Cyclic Peptides from Linear Unprotected Peptide Precursors through Thiazolidine Formation†

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Abstract: We describe a general method for the preparation of cyclic peptides by intramolecular thiazolidine formation from linear, unprotected peptide precursors. The precursors contain a protected 1,2-aminothiol from an N-terminal cysteine and a 1,2-amino alcohol or 1,2-diol as a masked aldehyde. Thiazolidine formation was effected by oxidation of the 1,2-amino alcohol or 1,2-diol by sodium periodate to give an aldehyde, followed by deprotection of the masked 1,2-aminothiol. The cyclization could be effected at concentrations as high as 20 mM and was free from any polymerized side products. Such high efficiency of macrocyclization may be attributed to the ring-chain" tautomerism of the open chain amino-aldehyde precursor that favors a macrocyclic thiazolidine ring. Thiazolidine formation was further exploited as a capture device to position the N and C termini covalently close together and then to allow a proximity-driven O to N acyl transfer through a novel tricyclic ring contraction to yield an all amide, end-to-end cyclic lactam. These macrocyclization methods have been applied to the synthesis of cyclic peptides containing 5 to 26 amino acids.

Introduction

Cyclic peptides have been extensively employed as a method of conformational constraint to achieve increased biological activity, selectivity, and metabolic stability.[1−3] Cyclic scaffolds and templates have been used to assemble various spatially defined functional groups for molecular recognition,[4] and cyclic peptide libraries[5] have been investigated to combine structural constraints with molecular diversity. In most cases cyclization has been achieved by either cyclic lactam or disulfide formation. Cyclic lactam formation requires the protection of selected functional groups and is usually performed in an organic solvent,[6] either at high dilution[7a] or using pseudo-dilution on a solid support.[7b] Disulfide formation proceeds in aqueous conditions at very dilute to moderately high concentrations. However, its limitation is that the disulfide bridges are not always metabolically stable *in vivo*.

These two major methods also provide fundamentally different cyclization processes. In lactam cyclization, the acylating moiety is highly enthalpically activated and is susceptible to nucleophilic attack. This procedure requires protecting groups to mask the peptide side chains and high dilution to prevent polymerization. In contrast, disulfide cyclization is an equilibrating process that can be performed with unprotected peptides at relatively high concentration and employs redox conditions that permit disulfide exchange[8,9] to favor intermolecular disulfide formation. Thus, it would be highly desirable to develop a cyclization method of unprotected peptides in aqueous solution that has the advantages of disulfide formation but forms a nondisulfide bond.

Recent advances in the orthogonal coupling of unprotected peptide segments have provided approaches that utilize a weakly activated acyl moiety coupled with proximity-driven amide formation.[10,11] In such an approach, addition to the weak acyl

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† Abbreviations: BOP, benzotriazole-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; DBU, 1,8-diazabicyclo[5.4.0.]undec-7-ene-(1,5-5); DCC *N*,*N*′-dicyclohexylcarbodiimide; DCM, dichloromethane; DCU, *N*,*N*′-dicyclohexylurea; Dde, 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl; DIEA, *N*,*N*-diisopropylethylamine; DMAP, 4-(dimethylamino)-pyridine; DME, ethylene glycol dimethyl ether; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, *N*-hydroxybenzotriazole; MALDI-MS, matrix-assisted laser desorption mass spectrometry; RP-HPLC, reversed-phase high-performance liquid chromatography; SPro, thiaproline; StBu, *S*-*tert*-butylsulfenyl; TLC, thin layer chromatography; TFA, trifluoroacetic acid; TCEP, tris(2-carboxyethyl)-phosphine; TIS, triisopropylsilane.

