alcohol molecule is then eliminated to yield a stable nonisomeric heterocycle (**4** or **5**). By positioning the acyl group and the amine moiety in close proximity, intramolecular acyl migration can be effected to give an amide bond in compound **6** or **7**. This principle has been successfully demonstrated in an open-chain system of an amino aldehyde peptide containing an N-terminal cysteine and a C-terminal glycoaldehyde ester (Figure 1, pathway 1). An open chain of an N-terminal cysteinyl peptide thioester can be considered as a variation of this system since the initial step in transthioesterification is also a reversible reaction, particularly in the presence of an excess of a small thiol compound such as 3-mercaptopropionic acid. Upon formation of the cyclic intermediate **3** which is a cyclic isomer of the open-chain compound **5**, an alkanethiol leaving group is eliminated to give a cyclic thiolactone (**5**) (Figure 1, pathway 2). This nonisomeric heterocycle will subsequently rearrange into a lactam (**7**) through a five-membered ring *S*- to *N*-acyl transfer. In this way, racemization may be greatly minimized, and cyclization can be carried out in high concentration.

In this paper, we describe and define the scope of the intramolecular transthioesterification for the cyclization of unprotected cysteinyl peptide thioesters under the influence of ring−chain tautomerization equilibrium and their application for the synthesis of a bicyclic peptide and two cyclic peptide dendrimers.

Results

Synthesis of Peptide Thioesters. All peptide thioesters were prepared directly by solid-phase peptide synthesis[33] on a methylbenzhydrylamine (MBHA) resin (**8**) using Boc (*tert*-butyloxycarbonyl) chemistry with the exception of **15** for which Boc-Gly-4-[[(oxymethyl)phenyl]acetamido]methyl (Boc-Gly-Pam) resin was used (Figure 2). The thioester resin **9** was prepared according to Aimoto et al.[34] by converting the C-terminal Boc-amino acid to its 3-mercaptopropionic acid ester and then attaching it onto the resin by *N*,*N*′-diisopropylcarbodiimide (DICI)/1-hydroxybenzotriazole (HOBt). After assembly of the sequence on the solid support by using the (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) coupling protocol, the peptide thioester **10** was cleaved from the resin by HF/anisole (9:1, v/v) at 4 °C. Thus, the N-terminal cysteinyl peptides obtained were alkyl thioesters **11**−**16** containing 3′-propionamide (Table 1). All peptides were purified by reversed phase HPLC (RP-HPLC) and characterized by matrix-assisted laser desorption ionization MS (MALDI-MS). Peptide alkyl thioesters were found to be relatively stable and could be purified under acidic conditions. Thus, they have the advantage over their corresponding aryl thioesters with respect to the susceptibility to hydrolysis in aqueous solutions.

Table 1. Summary of Yields and MALDI-MS Data of Peptide Thioesters and Their Cyclic Products

sequence		thioester		cyclic peptide	
linear	cyclic	yield[a] (%)	MW[b]	yield[c] (%)	MW[b]
CGGFL (CL-5) (**11**)	**c11**	87	581.7	92	477.6
CYGGFL (CL-6) (**12**)	**c12**	85	744.9	91	641.0
CKYGGFL (CL-7) (**13**)	**c13**	82	873.1	93	768.9
CKAYGGFL (CL-8) (**14**)	**c14**	83	944.1	90	840.0
CAVSEIQFMHNLGK (CK-14) (**15**)	**c15**	72	1721	90	1559
CSNLSTCVLGKLSQEL (CL-16) (**16**)	**c16**	70	1780	82	1677
CSNLSTCVLGKLSQEL (CL-16) (**16**)	**17**	70	1780	75	1675

[a] Crude yield from solid-phase synthesis. [b] MW obtained by MALDI-MS and in agreement with the calculated MW. [c] Isolated yield after cyclization.

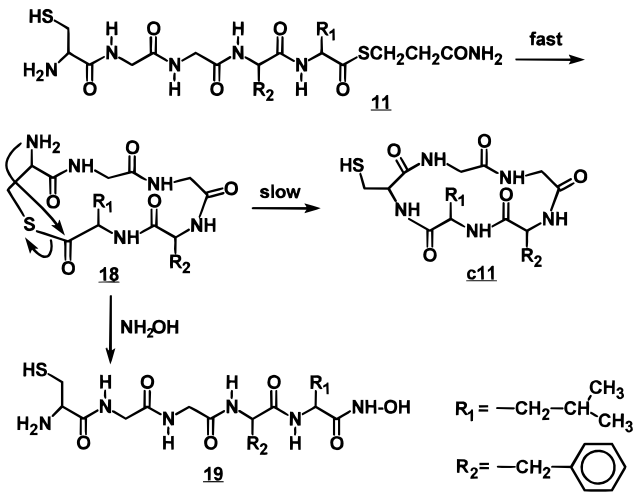

Figure 3. Products and a cyclic intermediate identified during the cyclization of Cys-Gly-Gly-Phe-Leu-$SCH_2CH_2CONH_2$ (**11**) (CL-5).

