Lactone and Lactam Library Synthesis by Silver Ion-Assisted Orthogonal Cyclization of Unprotected Peptides†

Lianshan Zhang‡ and James P. Tam*

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

Received November 6, 1998

Abstract: An orthogonal cyclization strategy has been developed to prepare cyclic peptides individually or as a mixture in aqueous solutions. In this strategy, we propose a dual activation by entropy and enthalpy through Ag^+ ion coordination of the reactive ends of an unprotected peptide thioester to permit long-range acyl migration. As a result, both lactamization and lactonization of linear peptides can be realized simultaneously or selectively by controlling the reaction conditions. At pH 4, lactonization is favored due to the strong protonation of the amino moieties. However, at pH 5−5.7 lactamization of α-amine of the N-terminal amino acid is favored, particularly with DMSO as a cosolvent. At pH > 6, lactamization of the ϵ-amine of lysine becomes significant. Furthermore, the Ag^+ ion complexation with the peptide thioester precursor in aqueous buffered solutions may facilitate entropy-favored ring−chain tautomerization, which in turn promotes formation of monomeric cyclic products and thereby reduces unwanted oligomerization. Evidence supporting Ag^+ ion-directed ring−chain tautomerization includes product distribution profiles, the concentration-independent nature of the cyclization, and lack of competition by intermolecular coupling with other unprotected peptide segments. The N-terminal amino acid has been found to exert a profound effect in conferring high specificity toward end-to-end cyclization. Examination of 23 linear peptide precursors containing different N^{α}-amino acids showed that the probability of lactamization is almost 20-fold greater in α-amines than ϵ-amines with N-terminal Gly, Asn, Asp, and Ser. Unprotected linear peptides ranging from 5 to 16 amino acid residues were cyclized individually or as a mixture with good to excellent yields. More importantly, this strategy paves the way for a new approach to synthesizing cyclic peptide libraries in which unprotected peptides are cyclized and released from the resin at the same time. The obtained libraries can, therefore, be used directly for bioassays without further chemical manipulations. This method is also useful for the synthesis of bicyclic peptides containing both sulfur−sulfur and lactam linkages.

Introduction

Cyclic peptides are useful tools in peptide research. When peptides are cyclized, the resulting conformational constraints simplify structures to mimic bioactive conformations, thus improving selectivity and bioactivity.[1−4] As part of the paradigm of discovering non-peptide drug leads, cyclic peptides also have found increased use in the synthesis of peptide libraries.[5,6] Recently, cyclic peptides have also been employed as building blocks for the synthesis of macromolecules such as peptide dendrimers[7] and synthetic vaccines[7,8] and as precursors for the synthesis of polyazamacrocyles.[9]

Current methods for peptide cyclization generally involve a partially or fully protected linear precursor which is then cyclized in organic solvents through various combinations of orthogonal protecting groups and on- or off-resin cyclization schemes.[1−4,10−12] In a typical end-to-end cyclization, protecting groups are used to mask all functionalities. After selectively

* To whom correspondence should be addressed. Phone: (615) 343-1465. Fax: (615) 343-1467. E-mail: Tamjp@ctrvax.vanderbilt.edu.

† Abbreviations: AcOH, acetic acid; AgOTfa, silver trifluoroacetate; Boc, *tert*-butoxycarbonyl; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; DIC, diisopropylcarbodiimide; DIEA, diisopropylethylamine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DPPA, diphenylphosphoryl azide; EDAC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; MALDI-MS, matrix-assisted laser desorption mass spectrometry; RP HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid.

‡ Present address: Sphinx Pharmaceuticals, 840 Memorial Dr., Cambridge, MA 02139.

(1) (a) Hruby, V. J. *Life Sci.* **1982,** *31*, 189−199. (b) Hruby, V. J. *Trends Pharm. Sci.* **1985**, *6*, 259−262. (c) Al-Obeidi, F.; Castrucci; A. M. del L.; Hadley, M. E.; Hruby, V. J. *J. Med. Chem.* **1989**, *32*, 2555−2561.

(2) (a) Blout, E. R. *Biopolymers* **1981**, *20*, 1901−1912. (b) Kessler, H. *Angew. Chem.* **1986**, *25*, 997−999.

(3) Karle, I. L. *Biopolymers* **1996**, *40*, 157−180.

(4) Rizo, J.; Gierasch, L. M. *Annu. Rev. Biochem.* **1992**, *61*, 387−418.

(5) (a) Cwirla, S. E.; Balasubramanian, P.; Duffin D. J.; Wagstrom, C. R.; Gates, C. M.; Singer, S. C.; Davis, A. M.; Tansik, R. L.; Mattheakis, L. C.; Boytos, C. M.; Schatz, P. J.; Baccanari, D. P.; Wrighton, N. C.; Barrett, R. W.; Dower, W. J. *Science* **1997**, *276*, 1696−1699. (b) Wrighton, N. C.; Farrell, F. X.; Chang, R.; Kashyap, A. K.; Barbone, F. P.; Mulcahy, L. S.; Johnson, D. L.; Barrett, R. W.; Jolliffe, L. K.; Dower, W. J. *Science* **1996**, *273*, 458−464.

(6) Spatola, A. F.; Crozet, Y. *J. Med. Chem.* **1996**, *39*, 3842−3846.

(7) (a) Zhang, L.; Tam, J. P. *J. Am. Chem. Soc.* **1997**, *119*, 2363−2370. (b) Spetzler, J.; Tam, J. P. *Pept. Res.* **1996**, *9*, 290−6; (c) Pallin, T. D.; Tam, J. P. *Chem. Commun.* **1996**, 1345−1346.

(8) (a) Robey, F. A.; Fields R. L. *Anal. Biochem.* **1989**, *177*, 373−377. (b) Tolman, R. L.; Bednarek, M. A.; Johnson, B. A.; Leanza, W. J.; Marburg, S.; Underwood, D. J.; Emini, E. A.; Conley, A. J. *Int. J. Pept. Protein Res.* **1993**, *41*, 455−466. (c) Conley, A. J.; Conard, P.; Bondy, S.; Dolan, C. A.; Hannah, J.; Leanza, W. J.; Marburg, S.; Rivetna, M.; Rusiecki, V. K.; Sugg, E. E.; et al. *Vaccine* **1994**, *12*, 445−451.

(9) Aston, K. W.; Henke, S. L.; Modak, A. S.; Riley, D. P.; Sample, K. R.; Weiss, R. H.; Neumann, W. L. *Tetrahedron Lett.* **1994**, *35*, 3687−3690.

(10) (a) Manesis, N. J.; Goodman, M. *J. Org. Chem.* **1987**, *52*, 5331−5341. (b) Gilon, C.; Halle, D.; Chorev, M.; Selinger, Z.; Byk, G. *Biopolymers* **1991,** *31*, 745−750.

10.1021/ja983859d CCC: $18.00 © 1999 American Chemical Society
Published on Web 03/17/1999

deprotecting the α-amine and the carboxyl group, ring closure is achieved via a coupling reagent. Similar approaches have been employed for synthesizing end-to-side chain, backbone-to-side chain or side chain-to-side chain lactams.[10−12] In addition to protection schemes, these methods also require postcyclization deprotection steps. Medium-sized cyclic peptides can also be obtained by enzymatic cyclization of linear unprotected peptide esters using subtiligase.[13]

Recently, we and others have focused our efforts on development of the orthogonal cyclization strategy of using unprotected peptides as linear precursors and forming products that do not require postcyclization deprotection steps.[7,14−16] These methods differ in concept and mechanism from the conventional, enthalpic coupling approaches. First, they are based on an intersegmental orthogonal ligation strategy using two unprotected peptide segments.[17−19] Second, they exploit the principle of intramolecular, proximity-driven acyl migration to achieve regioselective amide bond formation without the use of the conventional coupling reagents. Thus far, two prominently established orthogonal ligation methods, thiazolidine and thioester, have been successfully applied to the syntheses of cyclic peptides and proteins.[7,14−16]

Unlike the intersegmental orthogonal ligation strategy, the orthogonal cyclization strategy has the advantage of undergoing ring−chain tautomerization because of the weakly activated acyl moiety.[7,14] In aqueous buffered solutions, ring−chain tautomerization occurs when a suitable N-terminal nucleophile can equilibrate with a C-terminal electrophile. Reversible cyclic intermediates favoring intramolecular cyclization reactions are formed, which promote formation of monomeric cyclic products. Such a reaction scheme can be successful without significant oligomerization even when relatively high concentrations of unprotected peptides are used in aqueous solution. Examples include linear precursors containing a Cys at the N-terminus with either a glycoaldehyde ester[14] to give a thiazolidine or a thioester at the C-terminus to give a Cys amide bond.[7] A limitation of these methods is the prerequisite for a cysteine residue or a cysteinyl mimetic at the N-terminus.[16a,16b] Thus, a more general scheme extending beyond N-terminal cysteinyl unprotected peptides is desirable.