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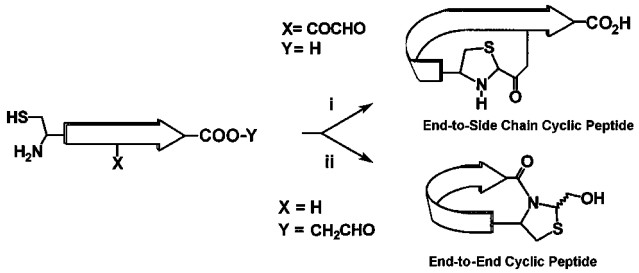

Figure 1. A general scheme for the preparation of cyclic peptides from a linear precursor bearing either (i) a side-chain aldehyde (X = COCHO) to give end-to-side chain cyclization or (ii) a C-terminal glycoaldehyde (Y = $COOCH_2CHO$) to give end-to-end all amide backbone cyclization.

moiety is the first step of covalent bond formation, which is usually reversible under aqueous conditions. A case in point is amine-aldehyde intramolecular ligation by the condensation of two peptide segments containing 1,2-aminothiol on one segment and an aldehyde on the other, which forms a thiazolidine ring under aqueous acidic conditions. The thiazolidine ligation method might also provide intramolecular cyclization when both 1,2-aminothiol and aldehyde are present on a single chain. The reversibility of the amine-aldehyde condensation also makes "ring-chain tautomerism" possible, favoring intramolecular cyclization to a ring.[12,13] Ring-chain tautomerisms have been well documented in small to macrocyclic member rings but have not been applied to macrocyclization of peptides. Nevertheless, if such a mechanism is feasible, it would offer the advantage of preventing random polymerization of unprotected peptide segments and would avoid the necessity of high dilution conditions during cyclization.

In this paper, we report a general method for the preparation of cyclic peptides under the influence of "ring-chain" tautomerism through thiazolidine formation from linear, unprotected aldehyde peptide precursors. Two types of thiazolidine bond formations are described. In the first type (Figure 1, path i), a thiazolidine ring is formed from a glyoxylaldehyde and a 1,2-aminothiol to generate an end-to-side chain cyclized product. In this study, the glyoxyaldehyde is generated from $NaIO_4$-mediated oxidation of a serine linked to the side chain lysine, whereas the 1,2-amino-thiol is derived from the cysteine placed at the amino terminus. Obviously, there is flexibility in placing two functional groups at different points in the peptide chain to generate different cyclic peptides. In the second type, the initial thiazolidine ring formation is an intermediate step that leads to a cyclic lactam as a final product (Figure 1, path ii). The mutually reactive functional groups, a C-terminal glycoaldehyde generated from $NaIO_4$-mediated oxidation of a diol precursor and an N-terminal 1,2-aminothiol, initially form a thiazolidine ring. This ring is used as a capture device to hold covalently the N- and C-termini close together, thus allowing a proximity driven O to N-acyl transfer that generates an end-to-end cyclized final product. The transfer proceeds through a tricyclic intermediate to give a thiaproline bond and provides a novel approach to the synthesis of cyclic lactams hitherto inaccessible through unprotected peptide precursors.

The model peptides used throughout our study ranged from 5 to 26 amino acid residues in length and were derived from the third variable (V3) loop on gp120 of human immunodeficiency virus (HIV-1).[14] A conserved tip of the V3 loop containing the Gly-Pro-Gly-Arg-Ala-Phe-Tyr sequence is known to be an important antigenic determinant recognized by neutralizing antibodies derived from different HIV-1 isolates and is of interest in the design of a peptide-based vaccine. This sequence adopts a multiple β-turn conformation[15] which may be stabilized by various forms of cyclization, and it is also known to mediate the membrane fusion events that determine viral infectivity.[16] Any cyclic peptide analogs that interfere with viral entry into host cells may provide useful leads for therapeutic agents.

Results

Synthetic Strategy To Generate a 1,2-Aminothiol and an Aldehyde on Unprotected Peptides. Our scheme requires a mild method for introducing a 1,2-aminothiol and an aldehyde onto an unprotected peptide for subsequent thiazolidine formation. A 1,2-aminothiol is available from an unprotected cysteine residue positioned at the N-terminus or attached to the N^{ϵ}-amine of lysine. Aldehydes do not occur naturally in amino acids and must be incorporated in the peptide chain in a masked form.