Concentration-Independent Cyclization and Ring−Chain Equilibrium. Four model peptides ranging from 5 to 8 residues (**11**−**14**) used for cyclizations via transthioesterification were analogs of enkephalin and contained the core sequence of Gly-Gly-Phe-Leu (Figure 3). Cysteine was placed at the N-termini and a thioester at the C-termini of the peptides to obtain an end-to-end cyclic peptide. After cleavage from the resin support, the crude unprotected cysteinyl peptide thioesters were found to be sufficiently pure (>87%) and were used directly for cyclization at pH ≥ 6.4 in phosphate buffer containing a 3−5 fold excess of 3-mercaptopropionic acid (Table 1). The roles of a thiol are to minimize polymerization and convert the cysteinyl *S*-acyl byproduct to the starting material.[22] In general, cyclization occurred when the pH of the cysteinyl peptide thioester solution was adjusted to >4. Water-soluble tris-(carboxyethyl)phosphine (TCEP)[35] was added to prevent disulfide formation and to accelerate the desired reaction.[22] The cyclization occurred cleanly and efficiently as shown by RP-HPLC (Figure 4) in yields ranging from 78 to 92% (Table 1). Usually, cyclization was completed within 4 h. The cyclization could be run in moderate to high concentrations at millimolar levels without affecting the yield. For example, cyclization of purified CL-7 (**13**) was carried out in 1, 3.5, and 7 mM at pH

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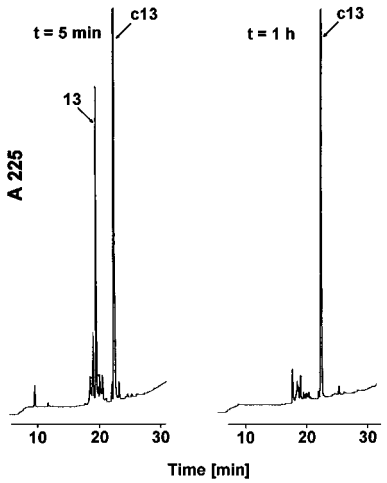

Figure 4. RP-HPLC profiles of the cyclization reaction of **13** (CL-7), Cys-Lys-Tyr-Gly-Gly-Phe-Leu-$SCH_2CH_2CONH_2$.

Table 2. Cyclization Rates of N-Terminal Cysteinyl Peptide Thioesters at Different pH Values

peptide sequence		ring size		$t_{1/2}$ (min)		
linear	cyclic	atoms	amino acid	pH 5.2	pH 6.4	pH 7.5
CGGFL (CL-5) (**11**)	**c11**	15	5	30	4.4	1.7
CYGGFL (CL-6) (**12**)	**c12**	18	6	10	1.5	0.5
CKYGGFL (CL-7) (**13**)	**c13**	21	7	29	5.0	0.5
CKAYGGFL (CL-8) (**14**)	**c14**	24	8	30	6.5	0.6
CAVSEIQFMHNLGK (CK-14) (**15**)	**c15**	42	14	162	40	2.2
CSNLSTCVLGKLSQEL (CL-16) (**16**)	**c16**	48	16	198	50	3.5

7.5. Oligomerization of the peptide which is often the case in cyclization using protected peptide segments was not detected by RP-HPLC. In all three cases, more than 90% of cyclic CL-7 (**c13**) was recovered as the isolated yield after RP-HPLC purification. Similar results were also obtained from CL-6 (**12**), CK-14 (**15**), and CL-16 (**16**). In the case of **15**, the reaction could be carried out in a concentration of 20 mM peptide, which was >200-fold higher than that of the conventional approach using active ester and high dilution.[31b] Thus, in the model studies, the cyclization appeared to be concentration-independent and conformed with the expected equilibrium behavior of ring−chain tautomerism.

Effect of Ring Size and pH. To determine the dependence of the cyclization rates on ring size, a series of cyclic analogs with 15−48-atom rings were synthesized, and their cyclization rates were assessed (Table 2). Peptides **11**−**14** with 5−8 residues cyclized with $t_{1/2}$ < 0.5 h at pH 5.6 while peptides **15** and **16** with large rings of 14 and 16 residues proceeded slowly with $t_{1/2}$ = 2.7 and 3.3 h. This result is consistent with the correlation of cyclization rates and the distance between the N- and C-termini of peptide chains with faster rates observed in smaller peptides.

The pH dependence of cyclization was also examined at pH 5.2, 6.4, and 7.5 (Table 2), and the cyclization rates were found to be faster as the pH increased. In the case of CL-6 (**12**), the $t_{1/2}$ was 10, 1.5, and 0.5 min when cyclization was performed at pH 5.2, 6.4, and 7.5, respectively. In general, the useful cyclization pH range is between 6 and 7.5. Below pH 5, the reaction is too slow, and side reactions occur at pH > 8 due to thioester hydrolysis at a rate of about 20%/h. The enhanced reactivity of the cyclization observed at higher pH is in line with the expected basicity of the unprotonated cysteine amino groups during the *S* to *N*-acyl transfer.

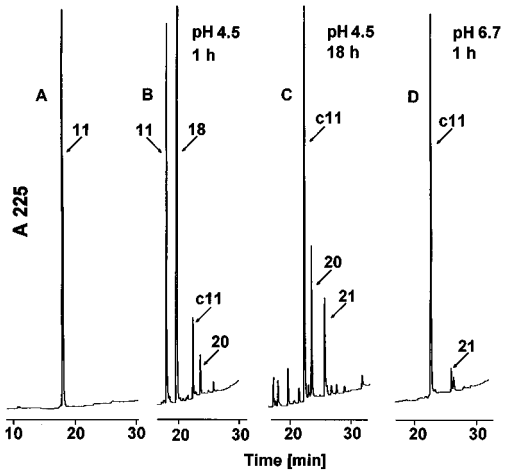

Figure 5. RP-HPLC profiles of product distribution during the cyclization of the linear precursor, Cys-Gly-Gly-Phe-Leu-$SCH_2CH_2CONH_2$ (**11**): (A) starting material **11**; (B) pH 4.5 after 1 h; (C) pH 4.5 after 18 h; (D) pH 6.7 after 1 h (**11**, starting material; **18**, cyclic thiolactone intermediate; **c11**, cyclic lactam product; **20**, byproduct formed by dimerization of the starting material via an amide bond; **21**, byproduct formed by dimerizatin of the product via a disulfide bridge.