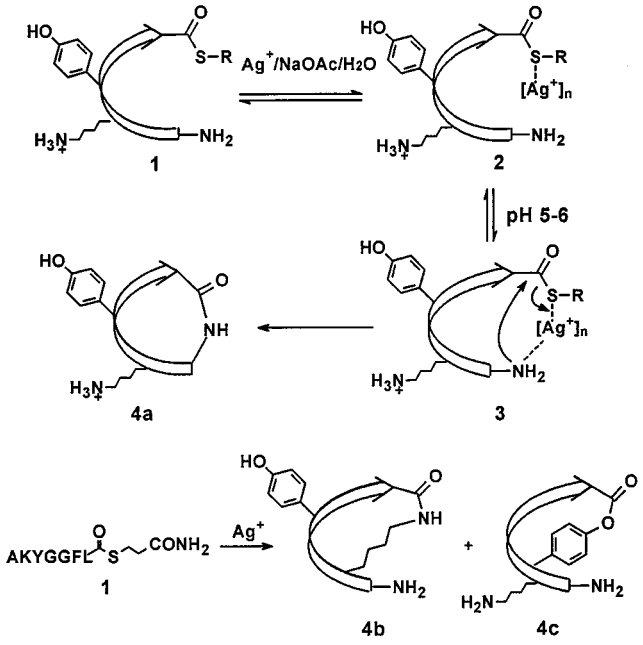

Figure 1. A proposed scheme for Ag^+ ion-assisted peptide cyclization in NaOAc buffered solutions to afford lactams **4a** and **4b** and lactone **4c**.

In this paper, we describe the development of a general orthogonal cyclization strategy (Figure 1) that exploits the concept of ring−chain tautomerization, **2** ⇔ **3**, for non-cysteinyl peptide thioesters. We propose that Ag^+ ion could serve a dual role for entropic and enthalpic activations in the macrocyclization process. Entropic activation of Ag^+ ion in bridging the N^{α}-amine and C^{α}-thioester as a Ag^+ ion-bridged complex, **3**, would favor an intramolecular acylation to form a monomeric product, **4a**. Complexation of Ag^+ ions with other side chain nucleophiles such as the N^{ϵ}-amine of lysine or the phenolic group of tyrosine would lead to the formation of cyclic products **4b** and **4c**. Enthalpic activation of the peptide thioester through Ag^+ ion complexation facilitates an acyl-transfer reaction.

Enthalpic and entropic activations of thioesters through Ag^+ ion have previously been described. In 1954, amide bond formation was demonstrated by Schwyzer and Hürlimann,[20a] who postulated that facile intermolecular aminolysis proceeded through enthalpic activation by a Ag^+ ion complex of the thioester and amine. Subsequent works by others have generally supported this view.[20b,c] In the 1980s, an effective segment coupling strategy of partially protected peptides was developed by Blake and Li,[21a] Yamashiro and Li,[21b] and Aimoto et al.[21c] using Ag^+ ion for the enthalpic activation of thioesters in their conversion to *N*-hydroxysuccinimide active esters. Furthermore, methods based on metal ion-assisted lactone cyclization of macrolides from thioester precursors have also been used successfully by Gerlach and Thalmann[22] and Masamune et al.[23] in nonaqueous media. To account for the high efficiency of the macrocyclization of the lactones, entropic activation through a cyclic metal ion complex was proposed as a reaction intermediate.[23]

(11) (a) McMurray, J. S. *Tetrahedron Lett*. **1992**, *33*, 4557−4560. (b) Kates, S. A.; Sole, N. A.; Johnson, C. R.; Hudson, D.; Barany, G.; Albericio, F. *Tetrahedron Lett*. **1993**, *34*, 1549−1552. (c) Richter, L. S.; Tom, J. Y. K.; Burnier, J. P. *Tetrahedron Lett*. **1994**, *35*, 5547−5550.

(12) (a) Schiller, P. W.; Nguyen, T. M.-D.; Miller, J. *Int. J. Pept. Protein Res.* **1985**, *25*, 171−177. (b) Felix, A. M.; Wang, C. T.; Heimer, E. P.; Fournier, A. J. *Int. J. Pept. Protein Res.* **1988**, *31*, 231−238. (c) Ehrlich, A.; Heyne, H. U.; Winter, R.; Beyermann, M.; Haber, H.; Carpino L. A.; Bienert, M. *J. Org. Chem.* **1996**, *61*, 8831−8838.

(13) Jackson, D. Y.; Burnier, J. P.; Wells, J. A. *J. Am. Chem. Soc.* **1995**, *117*, 819−820.

(14) Botti, P.; Pallin, D. P.; Tam, J. P. *J. Am. Chem. Soc.* **1996**, *118*, 10018−10024.

(15) (a) Tam, J. P.; Lu, Y.-A. *Tetrahedron Lett*. **1998**, *7*, 1583−1592. (b) Tam, J, P,; Lu, Y.-A. *Protein Sci*. **1998**, *7*, 1583−1592.

(16) (a) Tam, J. P.; Yu, Q. *Biopolymers* **1998**, *46*, 319−327. (b) Shao, Y.; Lu, W.; Kent, S. B. H. *Tetrahedron Lett*. **1998**, *39*, 3911−3914. (c) Camarero, J. A.; Muir, T. W. *Chem. Commun*. **1997**, 1369−1370. (d) Camarero, J. A.; Muir, T. W. In *Peptides Chemistry, Structure and Biology*, Proceedings of the 15th American Peptide Symposium; Tam, J. P., Kaumaya, P. T. P., Eds.; in press.

(17) (a) Kemp, D. S. *Biopolymers* **1981**, *20*, 1793−1804. (b) Fotouhi, N.; Galakatos, N.; Kemp, D. S. *J. Org. Chem.* **1989**, *54*, 2803−2817. (c) Kemp, D. S.; Carey, R. I. *Tetrahedron Lett.* **1991**, *32*, 2845−2848. (d) Kemp, D. S.; Carey, R. I. *J. Org. Chem*. **1993**, *58*, 2216−2222.

(18) (a) Liu, C. F.; Tam, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 6584−6588. (b) Liu, C. F.; Tam, J. P. *J. Am. Chem. Soc.* **1994**, *116*, 4149−4153.

(19) (a) Tam, J. P., Lu, Y.-A.; Liu, C. F.; Shao, J. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 12485−12489. (b) Dawson, P. E.; Kent, S. B. H. *J. Am. Chem. Soc.* **1993**, *115*, 7263−7266.

(20) (a) Schwyzer, R.; Hürlimann, C. *Helv. Chim. Acta*. **1954**, 37, 155−166. (b) Boopsingh, B.; Satchell, D. P. N. *J. Chem. Soc., Perkin Trans.* **1978**, *2*, 23. (c) Nambu, Y.; Endo, T.; Okawara, M. *Bull. Chem. Soc. Jpn.* **1981**, *54*, 3433.

(21) (a) Blake, J.; Li, C. H. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 4055−4058. (b) Yamashiro, D.; Li, C. H. *Int. J. Pept. Protein Res.* **1988**, *31*, 322−334. (c) Hojo, H.; Aimoto, S. *Bull. Chem. Soc. Jpn.* **1991**, *64*, 111−117.

(22) Gerlach, H.; Thalmann, A. *Helv. Chim. Acta* **1974**, *57*, 2661−2663.

(23) Masamune, S.; Kamata, S.; Schilling, W. *J. Am. Chem. Soc.* **1975**, *97*, 3515−3516.

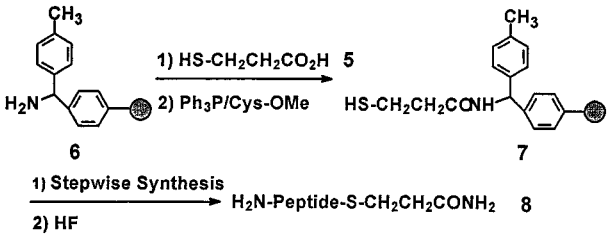

Figure 2. A simplified procedure for the on-resin synthesis of peptide thioesters.