Two different strategies were examined to obtain a 1,2-aminothiol and an aldehyde on unprotected peptides. In the one-step strategy, both the 1,2-aminothiol and the aldehyde were generated during the final cleavage step that removes both the side chain protecting groups and the peptide from the resin support. In the sequential strategy, the 1,2-aminothiol and the aldehyde were masked during the cleavage step to produce an unprotected linear precursor. These functional groups were then sequentially unmasked for the cyclization reaction.

Model studies in our laboratory showed that alkyl aldehydes, introduced as protected acetals, were generally unstable to the strong acid used to cleave the peptide from the resin. For example, 5,5-dimethoxyvaleric acid introduced onto the side chain of a lysine residue did not yield appreciable amounts of aldehyde following TFA cleavage from the resin support. A major side reaction was the conversion of the aldehyde to a mono ester by TFA.[17] Resin-linkers that yielded C-terminal aldehydes directly after TFA or HF cleavage of the peptide also failed to yield significant amounts of aldehyde product. In these cases, the major side reaction was the hydrolysis of the glycoaldehyde ester to the free carboxylic acid in a strong acid. Based on these limited preliminary results, we concluded that aldehydes were not sufficiently stable to the peptide-resin cleavage conditions to allow their practical use in a one-step strategy. Thus, our attention turned to a sequential strategy in which both the thiol and the aldehyde moieties are masked during the final resin cleavage step. This method offers the advantages of stability, ease of purification, and reduced side products for the synthesis of end-to-side chain and end-to-end cyclized peptides.

A Sequential Strategy for End-to-Side Chain Cyclization by Thiazolidine Bond Formation. A general scheme for end-to-side chain cyclization is depicted in Figure 2. In this scheme, a cysteinyl 1,2-aminothiol moiety was placed at the N-terminus and an unprotected serine on the side chain of an internal lysine.

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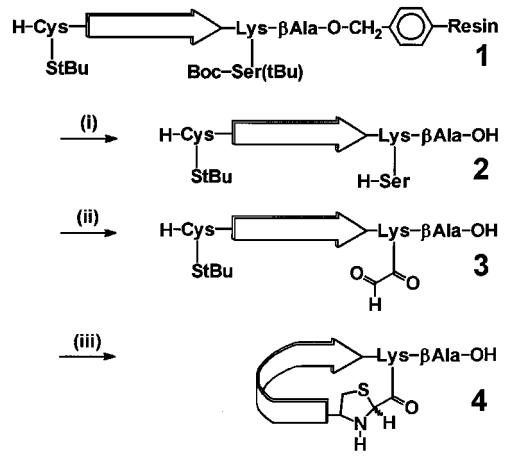

Figure 2. Side chain to N-terminal cyclization by thiazolidine formation of the linear peptide **2** C(StBu)GIGPGRAFGK(Ser)-βAla: (i) CF_3COOH, (ii) $NaIO_4$ pH 6.8, (iii) tris(carboxyethyl)phosphine, pH 4.2.

The 1,2-amino alcohol of the serine is an aldehyde precursor and is easily converted to a glyoxylaldehyde by oxidation with sodium periodate.[18] A *tert*-butylsulfenyl group was used to protect the cysteinyl thiol during the oxidation of the 1,2-aminoalcohol to the aldehyde. The thiol was easily deprotected by a trialkylphosphine derivative under acidic conditions[19] to effect the thiazolidine formation.