Thiolactone Intermediate. In cyclization via transthioesterification, the thiolactone is usually not observed when the reactions are performed at pH 6.5−7.5. This thiolactone rapidly rearranged through an S to N shift to the desired end-to-end cyclic peptide. To show that the cyclization does occur through a thiolactone intermediate and not by direct thioester displacement of the N^{α}-amine, a pentapeptide thioester, Cys-Gly-Gly-Phe-Leu-S-$CH_2CH_2CONH_2$ (**11**) (CL-5), was used as a model. In this case, the thiolactone intermediate **18** is a 16-membered ring, and a ring contraction via an S to N shift gives a 15-membered end-to-end cyclic peptide (**c11**) (Figure 3). Because of the ring strain, the contraction via *S*- to *N*-acyl shift is likely to be slow, and it should be possible to isolate the labile thiolactone intermediate **18**. Indeed, at pH 4.5, cyclization of this pentapeptide proceeded slowly and gave a major product eluting between the starting material **11** and the lactam **c11** in analytical RP-HPLC (Figure 5). There were several lines of evidence indicating that this peak was the thiolactone intermediate. These included the change of retention time in RP-HPLC (Figure 5B), the correct molecular weight in the MALDI-MS (found 499.5, calcd for $[M + Na - H]^+$ 499.5), and the susceptibility of the thiolactone intermediate to hydroxylamine at pH 9[36] (Figure 3). The corresponding hydroxylamine derivative **19** of this peptide was isolated and further confirmed by MALDI-MS (found 532.9, calcd for $[M + Na - H]^+$ 532.6). In our study of the intermolecular transthioesterification of two unprotected peptide segments, the *S*- to *N*-acyl transfer occurs spontaneously, and thus far, the thioester intermediate has not been observed. Therefore, it is interesting that we could confirm the thioester intermediate using this cyclic pentapeptide model. At pH <4, the cyclic intermediate underwent a new intermolecular transthioesterification with the starting material, giving rise to a dimer of CL-5 as a byproduct (**20**) (Figure 5C). The byproduct had a molecular mass of 1082 as detected by MALDI-MS (found 1082, calcd for $[M + Na - H]^+$ 1082). It was identified as CGGFLCGGFL-S-$CH_2CH_2CONH_2$. This side reaction could be avoided by performing the reaction at pH > 6 to accelerate the S to N transfer in the cyclic intermediate (Figure 5D).

(36) Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70−77.

Selectivity. Two factors affecting cyclization were examined, the first regioselectivity and the second structural propensity. The former involves competing side-chain functional groups such as the amine of lysine, thiol of cysteine, and imidazole of histidine at internal positions of the peptide sequence. To study the effect of structural propensity on the cyclization reaction, two groups of peptides were selected. The first group consisted of flexible enkephalin analogs with internal glycine. The second group, exemplified by the N-terminal peptides of human parathyroid hormone (hPTH) and salmon calcitonin, has an amphiphilic character and α-helix propensity.[37,38]

Two peptides, CL-7 **13** and CL-8 **14**, were used to determine the regioselectivity of the cyclization. Because the N^{ϵ}-amine of lysine near the N^{α}-amine terminus would be most likely to interfere with the *S*- to *N*-acyl transfer, a lysine residue was inserted near the N^{α}-termini of the peptides to compete for the S to N shift during cyclization. It is important to stress that both forms of cyclic peptides (end-to-end vs end-to-side-chain) are likely to be favorable because there is little difference in their ring sizes. The end-to-end cyclic product of CL-7 is a 21-membered ring while the lysine side-chain cyclic product is a 22-membered ring. Similarly, the ring sizes of both CL-8 products would differ only by 1 atom (24 vs 25). In both cases, no lysine side-chain cyclic peptides were observed, showing that the side-chain amino group of lysine was not involved in the cyclization reaction.

Using CL-7 (**13**) as an example, the following method was used to determine regioselectivity of the intramolecular acylation. Cyclization of CL-7 was carried out in 0.2 M phosphate buffer (pH 7.2) in the presence of 2 equiv of TCEP. The reaction was complete within 2 h. Only one major peak appeared, and the yield was 95% as indicated by analytical RP-HPLC. The cyclized product **c13** was characterized by MALDI-MS (found 770, calcd for $[M + H]^+$ 769) and amino acid analysis. To verify α- and ϵ-cyclization, the peptide was oxidized with HCO_3H to convert cysteine into cysteic acid and then treated with 2,4-dinitro-1-fluorobenzene (Sanger's reagent)[39] and hydrolyzed with 5.7 M HCl at 110 °C for 24 h. RP-HPLC analysis showed that no N^{α}-(dinitrophenyl)(DNP)-cysteic acid was found (t_R = 4.7 min), and only the desired N^{ϵ}-DNP-lysine was detected (t_R = 11.7; for HPLC conditions, see the Experimental Section), indicating the cyclization had occurred at the N-terminus. Enzymatic digestion of the product further confirmed the end-to-end cyclization since treatment of the peptide with trypsin gave a single peak with t_R = 16.6 min on RP-HPLC (for HPLC conditions, see the Experimental Section). The expected peptide, YGGFLCK, was further characterized by MALDI-MS (found 788, calcd for $[M + H]^+$ 788).

The N-terminal sequence of human parathyroid hormone (hPTH 1−13), Cys-Ala-Val-Ser-Glu-Ile-Gln-Phe-Met-His-Asn-Leu-Gly-Lys-S-$(CH_2)_2$CONH-CH_2CO_2H (**15**) (CK-14) obtained from a different thioester resin is a more stringent example for demonstrating regioselectivity because it is rich in side-chain functional groups, including a C-terminal lysine and an internal histidine. Cyclization of unprotected peptides such as CK-14 could potentially generate several cyclic products due to end-to-side-chain cyclization. The cyclization was performed essentially the same as described in the case of CL-7. Only one product (**c15**) was obtained when the cyclization was effected at pH 7.2. Treatment of the cyclic product with Sanger's reagent and subsequent hydrolysis of the peptide revealed that the cyclization had occurred in an end-to-end fashion because only N^{ϵ}-DNP-lysine was obtained by analytical RP-HPLC. The product was further confirmed by MALDI-MS (found 1559.2 ± 1, calcd for $[M + H]^+$ 1558.8). Trypsin digestion of (**c15**) (t_R = 20.27 min) gave the expected linear peptide as detected by analytical RP-HPLC (t_R = 18.47 min) and MALDI-MS (found 1578.4, calcd for $[M + H]^+$ 1577.8).