Thus far, Ag^+ ion activation of thioesters for either lactone or amide bond formation has been performed in nonaqueous media assisted by protecting groups that direct the reaction to a specific nucleophile with high efficiency.[22,23] Indeed, our preliminary results have shown that end-to-end or end-to-side chain cyclic peptides could be achieved in this manner under aqueous conditions.[24] However, a more efficient approach and a stringent test of chemoselectivity would be the use of a free peptide thioester containing a plethora of functional groups including α-amine, ϵ-amine, phenolic, and other side chain nucleophiles. Productive Ag^+ ion complexation with one of these nucleophiles and the thioester would yield a cyclic intermediate through ring−chain tautomerization, leading to either lactone **4c** or lactam **4a** and **4b** formation, thus minimizing the danger of oligomerization (Figure 1). Furthermore, it might be possible to obtain site-specific cyclization of different lactones or lactams if aqueous buffered solutions are used to control the complexation sites. Such a complex would fulfill the entropic principle and would also enthalpically activate the thioester for orthogonal coupling when thiolphilic Ag^+ ion is present. Our aims are to define the factors affecting the selective formation of various lactones and lactams under aqueous conditions and to apply the methodology to afford a cyclic peptide library based on single unprotected precursors.

Results

Preparation of Peptide Thioester Precursors. A simplified procedure based on the linker developed by Hojo and Aimoto[21c] was used for preparing peptide thioesters. In this method, 3-mercaptopropionic acid **5** was first incorporated onto MBHA resin **6** or Gly-Pam-resin to give a 3-mercaptopropionyl amide resin, **7**, as a cleavable linker. Since the 3-mercaptopropionyl moiety was introduced without the thiol protection group, disulfide and thioester byproducts were formed. However, they were quantitatively transformed to the desired alkanethiol resin **7** by treatment with cysteine methyl ester and triphenylphosphine in the presence of DIEA.[25] The alkylthiol resin **7** was then used for attachment to any activated Boc-amino acid to give the desired thioester resin (Figure 2). This method is general because it does not require individually premade amino acid thioesters to be attached to the resin support. After assembly of the sequence on the solid support, the peptide thioester **8** was cleaved from the resin by HF/anisole (9:1) at 4 °C. Unprotected peptide thioesters are relatively stable in aqueous solutions at pH 2−8 after purification by preparative RP-HPLC and are used as precursors for cyclization studies.

Model Peptide and Product Profile of Lactones and Lactams. To study the cyclization reaction of thioesters mediated by Ag^+ ion, a seven-residue analogue of Leu-enkephalin peptide, Ala-Lys-Tyr-Gly-Gly-Phe-Leu-SR (R = $CH_2CH_2CONH_2$) **1,** was designed as a model for the orthogonal cyclization experiments. This water-soluble peptide is a suitable model with two glycines in its sequence to provide the flexibility to facilitate various cyclization products. Ala at the N-terminus represents a standard, uncharged amino acid, while Lys and Tyr residues provide side chain nucleophiles to compete for the cyclization reactions. The ring sizes of the three possible cyclic products are relatively similar: a 21-member ring for the end-to-end lactam cyclization via the N-terminal α-amine of Ala **4a**, 22 atoms for the side chain-to-end lactam cyclization via the side chain ϵ-amine of Lys **4b**, and 20 atoms for the lactone cyclization via the phenolic oxygen of Tyr **4c** (Figure 1).

Table 1. Kinetics of Ag^+ Ion-Assisted Cyclization of AKYGGFL-Thioester **1** at pH 3.5−6

	$t_{1/2}$ (min) for Ag^+/peptide thioester **1** (mol/mol)				$t_{1/2}$ (min) for Ag^+/peptide thioester **1** (mol/mol)		
pH[a]	1	2	3	pH[a]	1	2	3
3.5	770	745	560	5.0	329	87	63
4.0	750	928	256	5.5	237	30	15
4.5	717	491	291	6.0	274	12	4

[a] 0.2 M sodium acetate buffers with different pH values were mixed with DMSO in a volume ratio of 1:1. α-Lactamization yields cyclic peptide **4a**, and ϵ-lactamization yields cyclic peptide **4b** (Figure 1).

The enthalpic activation by Ag^+ ion was determined by the cyclization kinetics at different pH. The linear precursor **1** was stable in aqueous buffer solutions at pH 3.5−7.0 for 24 h, as monitored by RP-HPLC. At least 2 equiv of AgOTfa (Table 1) was required for the complete disappearance of the starting material in 1−4 h and the appearance of four major products, three of which were cyclized peptides **4a**−**4c**. Increased cyclization rates were observed as the pH increased or with larger excesses of AgOTfa. Using 3 equiv of Ag^+ ion, the $t_{1/2}$ was 63, 15, and 4 min when cyclizations were performed at pH 5, 5.5, and 6, respectively. Below pH 4.5, the reaction was slow. The possible role of Ag^+ ion in entropic activation was examined in product profiles for the monomeric and oligomeric products. Productive Ag^+ ion-coordinated complexes derived from three nucleophiles and the C^α-thioester would lead to the formation of a lactone and lactams but little or no oligomeric products. Cyclization of **1** in 0.2 M acetate buffer at pH 5.4 after 4 h resulted in four compounds as determined by RP-HPLC (Figure 3, left panel). Two peptides were identified as lactams of end-to-end **4a** (55%) and side chain-to-end cyclic peptides (17%) **4b**, respectively. The third compound **4c** was found to be a peptide lactone (22%) resulting from the lactonization of the phenol group of Tyr, and the fourth compound was the hydrolysis product **4d** (5%). There was <1% dimerization detected by RP-HPLC. The cyclic peptides were all isomeric and gave the expected MW by MALDI-MS. The end-to-end cyclization was distinguished from the end-to-side chain cyclization by end-group analysis using Sanger's reagent,[26] while the lactam and the lactone could be identified by treatment with 1 M NH_2OH.[27]

The product profile of the model peptide **1** was sensitive to pH. Varying the pH of the reaction conditions strongly influenced the complexation sites of the Ag^+ ion and changed the product distributions (Table 2). At pH < 4.5, the amines became strongly protonated and formation of the lactone **4c** predominated. When the reaction was conducted at pH 6−8, cyclization of **1** afforded **4a** and **4b** in a ratio of 0.3, indicating

(24) Zhang, L.; Tam, J. P. *Tetrahedron Lett.* **1997**, *38*, 4375−4378.
(25) Wieland, T.; Bokelmann, E.; Bauer, L.; Lang, H. U.; Lau, H. *Liebigs Ann. Chem.* **1953**, *583*, 129−149.
(26) Sanger, F. *Biochem. J.* **1945**, *39*, 507−515.
(27) Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70−77.

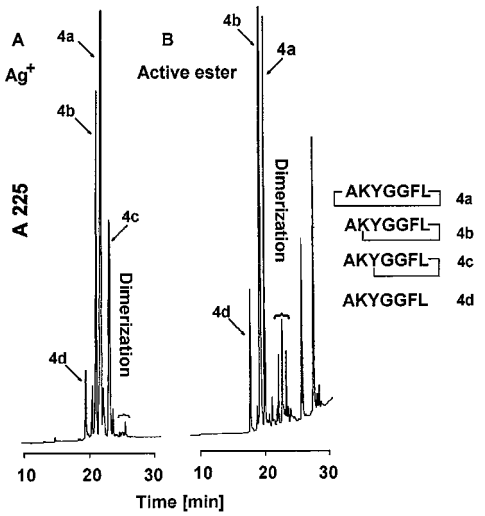

Figure 3. Comparative cyclization of AKYGGFL. (A) Ag^+ ion-assisted cyclization from the thioester precursor **1**. (B) Cyclization from the pentafluorophenyl ester precursor, AKYGGFL-OPfp. **4a**: end-to-end cyclic lactam. **4b**: end-to-side chain cyclic lactam. **4c**: end-to-side chain cyclic lactone. **4d**: free AKYGGFL.

Table 2. Product Distributions (%) of Ag^+ Ion-Assisted Cyclizations of AKYGGFL-Thioester **1** Performed at pH 4.5−6.5

pH	**4a**	**4b**	**4c**	pH	**4a**	**4b**	**4c**
4.5	10	4	65	5.9	60	25	10
5.0	50	12	30	5.7 (20% DMSO)	67	15	2
5.4	55	17	22	6.5 (20% DMSO)	20	75	0

that at higher pH the less hindered ϵ-amino group of lysine was the favored Ag^+ ion complexation site. The reaction could not be performed at basic pH > 8 under aqueous conditions because the Ag^+ ion precipitated into silver oxide. Thus, there is a pH window of 5.2−5.7 optimal for lactam formation of end-to-end cyclic peptides. To optimize the necessary Ag^+ ion-assisted acylation when using unprotected peptide precursors with a free lysine ϵ-amino, conditions that would specifically stabilize coordination of the α-amino nucleophile and the C-terminal thioester were sought. Two reaction conditions which significantly affected regioselectivity were found. First, the N-terminal amino acid possesses a stabilizing effect. This is discussed in detail below. Second, in organic solvents and in the absence of water, the Ag^+ ion complex was found to be relatively stable with the amines protonated as TFA salts. Thus, the stability of the complex could be enhanced by the addition of organic solvent. Two direct consequences were observed. With addition of 20−50% DMSO at pH 5.7, lactonization and hydrolysis were nearly suppressed, and the desired end-to-end cyclized peptide **4a** was obtained as the major product in 67% yield in 5 h. Thus, for all subsequent studies, cyclizations were performed at pH 5.2−5.7. In some cases, with 50% DMSO present, lactonization and thioester hydrolysis were suppressed to <2%.