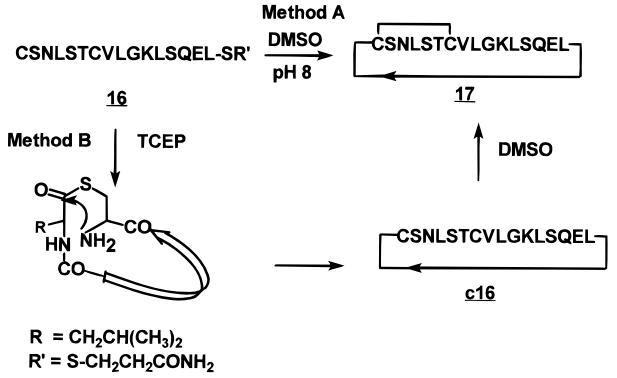

Figure 6. Synthesis of bicyclic calcitonin fragment 1−16 (**17**).

To determine whether the internal cysteine interferes with the desired reaction, a 16-residue peptide (**16**) corresponding to the N-terminal sequence of salmon calcitonin 1−16, Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu-S-$CH_2CH_2CONH_2$ (CL-16), was subjected to cyclization, which was carried out in 0.2 M phosphate buffer (pH 7.2) containing 50% DMF in the presence of 6 equiv of TCEP. *N*,*N*-Dimethylformamide (DMF) was used as a cosolvent to increase solubility. Analytical RP-HPLC showed the completion of the reaction after 3 h. A small amount of byproduct (<5%) was formed due to the hydrolysis of the peptide thioester as detected by RP-HPLC. Similar cyclization experiments without adding TCEP led to oligomerization of the cyclic peptide via disulfide bond formation. Results were improved when the reaction was performed at 4 °C overnight to suppress thioester hydrolysis. The cyclic peptide **c16** was purified by preparative RP-HPLC and identified by amino acid analysis and MALDI-MS (found 1677 ± 1, calcd for $[M + H]^+$ 1676.9). Treatment of **c16** (t_R = 22.42 min) with trypsin yielded the expected peptide as identified by analytical RP-HPLC (t_R = 19.24 min) and MALDI-MS (found 1695.4, calcd for $[M + H]^+$ 1695.0). End group analysis using Sanger's reagent, 2,4-dinitro-1-fluorobenzene, showed that no DNP-cysteic acid was found, indicating that only cysteine was the ligation site. The results revealed that the internal cysteine did not affect transthioesterification and the subsequent *S*- to *N*-acyl rearrangement.

Applications. An advantage of transthioesterification is the presence of a free thiol in the end product. Two applications could be visualized to exploit this advantage for the formation of peptides with unusual architectures. These included the synthesis of two cascade peptide dendrimers as well as a bicyclic peptide containing an end-to-end backbone and a disulfide bridge.

1. Bicyclic Peptide. To demonstrate the feasibility of forming bicyclic peptides, purified cyclic CL-16, cyclo(Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Gly-Lys-Ser-Gln-Glu-Leu) (**c16**), obtained as previously described was oxidized with 10% dimethyl sulfoxide (DMSO) in 0.2 phosphate buffer (pH 7.2) for 12 h (Figure 6). The bicyclic product **17**, an end-to-end cyclic peptide with a disulfide bridge, was obtained in a yield of 82% (method B, Figure 6). This bicyclic product could also be synthesized by a one-pot reaction, in which cyclization and disulfide bond formation of the linear thioester peptide **16**

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(38) (a) Motta, A.; Morelli, M. A.; Goud, N.; Temussi, P. A. *Biochemistry* **1989**, *28*, 7996−8002. (b) Meadows, R. P.; Nikonowicz, E. P.; Jones, C. R.; Bastian, J. W.; Gorenstein, D. G. *Biochemistry* **1991**, *30*, 1247−1254.
(39) Sanger, F. *Biochem. J.* **1945**, *39*, 507−515.

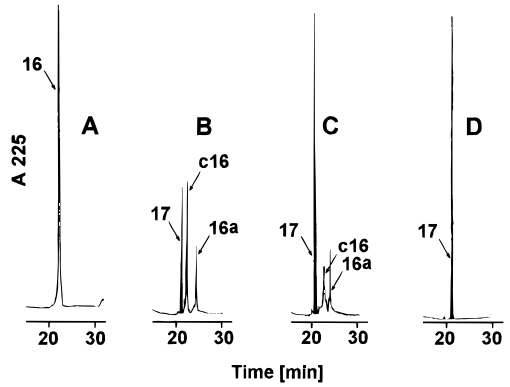

Figure 7. RP-HPLC profiles of the cyclization progress of **16**: (A) starting material **16**; (B) reaction after 1 h; (C) reaction after 8 h; (D) purified bicyclic product **17** (**16**, starting material; **17**, bicyclic product; **c16**, monocyclic intermediate; **16a**, byproduct resulting from dimerization of **16** via a disulfide bond.

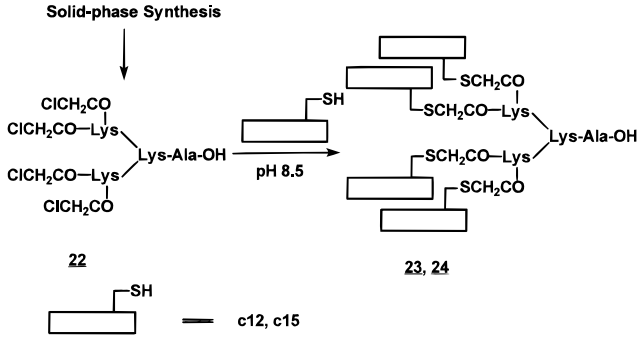

Figure 8. Synthesis of cyclic peptide dendrimers **23** and **24** through thioether linkage.

were achieved sequentially without isolation of the intermediate (method A, Figure 6). The reaction was monitored by analytical RP-HPLC (Figure 7). Interestingly, an intermediate was found during the reaction. This intermediate was characterized as the monocyclic peptide bearing free thiol groups (**c16)** by MALDI-MS (found 1677 ± 1, calcd for $[M + H]^+$ 1676.9), indicating that the amide bond formation via transthioesterification proceeded much faster than disulfide bond formation mediated by DMSO even though intramolecular disulfide formation was favored at this oxidative solution. After 18 h the bicyclic peptide was purified by preparative HPLC in a yield of 75%. No free thiol groups were present in the cyclic peptide, as detected by Ellman's reagent.[36] The product **17** was characterized by amino acid analysis and MALDI-MS (found 1675 ± 1, calcd for $[M + H]^+$ 1674.9). The bicyclic products prepared by both methods were identical in RP-HPLC behavior and MS.

2. Cyclic Peptide Dendrimers. To demonstrate the usefulness of cyclic peptides as building blocks for the construction of peptide dendrimers, we assembled cyclic CL-6 (**12**), an enkephalin derivative, onto a four-branched tetravalent lysinyl core peptide. The thiol group of the cyclic CL-6 was intended as a linking site. Thus, a tetravalent (chloroacetyl)lysinyl core peptide was synthesized by the solid-phase method (Figure 8). The assembly of **12** and the core peptide **22** through thioether formation was performed by incubation of 2.5 equiv of cyclic CL-6 with the core peptide in 0.2 M phosphate buffer/DMF (3:1, pH 8.5) containing 0.01 M EDTA under argon. The reaction was checked by RP-HPLC and was complete in 48 h. The desired tetravalent cyclic peptide dendrimer **23** was found as a major product and confirmed by MALDI-MS (found 3128.4 ± 1, calcd for $[M + Na - H]^+$ 3127.7. The byproduct was identified as a dimer of cyclic CL-6 via a disulfide bond.

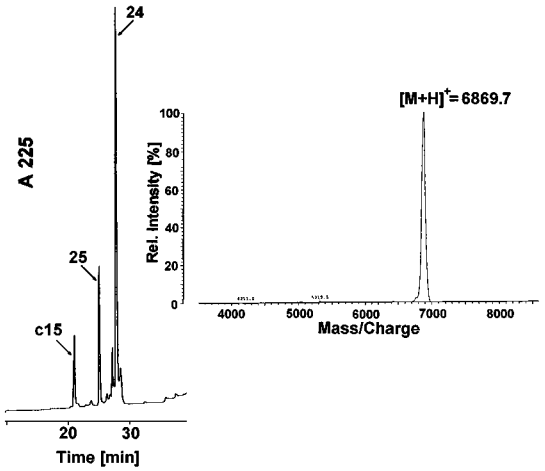

Figure 9. RP-HPLC profile of the conjugation of cyclic CL-14 (**c15**) to the tetravalent lysinyl core peptide **22** (left) and MALDI-MS analysis of the product **24**. **25** was identified as the dimer of **c15** via a disulfide bridge.

Using this condition, cyclic CK-14 also could be assembled to the tetravalent (chloroacetyl)lysinyl core peptide **22**. The reaction proceeded cleanly as shown in analytical RP-HPLC (gradient 30−100% buffer B in 30 min) and was complete in 40 h (Figure 9). The product **24** was isolated from the excess cyclic CL-14 in a yield of 80% based on the core peptide. TCEP was added to the reaction mixture prior to isolation of the product to convert the dimeric byproduct **25** formed via a disulfide bridge to its monomer. The monomer **c14** was recovered from the reaction and used in a recycling manner. The product was finally analyzed by RP-HPLC and confirmed by MALDI-MS (found 6869.7 ± 1, calcd for $[M + H]^+$ 6868.7).

Discussion

Intermolecular transthioesterification has been successfully applied to the formation of a peptide bond between two unprotected peptide segments.[21,22] We exploit this new chemistry for the cyclization of unprotected peptides. An unanswered question is whether transthioesterification can fulfill the requirements for forming an end-to-end cyclic peptide from an unprotected linear precursor with high efficiency and regiospecificity. Our results show that transthioesterification appears to meet these requirements. The ability of the cysteinyl peptide thioester to undergo ring−chain tautomerism may contribute to the high efficiency of cyclization and limit competing oligomerization. Ring−chain tautomerism is made possible by the weakly activated C^α-moiety which allows the thioester exchange between the C^α-thioester and the free thiol at the N-terminus. In this case, intramolecular addition of a free thiol to the carbonyl of the C^α-thioester followed by the elimination of a thiol molecule results in a nonisomeric heterocycle as a cyclic intermediate. This reaction occurs even in the presence of an excess of another small thiol molecule. Such a reversible intramolecular reaction is commonly observed in the synthesis of macrocycles and heterocyclic compounds.[32] The influence of ring−chain tautomerism is supported by evidence that the cyclization can be performed in different concentrations without affecting the yield. In the case of peptide CL-5, it is interesting that the *S*- to *N*-acyl transfer of the cyclic intermediate proceeds too slowly at pH 4, and that a cyclic thioester intermediate of this reaction can be isolated and characterized. Usually, the *S*- to *N*-acyl transfer occurs spontaneously at pH 7, and no cyclic thioester is observed.