Comparison of Ag^+ Ion-Assisted Cyclization with Other Enthalpic Activated Cyclization Methods. To show that in aqueous solutions the Ag^+ ion-assisted cyclization of peptide thioesters contains elements of both enthalpic and entropic activation, we compared product profiles of cyclized AKYGGFL-thioester **1** with three conventional cyclization methods (Table 3): pentafluorophenol (Pfp) active ester, water-soluble carbodiimide (EDAC), and DPPA. Three distinct differences were discerned. First, there was a 1.8−11-fold difference in the regioselectivity of intramolecular α/ϵ acylation of cyclic lactams **4a** and **4b**. Product analysis showed that the selectivity of α/ϵ acylation of AKYGGFL-thioester in Ag^+ ion-assisted cyclization was 1.8-, 4-, and 11-fold higher than in reactions mediated by EDAC, Pfp, and DPPA, respectively. However, these results could also be interpreted to mean that cyclization of the peptide, AKYGGFL, using DPPA provided higher selectivity for ϵ-lactamization as compared with Ag^+ ion-assisted α-amine cyclization. Second, there were insignificant amounts of detectable oligomeric products by the Ag^+ ion-assisted cyclization. In contrast, significant amounts of oligomeric products were observed with cyclization methods mediated only by enthalpic activation, noticeably reducing the cyclic peptide yields. For example, 7% dimer plus other oligomeric products were found using the Pfp method as detected by RP-HPLC (Figure 3, right panel). Finally, the strongest evidence for the entropic contribution of Ag^+ ion in assisting ring−chain tautomerization is that lactonization occurred during the reaction to give **4c** (22%). **4c** was observed presumably via Ag^+ ion coordination between the phenolic hydroxyl group and the thioester. No detectable lactonization, however, was found using conventional activation methods. Such results were expected because the reactions were performed in acetate-buffered solutions at pH 5.4 in the presence of a large excess of acetate nucleophile, which inhibits lactone formation in the conventional enthalpic pathways. Although lactone formation has been demonstrated in Ag^+ ion-assisted macrolide formation, the reactions are performed in nonaqueous solutions with hydroxyls as the only nucleophiles.[22,23]

Table 3. Selectivity and Yields of α- and ϵ-Lactamization of the Sequence AKYGGFL Using Different Activation Methods

activation method	selectivity (α/ϵ lactam **4a**/**4b**)	yield (%)	dimer (%)
Ag^+/thioester	3.5	60	<1
DPPA	0.31	17	<1
EDAC	1.9	27	<1.5
Pfp active ester	0.86	13	7

Other Supporting Evidence for Ring−Chain Tautomerization. There are additional lines of evidence supporting Ag^+ ion-assisted cyclization through ring−chain tautomerization. Competition experiments with peptides did not yield a significant amount of intermolecular ligation products. For example, reactions of AYGGFL-thioester and AgOTfa with three tetrapeptides XAKA (where X = A, L, and M) individually at pH 5.4 in the solution phase resulted in cyclic products without any significant amount of intersegmental ligated products of AYGGFL-XAKA observed in HPLC. On-resin cyclization of a mixture of 19 peptide thioesters with the sequence GYGXFL-thioester, where X represents all proteinogenic amino acids except cysteine, gave 19 expected cyclic peptides (detailed description later).

Cyclization of AKYGGFL-thioester **1** in different concentrations ranging from 1 to 10 mM did not affect the product profile, and the cyclization appeared to be concentration-independent. Thus, cyclization at relatively high concentrations is possible in the Ag^+ ion-assisted acylation process, which is usually not the case using conventional methods. Consistently high yields of cyclic products were achieved even with pentapeptides, which are prone to cyclodimerization. These combined results appear to support the occurrence of a nonclassical ring−chain tautomerization mediated by Ag^+ ion complexation, which can be exploited favorably for intramolecular reactions.

Effect of N- and C-Terminal Nucleophiles on End-to-End Cyclization. End-to-end cyclization is desirable because many

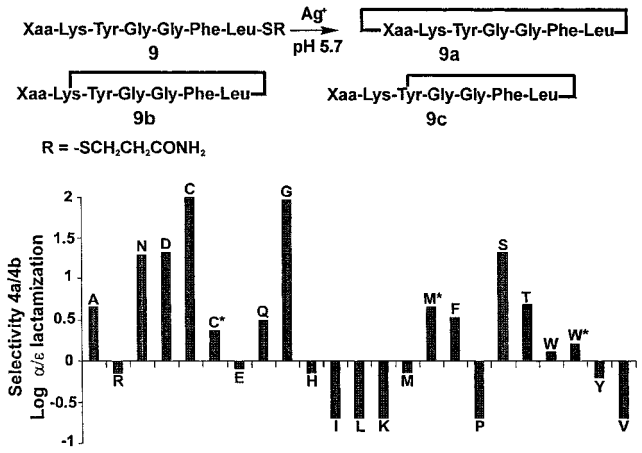

Figure 4. Effect of N-terminal amino acids on acylation selectivity at pH 5.5 in 25% DMSO. The ratios of α and ϵ lactamizations were determined by RP-HPLC, and the selectivity is expressed as log(α/ϵ lactamization) of **4a**/**4b**. The lactone **9c** is found less than 2% in most cases. A one letter code was used for all amino acids: A, Ala; R, Arg; N, Asn; D, Asp; C, Cys; C*, cystine; E, Gln; G, Gly; H, His; I, Ile; L, Leu; K, Lys; M, Met; M*, Met(0); F, Phe; P, Pro; S, Ser; T, Thr; W, Trp; W*, Trp (CHO); Y, Tyr; V, Val.

naturally occurring products are end-to-end cyclic peptides.[28] To increase further the selectivity for end-to-end over side chain-to-end lactam formation, we next studied the effect of N-terminal amino acids on Ag^+ ion-assisted cyclization. It is known that N-terminal amino acids are often the high-affinity binding sites of metal ions because of the presence of side chain functional groups that act as accessory coordinate sites.[29] Thus, peptides with N-terminal residues such as Ser/Thr, Asp/Asn, Glu/Gln, His, Met, and Trp may favor end-to-end cyclic products. In addition, there is some latitude in choosing N-terminal amino acids as cyclization sites since the sequences of end-to-end cyclic peptides are circularly permutated. Thus, by delineating factors that affect reactivity and selectivity, it is possible to choose the most appropriate N-terminal amino acids to yield end-to-end cyclic peptides. We, therefore, placed 23 amino acids, including all 20 genetically coded amino acids, at the N-terminus of the model heptapeptide Xaa-Leu-Tyr-Gly-Gly-Phe-Leu (Xaa = any amino acid) **9**.

The selectivity of end-to-end over end-to-side chain cyclization, i.e., the ratio of lactamization of N^α and ϵ-amines of **9a** and **9b**, was strongly affected by the N-terminal amino acids in buffered acetate aqueous solution at pH 5.5 (Figure 4). Lactone **9c** formation was inconsequential under the conditions used because it was suppressed to a minimum by the addition of 25% DMSO as a cosolvent. Four amino acids at the N^α-amino terminus, Gly, Ser, Asn, and Asp, showed the highest selectivity with an α/ϵ acylation ratio of >20. In comparison, this ratio is 3.5 for the standard Ala-peptide **1** without the heteroatomic side chain. N^α-Ser, Asn, and Asp containing O-coordinating sites at their side chains seem to stabilize the Ag^+ ion complex with the α/ϵ acylation ratio of 21, 20, and 21, respectively. N^α-Gly, the least sterically hindered amino acid which does not have a heteroatomic side chain, is an exceptional amino acid and gives an α/ϵ acylation ratio of >95 with no significant ϵ-amine acylated product. This is interesting because a reverse turn is required for peptide cyclization, and these N-capping amino acid residues may facilitate end-to-end over end-to-side chain cyclization.

The ratio of α/ϵ lactamization in N-terminal amino acids with side chain heteroatoms, such as S from α-Cys or α-Met and N from α-His or α-Trp, that are usually stronger coordinating sites than O from α-Asp was not greater than in the α-Ala-model peptide **1.** Decreasing the side chain nucleophilicity of the N-terminal amino acids such as conversion of N^α-Met to N^α-Met(O) and N^α-Trp to N^α-Trp(For) improved α/ϵ acylation. Similarly, N^α-Cys with the free SH complexed tightly with Ag^+ ion, leading to precipitation in aqueous solutions. Upon solubilizing the Ag^+ complex in high concentrations of organic solvents or in the presence of urea, end-to-end cyclization was both selective and accelerated (log(α/ϵ lactamization) = 2). Protecting the side chain of N^α-Cys as a disulfide reduced the affinity of Ag^+ ion complexation and significantly improved solubility. A protected N^α-cysteine, such as a disulfide, would be useful in the Ag^+ ion-assisted reaction. An example of the use of this strategy is in the synthesis of a bicyclic salmon calcitonin peptide segment, which is described below. However, with N^α-Cys-peptide, there is an alternative, more facile approach by exploiting thiol−thioester exchange, which as previously reported leads to the desired end-to-end cyclic product.[7]

Compared with Ala-peptide **1**, Ag^+ ion-assisted cyclization of peptides of N^α-Lys and N^α-Arg with strong basic amine-substituted side chains which are protonated at pH 5.5 did not show improved selectivity of α/ϵ acylation**.** Hindered amino acids such as Val, Ile, and Pro were found to give <1 α/ϵ acylation. Thus, cyclizations of N-terminal Ile, Val, or Pro peptides containing internal side chain amino groups resulted mainly in end-to-side chain cyclic products.

Scope and Limitations. Unlike the N-terminal amino acid, the C-terminal amino acid did not have the same profound effect on the selectivity of α/ϵ lactamization. However, there were two other effects noted upon varying the C-terminal amino acid. Hindered amino acids at the C-termini such as Ile, Pro, and Val derived from **9** (AKYGGFX, X = A, F, I, P, or V) were found to form cyclic peptides 4−10-fold more slowly than Ala. These slower kinetics not only produced lower yields, but also 7% racemization in the case of C-terminal Phe. Lys and Arg formed side chain lactams and should be avoided as C-terminal residues for cyclization. For example, the C-terminal Lys of AVSEIQFMHNLGK-thioester **10** formed the seven-membered ring lactam **10b** in the Ag^+ ion-assisted cyclization (<5% of the desired α-lactam **10a** was found). To a lesser extent, the C-terminal Arg of GDfVR-thioester (f = D-phenylalanine) **11** formed the lactam **11b** with its side chain in 12% yield. The desired cyclo(GDfVR) **11a** was only obtained in 30% yield, while 32% of **11** underwent hydrolysis. However, internal Arg was generally found not to be involved in the cyclization reaction tested thus far. An example is the cyclization of DfVRG-thioester **12**, resulting in the end-to-end cyclized DfVRG **12a**[31] in 90% yield with no detectable Arg side chain participation in the lactam formation. Thus, with these exceptions, most amino acids are suitable for the Ag^+ ion-assisted cyclization reaction. Although not tested, our experience in segment ligation indicates that Asn, Glu, and Gln likely pose

(28) (a) Hotchkiss, R. D.; Dubos, R. J. *J. Biol. Chem.* **1940**, *136*, 803−804. (b) Wieland, T. *Naturwissenschaften* **1987**, *74*, 367−373. (c) Wieland, T.; Lüben, G.; Ottenheym, H. C. J.; Faesel, J.; de Vires, J. X.; Konz, W.; Prox, A.; Schmid, J. *Angew. Chem., Int. Ed. Engl.* **1968**, *7*, 204−208.

(29) (a) Belew, M.; Porath, J. *J. Chromatogr.* **1990**, *516*, 333−354. (b) Sigel, H.; Martin, R. B. *Chem. Rev.* **1982**, *82*, 385−426.

(30) (a) Richardson, J. S.; Richardson, D. C. *Science* **1988**, *240*, 1648−1652. (b) Chakrabartty, A.; Doig, A. J.; Baldwin, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 11332−11336. (c) Kamtekar, S.; Schiffer, J. M.; Xiong, H.; Babik, J. M.; Hecht, M. H. *Science* **1993**, *262*, 1680−1685.

(31) (a) Gurrath, M.; Müller, G.; Kessler, H.; Aumailley, M.; Timpl, R. *Eur. J. Biochem.* **1992**, *210*, 911−921. (b) Müller, G.; Gurrath, M.; Kessler, H.; Timpl, R. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 326−328.

Table 4. Scope of Selective End-to-End Cyclization of Peptides Containing the Core Sequence, YGGFL, and Dichotomins (DM) A−G

compd no.	sequence	yield (%)	MALDI-MS $[M+H]^+$
13	GYGGFL	87	594
14	GKYGGFL	94	722
15	GAKYGGFL	89	794
16	GDAKYGGFL	82	909
17	GADAKYGGFL	84	980
18	GTFLY	89	583
19	GTFLYV (DM A)	94	682
20	GTFLYT (DM B)	85	684
21	GTFLYA (DM C)	79	654
22	GVGFYI (DM D)	82	638
23	GTAFA (DM E)	87	511
24	SVYPYFVLP (DMF)	85	1067
25	SPLPIPPFY (DM G)	80	1013

problems because of their side chain assistance in imide formation and hydrolysis.

Peptide Lengths and Conformation. We next examined peptides with different lengths and conformational preferences. The advantage of choosing Gly, Ser, Asp, and Asn as intramolecular ligation sites could be demonstrated by synthesizing different lengths of cyclic peptides. Using the YGGFL as a core sequence, peptides **13**−**17** ranging from 6 to 10 residues were cyclized in conditions favoring end-to-end linkage in 89−94% yield (Table 4). For example, an end-to-end cyclic product, **14a**, with the sequence GKYGGFL, was obtained from GKYGGFL-thioester **14** in 94% yield. When the length was increased to an octapeptide, GAKYGGFL-thioester **15**, the yield of end-to-end cyclic peptide **15a** was 89%. Similarly, in applying this technique to the preparation of naturally occurring cyclic peptides, we synthesized dichotomin A−F isolated from *Stellaria dichotoma* var. *lanceolata* **18**−**25**, some of which are newly discovered cyclic peptides from higher plants that show cell growth inhibitory activities.[32] Using the Ag^+ ion-assisted cyclization method, we synthesized two pentapeptides, four hexapeptides, and two nonapeptides, all of which were obtained as end-to-end cyclic peptides in yields ranging from 79% to 94% based on the HPLC (Table 4). In addition to MALDI-MS, these end-to-end cyclizations were confirmed by end-group analysis as cyclic peptides[26] and were treated with 0.5 N NH_2-OH at pH 9 to rule out the possibility that they were lactone products.[27]

Finally, we tested Ag^+ ion-assisted cyclization by the synthesis of a 16-residue bicyclic salmon calcitonin analogue with the sequence CSNLSTCVLGKLSQEL **28**. The synthesis of this bicyclic peptide provided an opportunity to compare Ag^+ ion-assisted cyclization with cyclization mediated by transthioesterification. The N-terminal cysteine could participate in a thiol−thioester exchange reaction in the presence of a trialkylphosphine. A cyclic thioester is formed which undergoes an S to N acyl transfer to yield the cyclic peptide (Figure 5). For Ag^+ ion-assisted cyclization (method A), cysteine residues of CSNLSTCVLGKLSQEL-thioester **26** were first oxidized with DMSO at pH 4, resulting in a cyclic peptide, **27**, with a disulfide linkage. The purified peptide **27** was then cyclized by the Ag^+ ion-assisted method at pH 5.4 in the presence of 50% DMSO (Figure 5). After 6 h, the product **28** was isolated in 82% yield and characterized by amino acid analysis and MALDI-MS (found 1675 ± 1, calcd for $[M+H]^+$ 1674.9). In method B, linear precursor **26** was first cyclized via an amide bond in the presence of trialkylphosphine[7a,16a] to form monocyclic lactam **29**. After oxidation by DMSO, the bicyclic product **28** was obtained in 72% yield. The bicyclic products prepared by both methods were identical in RP-HPLC and MALDI-MS (Figure 6).

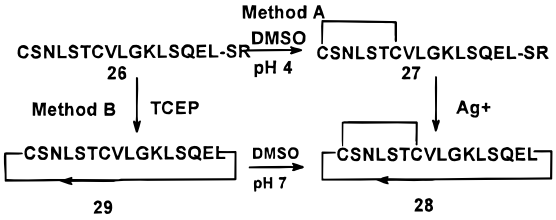

Figure 5. A comparative synthesis of a bicyclic peptide **28**. (A) Ag^+ ion-assisted orthogonal coupling in which cysteine was oxidized to cystine prior to cyclization. (B) Cyclization by transthioesterification and intramolecular acyl transfer.