Previous methods for cyclization of peptides in solution rely on a combination of enthalpic activation of the C^α-carboxy

group and a protecting strategy for side chains. In addition, cyclization reactions need to be carried out in high dilution to minimize unwanted competing intermolecular oligomerization. In contrast, our method uses a combination of a weakly activated thioester and a chemoselective transthioesterification, resulting in a proximity-driven acyl transfer that avoids the limitations of conventional methods. Furthermore, because the intramolecular transthioesterification is favored under equilibrating conditions of ring−chain tautomerizaton, moderate to high concentrations of peptides can be used for the cyclization reaction.

The high regiospecificity of cyclization achieved through transthioesterification is demonstrated in our model peptides in which there are no significant side reactions in the peptides containing side-chain functionalities such as the amine of lysine, thiol of cysteine, guanidine of arginine, and imidazole of histidine at internal positions of the peptide sequence. The selectivity of thiol over amines at side chains is understandable because the thiol group is a stronger nucleophile at nearly neutral pH. The selectivity of the thiol at the N-terminus over the internal cysteinyl thiol, although not readily apparent, could be attributed to two factors. First, N-terminal cysteine contains an α-amine in a 1,2-relationship which can serve as a general base to facilitate transthioesterification while the internal cysteine does not have this advantage. The general base assistance of an N^{α}-amine in the rate acceleration of disulfide formation on N^{α}-cysteinyl peptides have been shown to be 3−10-fold faster than the corresponding disulfide formation between internal cysteinyl thiols.[40] This also provides an explanation as to the great susceptibility of N^{α}-cysteinyl peptide to form a disulfide bond homodimer as compared to those peptides with an internal cysteine residue. Second, the transthioesterification between N-terminal cysteine and thioester will lead to a proximity-driven *S*- to *N*-acyl transfer to form a stable peptide bond whereas transthioesterifications between thiols of internal cysteines are likely to be reversible. The reversibility of S-acylation of an internal cysteine has been demonstrated and can be suppressed with the addition of an excess of thiol compounds such as 3-mercaptopropionic acid.[22]

Although peptides ranging from 5 to 16 residues in our study are cyclized in relatively high concentrations with high yields, the structural factor contributing to high efficiency of cyclization has not been throughly worked out. In this study, we cyclized without any difficulties peptides containing internal glycine, which favors reverse turns, as well as peptides with high α-helical propensity such as the N-terminal sequences of human parathyroid hormone (CK-14) and salmon calcitonin (CK-16). In a related study, we also used ring−chain tautomerism to cyclize peptides ranging from 5 to 26 residues with a β-strand−turn−β-strand structure[18a] as well as circular permutated peptides with >30 amino acid residues.[41] Small to medium flexible peptides appear amenable to ring−chain tautomeric equilibrium with a low risk of oligomerization. However, it remains to be determined whether the approach is applicable to the cyclization of larger and rigidified peptides, or whether a strong denaturing condition, such as the presence of a high concentration of urea or guanidine, is required to force such peptides to cyclize with high efficiency.

Cyclic peptides with a thiol moiety are useful building blocks for further transformations. Thiols can be transformed into other functional groups, providing a useful entry for the synthesis of cyclic peptides containing unusual amino acids.[42] Furthermore, cyclic peptides bearing a free thiol can be utilized for assembly of cyclic peptide dendrimers or synthetic protein constructs. Such complex constructs of defined structure are beginning to find application as vaccine candidates, as immunodiagnostic agents, and as drug delivery vehicles. Two tetravalent cyclic dendrimers were synthesized as examples to demonstrate the usefulness of this method. The cyclic peptide dendrimers obtained in this way were of high homogeneity. A bicyclic peptide with interesting structural complexity was also prepared. It contained an end-to-end backbone and a disulfide bridge. Several natural products with interesting medicinal properties such as circulins[43a] and kalata[43b,c] belong to this category, and our approach may be useful for their syntheses.[41]

Experimental Section

Solid-Phase Peptide Synthesis. All peptide thioesters were prepared by SPPS according to the procedure described by Aimoto et al.[34] using 4-methylbenzhydrylamine (MBHA) or Boc-Gly-[[4-(oxymethyl)phenyl]-acetamido]methyl (Boc-Gly-Pam) resin at a substitution level of 1.1 mmol/g. The first amino acid thioester was incorporated on the resin in its propionic acid form. Typically, 0.3−0.4 g of resin was used for each synthesis. All amino acids were protected with an N^{α}-*tert*-butoxycarbonyl (Boc) group. The side-chain protections were as follows: Arg(Tos), Asp(OcHex), Cys(4-MeBzl), Glu(OcHex), His(Tos), Lys(2-ClZ), Ser(Bzl), and Thr(Bzl). Each synthesis cycle consisted of (i) a 25-min deprotection with 55% trifluoroacetic acid/CH_2Cl_2 and (ii) coupling with 4 equiv each of Boc-amino acid and BOP or HBTU in the presence of 6 equiv *N*,*N*-diisopropylethylamine (DIEA) in DMF for 1 h. All couplings were monitored by the ninhydrin test, and a double coupling was used with 3 equiv each of Boc-amino acid and *N*,*N*′-diisopropylcarbodiimide (DICI)/1-hydroxy-7-azabenzotriazole (HOAt)[44] if necessary.

After assembly of the sequence using BOP/*N*,*N*-diisopropylethylamine (DIEA, 1:1.5) as coupling reagent, the peptide thioesters were cleaved from the resin by HF/anisole (9:1) at 4 °C for 1 h. After HF was removed, the resulting residue was washed with diethyl ether and then extracted with 60% acetonitrile in H_2O containing 0.045% trifluoroacetic acid (TFA). All crude peptides were purified on a preparative C_{18} reversed-phase (RP) HPLC column (250 × 22 mm) with a linear gradient of H_2O containing 0.045% TFA and 60% acetonitrile in H_2O containing 0.039% TFA at a flow rate of 10 mL/min. The major fractions were lyophilized, and the total yields ranged from 70 to 87%, based on the first amino acid loading to the resin (Table 1). In general, such peptide thioesters can be purified and stored under appropriate conditions without reaction or loss of the thioester moiety.

Characterization of Peptide Analogs. Amino acid analysis of each synthetic peptide was carried out in 5.7 M HCl at 110 °C for 24 h and was found to agree with the theoretical ratio. Analytical HPLC for all peptides was performed on a Vydac column (250 × 4.6 mm) with a 1-min isocratic gradient of 10% buffer B and a 30-min linear gradient of 10−100% buffer B (gradient 1) at a flow rate of 1 mL/min (buffer A, 0.045% TFA in H_2O; buffer B, 0.039% TFA in 60% CH_3CN in H_2O). All synthetic peptides were finally characterized by MALDI-MS (Table 1).

Verification of regiospecificity of cyclization reactions. I. Determination of N^{α}-DNP-cysteic Acid and N^{ϵ}-DNP-Lys To Verify the Coupling Site. (i) Preparation of N^{α}-DNP-cysteic Acid and N^{ϵ}-DNP-Lys Standards. (a) N^{α}-DNP-cysteic Acid. L-Cysteic acid monohydrate (18.7 mg, 0.1 mmol) was dissolved in 0.2 M phosphate

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buffer (400 μL, pH 6.9). This solution was added to 2,4-dinitro-1-fluorobenzene (18.6 mg, 0.1 mmol). The mixture was heated at 40 °C for 2 h. After removal of the solvent *in vacuo*, the residue was washed several times with diethyl ether. The product had a t_R of 4.7 min by analytical HPLC as detected at 408 nm (gradient 2: 1 min 30% B, isocratic; 30−100% within 30 min). The molecular mass of N^{α}-DNP-cysteic acid was confirmed by MALDI-MS (found 336.3, calcd for $[M + H]^+$ 336.2).

(b) N^{ϵ}-DNP-Lys. N^{α}-Boc-Lys-OH (24.6 mg, 0.1 mmol) was suspended in 0.2 M phosphate buffer (400 μL, pH 6.9). To the suspension was added 2,4-dinitro-1-fluorobenzene (18.6 mg, 0.1 mmol). The mixture was heated at 40 °C for 2 h. After removal of the solvent *in vacuo*, the residue was treated with TFA for 10 min. The product was isolated by preparative HPLC and confirmed by MALDI-MS (found 313.5, calcd for $[M + H]^+$ 313.3). N^{ϵ}-DNP-Lys had a t_R of 11.7 min (HPLC conditions as described above).

(ii) Determination of N^{α}-DNP-cysteic Acid and N^{ϵ}-DNP-Lys through Analytical HPLC. A 100 nmol sample of cyclic peptide was dissolved in 250 μL of 88% formic acid. The solution was cooled to 4 °C, and then 500 μL of a premade mixture of 88% formic acid and 30% hydrogen peroxide (9:1) was added. The mixture was allowed to stand for 2 h in an ice bath. The mixture was diluted with 10 mL of H_2O and lyophilized. The sample was then treated with 2,4-dinitro-1-fluorobenzene as described above and hydrolyzed with 5.7 M HCl at 110 °C for 24 h. The hydrolysate was analyzed with RP-HPLC using gradient 2. No N^{α}-DNP-cysteic acid was detected in cyclic peptides obtained from CL-5, CL-6, CL-7, CL-8, CK-14 and CL-16, indicating the coupling proceeded through the α-amino terminus of cysteine.

(II) Enzymatic Digestion of Cyclic Peptides. c13, c15, and c16. To a solution of cyclic peptide (0.15 μmol) in 0.01 M Tris buffer (pH 7.3, 0.5 mL) was added 10 μg trypsin of dissolved in the same buffer (2 mg/1 mL). The mixture was shaken for 3 h, and an aliquot was taken for analytical RP-HPLC using gradient 1. As expected, all three cyclic peptides were digested into linear peptides. MALDI-MS showed these to be expected products corresponding to Lys-Xaa bond hydrolysis. Anal. RP-HPLC (t_R in min): t_R = 20.98 (**c13**), t_R = 16.65 (**c13** after digestion); t_R = 20.27 (**c15**), t_R = 18.47 **(c15** after digestion), t_R = 22.42 (**c16**), t_R = 19.24 (**c16** after digestion). MALDI-MS: 788.8 (calcd for YGGFLCK 786.7, t_R = 16.65), 1578.4 (calcd for CAVSEIQFMHNLGK 1577.8, t_R = 18.47), and 1695.4 (calcd for LSQELCSNLSTCVLGK 1695.0, t_R = 19.24).

General Procedure for the Cyclization of Enkephalin Analogs. Linear precursors **11**−**16** were dissolved in 0.2 M Na_2HPO_4 0.1 M citric acid buffer (pH 7.2) and diluted with the same buffer to the desired concentration which ranged from 1 to 7 mM. A 1−2 equiv sample of TCEP was added to the solution. Aliquots were withdrawn for analytical HPLC. The cyclizations were usually complete within 2 h. The cyclized peptides were isolated by preparative HPLC. Analytical data and cyclization yields are summarized in Table 1.

Isolation and Characterization of Cyclization Intermediate 18 and Byproduct 20. Cys-Gly-Gly-Phe-Leu-S-$(CH_2)_2CONH_2$ (**11**) (0.58 mg, 1 μmol) was dissolved in 0.02 M Na_2HPO_4/0.01 M citric acid buffer (370 μL, pH 6) containing TCEP (1.2 mg, 4.2 μmol). The reaction pH was approximately 4.5. The reaction was allowed to proceed for 8 h. The cyclization intermediate **18** was then isolated by preparative HPLC. MALDI-MS: 477.6 (calcd for $[M + H]^+$ 478.6), 499.5 (calcd for $[M + Na - H]^+$ 499.5), 515.9 (calcd for $[M + K - H]^+$ 516.6). **18** was treated with 1 M NH_2OH (pH 9) to give Cys-Gly-Gly-Phe-Leu-NH-OH **(19)**. **19** was further confirmed by MALDI-MS (found 532.9, calcd for $[M + Na - H]^+$ 532.6). The dimeric byproduct **20** was also isolated from the reaction and characterized by MALDI-MS (found 1082, calcd for $[M + Na - H]^+$ 1082).