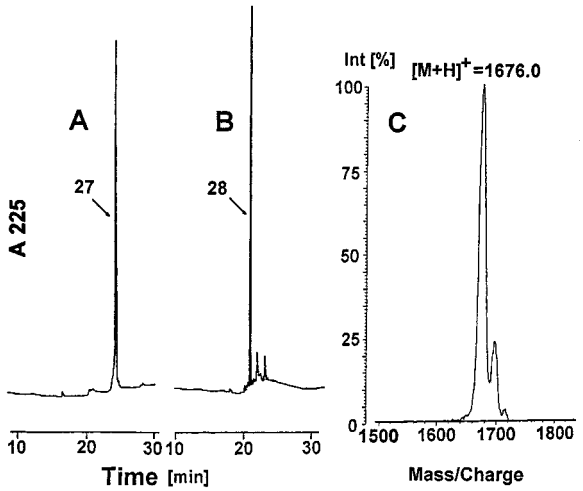

Figure 6. RP-HPLC analysis of Ag^+ ion-assisted cyclization of a bicyclic 16-residue peptide, **28**. (A) Starting material of the thioester **27**. (B) Cyclization after 6 h. Peak B was identified as the desired product **28**. (C) MALDI-MS of **28**.

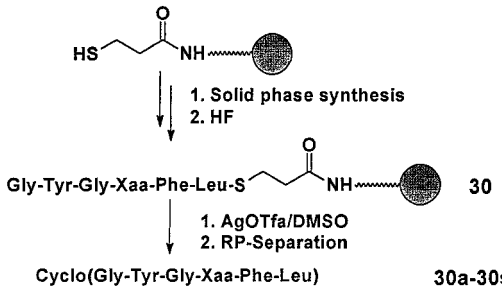

Figure 7. An on-resin cyclization scheme for the synthesis of peptide libraries from a mixture of 19 peptide thioester resins (Gly-Tyr-Gly-Xaa-Phe-Leu thioester).

On-Resin Synthesis of Cyclic Peptide Libraries. To demonstrate the utility of the Ag^+ ion-assisted cyclization method for preparing cyclic peptide libraries, peptide thioesters were synthesized on 3-mercaptopropionyl-derivatized TentaGel[33] (Figure 7). A peptide mixture of 19 peptides with the sequence Gly-Tyr-Gly-Xaa-Phe-Leu **30** (Xaa = all 19 proteinogenic amino acids except cysteine) was synthesized according

(32) (a) Morita, H.; Kayashita, T.; Shishido, A.; Takeya, K.; Itokawa, H.; Shiro, M. *Tetrahedron* **1996**, *52*, 1165−1176. (b) Morita, H.; Shishido, A.; Kayashita, T.; Takeya. K.; Itokawa, H. *J. Nat. Prod.* **1997**, *60*, 404−407.

(33) Rapp, W.; Zhang, L.; Bayer, E. In *Innovation and Perspective in Solid-Phase Synthesis, Peptides, Polypeptides and Oligonucleotides;* Epton, R., Ed.; SPCC (UK) Ltd.: Birmingham, 1990; p 205.

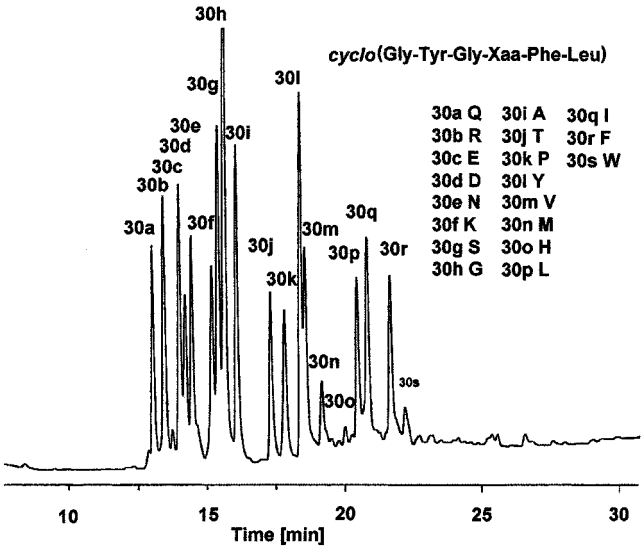

Figure 8. RP−HPLC profile of on-resin cyclization of a peptide thioester mixture consisting of 19 peptides with sequences of cyclo-(GYGXFL).

to the procedure by Ostresh et al.[34] The side chain protecting groups were cleaved by HF at 4 °C prior to cyclization. Cyclization was conducted in 0.2 M sodium acetate buffer at pH 5.5 in the presence of 50% DMSO. All 19 cyclic peptides were distinctly obtained in HPLC. The cyclic peptides were separated by RP-HPLC (Figure 8) and identified by MALDI-MS. Cyclic peptides where Xaa = His and Trp were found in lesser amounts due to the inherent error of competitive coupling of a mixture of 19 amino acids to afford the His- and Trp-containing linear peptide precursors. More importantly, RP-HPLC and MALDI-MS investigation indicated no significant level of oligomerization had occurred in the cyclization process (Figure 8). To further verify this method, a peptide library of 19 × 19 peptides in the format of Gly-Tyr-Xaa-Yaa-Phe-Leu **31** was synthesized. After cyclization in 0.2 M sodium acetate buffer at pH 5.5 in the presence of 50% DMSO, the products were separated in 1 min portions by RP-HPLC. MALDI-MS of the peptide mixtures clearly indicated that no oligomer was formed. Similar results were obtained when the effect of conformation on the cyclization was tested using a peptide library based on the model peptide **1** in which the Gly-Gly dipeptide sequence was replaced by a library of 361 dipeptides. This experiment also suggests that for flexible peptides the intramolecular reaction of Ag^+ ion-assisted peptide cyclization is entropically favored over the intersegmental ligation among these free peptide thioesters to yield various hetero- or homo-oligomers.

Discussion

Unprotected peptide thioesters are generally resistant to aminolysis at pH 4−8 in buffered aqueous solutions. However, aminolysis can occur in these conditions when thioesters are enthalpically activated by Ag^+ ions. Our working hypothesis is to test whether the Ag^+ ion-assisted orthogonal ligation strategy would provide the entropically activated intramolecular acyl migration to achieve chemoselectivity through ring−chain tautomerization[7,14−19] This is desirable because the absence of protecting groups would result in the untenable consequence of random acylation and oligomerization after enthalpic activations.

The action of the Ag^+ ion as an entropic factor facilitating coordination of a reactive nucleophile either at the N-terminus or on a side chain with the C-terminal peptide thioester through ring−chain tautomerization (Figure 1) has two consequences. First, it permits the two reactive nucleophiles of an unprotected peptide thioester to be oriented in close proximity, thereby reducing the entropy barrier to cyclization. Since the complexation sites can be manipulated by adjusting the pH and the conditions of the aqueous reaction media, both lactones and lactams can be achieved with high regiospecificity. The combination of enthalpic and entropic activations by Ag^+ ion permit a long-range acyl migration, resulting in lactonization or lactamization of peptide thioesters generally not accessible without Ag^+ ion assistance in aqueous media. Entropic activation by the Ag^+ ion coordination of two reactive ends would facilitate metal ion-promoted ring−chain tautomerization, favoring an intramolecular acylation process. Previously, we have shown that ring−chain tautomerization in other orthogonal cyclization methods can greatly minimize polymeric products and permit cyclization at relatively high concentrations.[7a,14] The present results based on competition experiments of model peptides and comparison with other conventional cyclization methods generally support the conclusion for the role of Ag^+ ion in promoting ring−chain tautomerization.

The selectivity for cyclization is strongly affected by pH and which N-terminal amino acids are used. At pH 5.2−5.7, end-to-end cyclization through the α-amines is favored. Furthermore, we have found that the α-amines of the less sterically hindered amino acids, Gly, Ser, Asp, and Asn, strongly favor end-to-end over end-to-side chain cyclization with a α/ϵ lactaminzation ratio of >20. A plausible explanation is that additional O-coordinating sites present at the side chains of Ser, Asn, and Asp would stabilize the Ag^+ ion complexation between the N-terminal amine and C-terminal thioester, thus providing high regioselectivity in α-acylation. Gly, being the least hindered amino acid, is exceptionally selective for end-to-end cyclization. The flexibility of the α-glycyl amide bond may mimic the α-Asn side chain as the O-coordinating site to favor end-to-end cyclization. Interestingly, Gly, Ser, Asp, and Asn are frequently found in either the reverse turn or the end of an α-helix and are known as N-cap amino acids.[30] In contrast, the presence of bulky and sterically hindered α-amines, such as Ile, Val, and Pro, favors side chain-to-end cyclization, forming either lactone or side chain lactam products. The usefulness of choosing Gly and Ser as cyclization sites has been demonstrated in the synthesis of dichotomin A−F. Even penta- or hexapeptides were obtained in good yields. This is remarkable because cyclization of penta- or hexapeptides is known to be associated with dimerization, cyclodimerization, and racemization.[36]

The pK_a of the N^{α}-amino acid may also contribute to the observed selectivity. The pK_a of a hexaglycine is known to be 6.7, while that of the ϵ-amine of an internal Lys is about 9.6. The ΔpK_a of 3.1 may account for the significant selectivity between the lactamization of α- and ϵ-amines at pH <5.7 used in our experiments. The relative pK_a of α-amines decreases with electronic donating side chain substitution on the α-amine and hence the decrease in selectivity of α/ϵ lactamization of model peptides **9** in the series of Gly > Ala > Leu from 100 > 4 >0.8 (Figure 4). However, electron-withdrawing side chain substitutions somewhat increase the selectivity as observed from

(34) Ostresh, J. M.; Winkle, J. H.; Hamashin, V. T.; Houghten, R. A. *Biopolymers* **1994**, *34*, 1681.