Synthesis of cyclo(Cys-Ala-Val-Ser-Glu-Ile-Gln-Phe-Met-His-Asn-Leu-Gly-Lys) (c15). Cys-Ala-Val-Ser-Glu-Ile-Gln-Phe-Met-His-Asn-Leu-Gly-Lys-S$(CH_2)_2CONHCH_2CO_2NH_2$ (**15**) (6 mg, 3.4 μmol) was dissolved in 0.2 M Na_2HPO_4/0.1 M citric acid buffer (2 mL, pH 7.5) containing TCEP (1.04 mg, 3.6 μmol). The mixture was vortexed for 6 h. Analytical HPLC indicated the completion of the cyclization (t_R = 20.27 min, gradient 1). A 5 mg sample of the cyclic product was isolated from the reaction. Yield: 88%. MALDI-MS: found 1560 ± 1 (calcd for $[M + H]^+$ 1559.8).

Synthesis of cyclo(Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu) (c16). The linear precursor, Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu)-S-CH_2CH_2-$CONH_2$ (**16**) (18 mg, 10 μmol, CL-16), was dissolved in 0.2 M phosphate buffer (pH 7.2) containing 50% DMF and 6 equiv of TCEP in three dilutions: 0.1, 0.2, and 0.6 mM. Analytical HPLC showed the completion of the reaction within 3 h in all three cases (t_R = 22.42 min, gradient 1). A small amount of byproduct (<5%) resulting from hydrolysis of the peptide thioester was detected. The cyclic peptide was finally purified by preparative HPLC and identified by MALDI-MS (found 1677 ± 1, calcd for $[M + H]^+$ 1676.9). Yield: 82%.

Synthesis of Bicyclic Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu (17). (i) Method A. The linear peptide, Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu-S-$CH_2CH_2CONH_2$ (**16**) (12 mg, 6.74 μmol), was dissolved in H_2O (20 mL). To this solution was added DMSO (5 mL). The pH of the reaction was adjusted to 9.5 for 10 min using 10% Na_2CO_3 and readjusted to 6.7 using acetic acid. The reaction was monitored by analytical HPLC (Figure 7). After 18 h, the bicyclic peptide was purified by preparative HPLC. No free thiol groups were detected by Ellman's reagent. The product was isolated by preparative HPLC to give a yield of 75%. The bicyclic peptide **17** was analyzed by RP-HPLC (t_R = 21.19 min, gradient 1) and characterized by amino acid analysis and MALDI-MS (found 1675 ± 1, calcd for $[M + H]^+$1674.9).

(ii) Method B. Cyclic CL-16 (**c16**) (3.2 mg, 0.6 μmol) was dissolved in H_2O (30 mL). The pH of this solution was adjusted to 5 with $(NH_4)_2CO_3$. DMSO (3 mL) was added to the solution. The progress of the oxidation was monitored by analytical RP-HPLC. The reaction was finished in 6 h. The product was purified by RP-HPLC and characterized by MALDI-MS (found 1675 ± 1, calcd for $[M + H]^+$ 1674.9).

Kinetic Study. Kinetic analysis was accomplished by RP-HPLC determination of the reaction progress. All reactions were carried out at a concentration of 2 mM in 0.2 M phosphate buffer (pH 5.2, 6.4, and 7.5) in the presence of TCEP. 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 of ln $[C]/[C_0]$ versus time (where $[C]$ is equal to the concentration of the linear thioester precursor and $[C_0]$ is equal to the amount of the linear thioester precursor at time 0) were used to calculate the rate and half-life of the cyclization reaction.

Preparation of Chloroacetyl Tetravalent Lysinyl Core Peptide 22. The tetravalent lysinyl core peptide was synthesized as described previously. The synthesis of the first level of carrier core to form Boc-Lys(Boc)-Ala-OCH_2-Pam resin was achieved using a 4-fold excess of Boc-Lys(Boc) dicyclohexylamine salt via (benzotriazol-1-yloxy)tris-(dimethylamino)phosphonium hexafluorophosphate (BOP) in dichloromethane. The second level of lysine was generated by the same protocol. After Boc deprotection, tetravalent chloroacetyl moieties were introduced to the core peptide by using a 10-fold excess of chloroacetic acid via DICI/HOBt activation. The tetravalent (chloroacetyl)lysinyl core peptide **22** was cleaved from the resin by HF/anisole (9:1) and finally purified by RP-HPLC. MALDI-MS: found 780.5, calcd for $[M + H]^+$ 780.5; found 802.3, calcd for $[M + Na - H]^+$ 802.5.

Synthesis of Cyclic Peptide Dendrimers 23 and 24 through Thioether Formation. General Procedure. Cyclic peptide (2.8×10^{-2} mmol) was dissolved in 0.2 M phosphate buffer containing 0.01 M EDTA (1.25 mL, pH 7.4). The solution was purged with argon for 10 min. To this solution was added the tetravalent (chloroacetyl)lysinyl core peptide (1.1 mg, 1.4×10^{-3} mmol) predissolved in DMF (0.75 mL). The mixture was allowed to vortex for 24 h. The product was then isolated by RP-HPLC. Yields for cyclic CL-6 and cyclic CK-14 dendrimers were 3.5 and 7.7 mg, respectively. Both cyclic dendrimers gave a single peak in analytical HPLC. The products were finally characterized by MALDI-MS: cyclic CL-6 dendrimer **23** (found 3218.4, calcd for $[M + Na - H]^+$ 3218.4); cyclic CK-14 dendrimer **24** (found 6869.7, calcd for $[M + H]^+$ 6869.9).

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