(35) Zhang, L.; Tam, J. P. *Tetrahedron Lett.* **1997**, *38*, 3−6.

(36) (a) Dale, J. *Angew. Chem.* **1966**, *78*, 1070. (b) Kessler, H.; Kutscher, B. *Liebigs Ann. Chem.* **1986**, 869. (c) Kessler, H.; Kutscher, B. *Liebigs Ann. Chem.* **1986**, 914.

the series Asp/Asn/Ser > Thr > Ala from 21 > 4.5 > 4, while His, Met, Trp, and Cys are surprisingly worse than Ala.

N-terminal amino acids with side chain heteroatoms, such as S from α-Cys or α-Met and N from α-His or α-Trp, that are usually stronger coordinating sites than O from α-Ser or α-Asp do not afford greater selectivity of α-lactamization than in the α-Ala-model peptide **1.** Decreasing the side chain nucleophilicity of these heterocyclic N-terminal amino acids such as conversion of α-Met to α-Met(O), thiol to disulfide, and α-Trp to α-Trp-(For) generally improved α/ϵ acylation. A possible explanation is that these amino acids contain two strong Ag^+ ion binding sites, the α-amine and the C-terminal thioester. Thus, Ag^+ strongly complexed at the amino terminus may act as a pseudo protecting group of the α-amine that minimizes α-lactamization.

In summary, the reactivity of N^{α}-amino acids is affected by a combination of several factors: steric hindrance, side chain assistance, and the relative pK_a of the N^{α}-amino acid. However, the steric factor appears to be the most predictive factor since it correlated well with the bulkiness of the N^{α}-amino acid since the time required for completion increased from 2 to >10 h from Gly to Ile. Thus, hindered aliphatic amino acids such as Ile, Val, and Pro, which require long reaction times, should be avoided whenever possible if end-to-end cyclization is desired. In contrast, the least hindered amino acid Gly and those small amino acids such as Ser, Asn, and Asp favor end-to-end cyclization.

This work has also demonstrated the utility of metal ion-assisted cyclization for efficient, high-yield synthesis of cyclic peptide libraries. Cyclization using unprotected peptide precursors has significant advantages. These include high yield, concentration independence, and simplicity. Since the orthogonal ligation strategy produces end products which do not require any postdeprotection steps and are assay-ready, it increases the efficiency of the high-throughput requirement for a peptide library. However, we have gone a step further. On the basis of a single precursor and a single synthesis, Ag^+ ion-assisted cyclization permits the synthesis of a range of cyclic peptides as end-to-end or side chain-to-end lactams and lactones. Thus, depending on the conditions, a range of cyclic peptides could be obtained as mixtures with different ring sizes and constraints. Under controlled conditions, it is also possible to achieve selective formation of specific cyclic peptides. For example, even in a mixture of 19 or 361 N-terminal glycyl peptides **30** and **31**, end-to-end cyclic peptides were selectively obtained with no discernible oligomerization or mixed dimers. To obtain the same range of products by conventional methods, several syntheses with different tiers of orthogonal protecting groups would be required. These advantages of Ag^+ ion-assisted cyclization should be useful for library applications.

Thus far, orthogonal ligation and cyclization based on entropic activation methods employ covalent intermediates through a triangulation strategy, whereby the first bond formation deviates from the intended course. Instead, first bond formation occurs between the weakly activated acyl moiety and a side chain hydroxyl or thiol nucleophile at the N-terminus to form a covalent branched *O*- or *S*-ester intermediate that permits a subsequent, short-range *O*- or *S*-acyl migration via a five- or six-member ring to the α-amine to be on course in forming the amide bond.[14−19] Similar triangulation has been exploited for ligation at the N-terminal His by using the side chain imidazole as the nucleophile to capture an activated acyl moiety to permit a six-member *N*,*N*-acyl migration.[35] However, the Ag^+ ion-mediated cyclization proposed in this study is a long-range acyl migration via an intermediate of >15 member ring atoms. Thus, our results are interesting because they describe a new approach that can supplant entropic activation via a metal ion-coordinated complex to permit the occurrence of long-range acyl migration in the orthogonal ligation strategy. More importantly, this method extends the number of possible orthogonal ligation sites to other N-terminal amino acids besides Cys and His.[7,14−19,35]

As a working concept, the Ag^+ ion-assisted method for providing long-range acyl migration may provide a general and useful approach, not only for intramolecular cyclization but also for intersegmental ligation. For example, using transthioesterification as a capture mechanism, a branched peptide intermediate would be formed, which would subsequently rearrange to a native, open-end peptide by the metal ion-assisted long-range acyl migration as proposed in Figure 1.

An unresolved question of the present study is the effect of peptide conformation. In this work, we report experiments with medium-sized peptides ranging from 5 to 16 amino acids. It remains to be determined whether similar results can be obtained from rigid peptides with preferred secondary structures and whether the described methods are applicable to large circularly permutated peptides and proteins whose N and C-termini are in close proximity.

In conclusion, we have developed a simplified strategy based on metal ion-assisted capture using unprotected peptide precursors to prepare moderate-sized cyclic peptides of lactones and lactams. Furthermore, we have shown that Ag^+ ion-assisted ring−chain tautomerization minimizes oligomerization. These methods have been shown to be useful concepts for peptide library methods in which cyclic peptides are released from the resin after cyclization and directly applied for high-throughput assays without further deprotection steps.

Experimental Section

Preparation of HS-CH_2CH_2CO-MBHA-Resin 7. General Procedure. 3-Mercaptopropionic acid **5** (1.2 mL, 13 mmol), HOBt (1.8 g, 13 mmol), and DIC (2 mL, 13 mmol) were sequentially added to a suspension of 4-methylbenzhydrylamine (MBHA) resin **6** (6 g, 3.2 mmol) in DMF (60 mL). The mixture was shaken at room temperature for 30 min, and a ninhydrin test of the resin indicated that no free amino groups were present. The resin was washed with DMF, DCM, CH_3OH, DCM, and DMF. The resulting resin was treated with a mixture of cysteine methyl ester hydrochloride (0.55 g, 3.2 mmol), triphenylphosphine (0.85 g, 3.2 mmol), and DIEA (0.68 mL, 3.9 mmol) in DMF/DCM (3:1, 60 mL). After 2 h, the resin was thoroughly washed with DMF, DCM, CH_3OH, and DCM and dried in vacuo. Yield: 6.3 g.

Preparation of *tert*-Butoxycarbonyl (Boc) Amino Acid Thioester Resin. General Procedure. To **7** (1 g, 0.52 mmol) was added Boc-amino acid (2.1 mmol) preactivated with BOP (0.88 g, 2.1 mmol) and DIEA (0.55 mL, 3.2 mmol) for 5 min. The mixture was shaken at room temperature for 2 h, and free thiol group was monitored by Ellman's reagent.[27] In general, a 2 h reaction time led to reaction completion. The resin was washed with DMF, DCM, CH_3OH, and DCM and used for stepwise peptide synthesis.

Solid-Phase Peptide Synthesis. All peptides were synthesized on thioesters **8** to afford peptide thioesters after HF cleavage. Typically, 0.3−0.4 g of resin was used for each synthesis. All amino acids were protected with the 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 2 min deprotection with 99% trifluoroacetic acid and (ii) coupling with 4 equiv of Boc-amino acid and BOP or HBTU in the presence of 6 equiv of DIEA in DMF for 30 min. All couplings were monitored by the ninhydrin test, and a double coupling was used with 3 equiv each of Boc-amino acid and DICI/HOAt[37] if necessary.

(37) Carpino, L. A. *J. Am. Chem. Soc.* **1993**, *115*, 4397−4398.

After assembly of the sequence using BOP/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% TFA. Crude peptides were purified on a preparative C_{18} reversed-phase 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.

Characterization of Peptide Analogues. Amino acid analysis of each synthetic peptide was carried out in 5.7 M HCl at 110 °C for 24 h and agreed with the theoretical ratio. Analytical HPLC for all peptides was performed on Vydac columns (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.

Verification of the Regiospecificity of Cyclization Reactions. (1) Determination of N^{α}-DNP-Amino Acids and N^{α}-DNP-Lys through Analytical HPLC. N^{α}-DNP-amino acid standards were obtained from Sigma Chemical (St. Louis, Mo), and N^{α}-DNP-Lys was prepared according to the procedure described previously.[7a] The retention times of N''-DNP-amino acids were determined by analytical HPLC as detected at 408 nm (gradient 2: 1 min 30% B, isocratic; 30−100% within 30 min). t_R (min) = 5.16 for cysteic acid, 12.93 for Asp, 11.59 for Ser, 14.77 for Gly, 18.45 for Ala, 10.27 for Asn, 10.73 for Arg, 11.37 for Gln, 14.70 for Glu, 15.06 for Thr, 27.60 for Leu, 27.15 for Phe, 26.22 for Trp, 24.51 for Val, 23.92 for Met, and 27.49 for Ile. N^{α}-DNP-Lys had a t_R of 11.37 min.

The cyclic samples were treated with 2,4-dinitro-1-fluorobenzene to yield DNP derivatives as described previously[7a] and hydrolyzed with 5.7 M HCl at 110 °C for 24 h. The hydrolysate was analyzed with RP-HPLC using gradient 2. The absence of N^{α}-DNP-amino acid at the N-terminus of the corresponding precursor indicated end-to-end cyclization.

(2) Enzymatic Digestion of Cyclic Peptides. A typical example of enzymatic digestion is illustrated with **28.** 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 of trypsin 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 a linear gradient of 10−100% buffer B over 30 min. As expected, **28** digested into its linear peptide, indicating that the lysine side chain was free and unmodified. MALDI-MS showed it was the expected product corresponding to Lys-Xaa bond hydrolysis. Anal. RP-HPLC (t_R in minutes): t_R = 22.42 (**28**), t_R = 19.24 (**28** after digestion). MALDI-MS: 1695.4 (calcd for LSQELCSNLSTCVLGK 1695.0, t_R = 19.24).

(3) Racemization. Cycilc products were hydrolyzed with 6 N HCl at 110 °C for 24 h, and amino acids were derivatized with Marfey's reagent.[38] Racemization of amino acids was then determined by RP-HPLC.

General Procedure for the Cyclization of Enkephalin Analogues 4a−d and 13−17 and Determination of the Specificity of the N-Terminus Amino Acid. Linear precursors Xaa-Lys-Tyr-Gly-Gly-Phe-Leu-SR (R = $-CH_2CH_2CONH_2$) were dissolved in 0.5 M sodium acetate buffer (pH 4.5−5.4) in concentrations ranging from 2 to 20 mM. A 1−3 equiv sample of AgOTfa in DMSO was added to the solution so that the final concentrations ranged from 1 to 10 mM. Aliquots were withdrawn for analytical HPLC. The cyclizations were usually complete within 4 h. Cyclized peptides were isolated by preparative HPLC and characterized by MALDI-MS.

Cyclization of AVSEIQFMHNLGK-$SCH_2CH_2CONHCH_2CO_2H$ 10. A 1.6 mg (0.1 mmol) sample was dissolved in 0.5 mL of 0.2 M sodium acetate buffer. To the solution was added 0.65 mg of AgOTfa (0.3 mmol) dissolved in 0.5 mL of DMSO. The reaction was complete after 6 h. One major peak was detected in analytical RP-HPLC (t_R = 19.5 min, gradient: 10% buffer B isocratic for 1 min; 10−100% buffer B within 30 min). MALDI-MS analysis indicated the product was a cyclic peptide (found 1455.0, calcd for $[M + H]^+$ 1455.7). End group analysis using Sanger's reagent revealed that the product was the Lys side chain-cyclized peptide **10b** since only DNP−Ala was detected in analytical HPLC.

Synthesis of Cyclo(RGDfV) 12a.[31] (1) From DfVRG-$SCH_2CH_2CONH_2$ 12 (f = D-Phe). A 6.8 mg (0.01 mmol) sample of **12** was dissolved in 4 mL of 0.2 N sodium acetate buffer. To the solution was added AgOTfa (6.6 mg, 0.03 mmol) in 1 mL of 0.2 N sodium acetate. The mixture was allowed to vortex for 6 h. The reaction was terminated by adding NaCl solution, and the final product **12a** was isolated in a yield of 90%. MALDI-MS: found 574.2, calcd for $[M + H]^+$ 574.6. No DNP−Asp was found in the end group analysis using Sanger's reagent.[26]

(2) From GDfVR-$SCH_2CH_2CONH_2$ 11. Cyclization of **11** was performed in a procedure analogous to that described for **12**. The desired product **12a** was obtained in 30% yield. Twelve percent of the Arg side chain-cyclized peptide **11b** was isolated and identified by end group analysis using Sanger's reagent. Another 32% was characterized as GDfVR resulting from the hydrolysis of the starting peptide thioester **11**.

Synthesis of Dichotomins A−G 18−25. For the synthesis of dichotomins A−G, the following linear peptide thioester precursors were used, and the corresponding product is indicated in parentheses: Gly-Thr-Phe-Leu-Tyr-Val-X (**19**), Gly-Thr-Phe-Leu-Tyr-Thr-X (**20**), Gly-Thr-Phe-Leu-Tyr-Ala-X (**21**), Gly-Phe-Tyr-Ile-Gly-Val-X (**22**), Gly-Tyr-Ala-Phe-Ala-X (**23**), Leu-Pro-Ser-Val-Tyr-Pro-Tyr-Phe-Val-X (**24**), and Ser-Pro-Leu-Pro-Ile-Pro-Pro-Phe-Tyr-X (**25,** X = $-SCH_2CH_2CONH_2$). All linear precursors were obtained from solid-phase synthesis and characterized by MALDI-MS (Table 4). Typically, peptide thioesters were dissolved in a mixture of 0.2 M sodium acetate buffer and DMSO (1:1) in a concentration of 2 mmol. To the peptide solution were added equivolumes of 0.2 M sodium acetate buffer and DMSO (1:1) containing 3 equiv of AgOTfa. The reaction was allowed to vortex overnight. The final product was purified by preparative RP-HPLC and characterized by MALDI-MS (Table 4).

Synthesis of Bicyclic Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu 28. (1) Disulfide Formation of Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu-S-$CH_2CH_2CONH_2$ 26. The linear peptide, Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu-S-$CH_2CH_2CONH_2$ **26** (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 4.5 using 10% Na_2CO_3. The reaction was monitored by analytical HPLC. After 4 h, the cyclic peptide via a disulfide bridge **27** was isolated by preparative HPLC in 85% yield.

(2) End-to-End Cyclization. 27 (3.2 mg, 0.6 μmol) was dissolved in 0.5 M sodium acetate buffer (0.5 mL, pH 5.5). AgOTfa (0.4 mg, 1.8 μmol) in DMSO (0.5 mL) was added to the solution. The progress of the reaction was monitored by analytical RP-HPLC. The reaction was complete in 6 h, and the product **28** 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 in a concentration of 2 mM in 0.2 M sodium acetate (pH 3.5−6.0)/DMSO (1:1) in the presence of 1−3 equiv of AgOTfa. 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.

Synthesis of Cyclic Peptide Libraries. For the synthesis of cyclic peptide libraries, 3-mercaptopropionic acid was first coupled to aminofunctionalized TentaGel (0.29 mmol/g) using DIC/HOBt and treated with a mixture of cysteine methyl ester hydrochloride, triphenylphosphine, and DIEA as described above. Two separate libraries, cyclo(Gly-Tyr-Gly-Xaa-Phe-Leu) and cyclo(Gly-Tyr-Yaa-Xaa-Phe-Leu), where Xaa and Yaa represent all proteinogenic amino acids except cysteine, were synthesized. Gly and Leu were chosen as N-terminal

(38) Marfey, P. *Carlsberg Res. Commun.* **1984,** 591−596.

and C-terminal cyclization sites, respectively. Thus, Leu was introduced onto the resin by BOP/DIEA. The incorporation of Xaa and Yaa was followed by the procedure of Ostresh et al.[34] to achieve approximately equimolar representation of each peptide using a 10-fold excess of amino acid and DICI/HOBt coupling. After the assembly of the linear precursors, the peptide resin was treated with HF/anisole (9:1) at 4 °C for 1 h. The resin was washed with diethyl ether, ACN/H_2O (1:1), and methanol and dried overnight. For the final cyclization, 25 mg (5 μmol) of peptide resin was suspended in 0.5 M sodium acetate (0.5 mL). To the suspension was added 3 equiv of AgOTfa (3.3 mg) in DMSO (0.5 mL). The mixture was allowed to vortex for 6 h. The cyclic peptide libraries were desalted by RP-HPLC.

Acknowledgment. This work was supported in part by U.S. Public Health Service NIH Grants GM57145 and CA35644.

JA983859D