The linear peptide precursor **1** was synthesized on a Wang resin using standard Fmoc/tBu methodology. For the introduction of the Boc-Ser(tBu) on the side chain of the resin-bound lysine, a non *tert*-butyl derived protecting group compatible with the Fmoc chemistry is required. We found that the Dde-Lys-(Fmoc)-OH[20] derivative of lysine was suitable after incorporating Dde-Lys(Fmoc)-OH onto the peptide bound resin. The Fmoc group was selectively removed using 1% DBU[21] in DMF and Boc-Ser(tBu)-OH was coupled to the unprotected resin-bound lysyl side chain as an amide. The N^{α}-Dde group was then removed with 1% hydrazine hydrate in DMF[20] and the rest of the peptide assembled from the N^{α}-amine in the usual manner. Treatment of the assembled peptide-resin **1** with TFA cleaved the peptide from the resin support and released the linear peptide precursor **2** in solution. At this point, the thiol moiety of the N-terminal cysteine was still protected as the StBu derivative, but the rest of the peptide did not contain any protecting groups.

Oxidation of the 1,2-amino alcohol of the serine in **2** by $NaIO_4$ at pH 6.8 for 2 min gave the desired glyoxylaldehyde **3** in quantitative yield. Purification of aldehyde **3** by RP-HPLC was necessary due to the presence of formaldehyde generated as a byproduct in the $NaIO_4$-mediated oxidation. Removal of the StBu protecting group of **3** to yield a free cysteine and the subsequent cyclizations were carried out concomitantly using 5 equiv of a water-soluble trialkylphosphine, tris(2-carboxyethyl)phosphine[22] (TCEP). Although thiazolidine formation is fast at pH 4−6, TCEP-mediated deprotection was found to be slow at acidic pH. Thus, the cyclization required 72 h to achieve 95% completion with 2% of the StBu moiety unremoved and 2% of resulting aminothiol uncyclized (Figure 3). No polymeric product from the thiazolidine formation was observed in analytical RP-HPLC. When the second step was performed in neutral or slightly basic conditions (pH 8.0), the StBu group was removed more quickly, and the cyclization was complete within 48 h. However, the cyclic peptide **4** was isolated in only 70% yield due to deleterious side reactions at the higher pH. When the end-to-side chain cyclization of **2** was performed at 0.07, 0.7, and 7 mM concentrations in aqueous sodium acetate buffer at pH 4.5, no polymerization was observed, and the reaction was 95% complete after 96, 72, and 72 h, respectively. Interestingly, cyclizations at higher concentrations of 0.7 and 7 mM were slightly faster than at 0.07 mM, possibly due to the higher concentration of phosphine derivative present in each reaction and consequently higher rate of thiol deprotection. A tryptic digest, hydrolyzing the Arg-Ala bond, and the subsequent MS analysis of the fragments produced, demonstrated that the product was cyclic. The cyclic peptide **4** gave the expected linear thiazolidine, while the linear precursor **2** gave two products confirmed as the expected C and N terminal fragments.

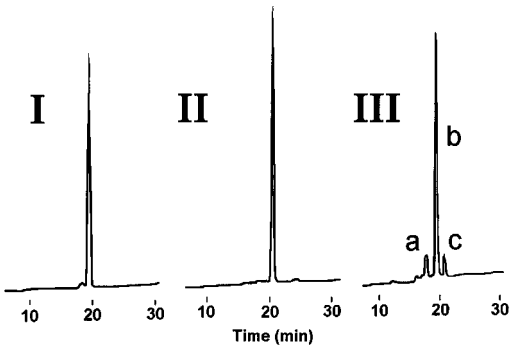

Figure 3. RP-HPLC analysis of the side chain to N-terminal cyclized peptide **4**: panel I, linear starting peptide **2** (see Figure 2); panel II, purified peptide-aldehyde **3**; panel III crude product after thiazolidine formation, peak **a** uncyclized peptide-aldehyde, thiol deprotected; peak **b** desired cyclic product **4**, peak **c** StBu protected peptide **3**. See Experimental Section for HPLC conditions.

End-to-End Cyclization by Thiaproline Formation. A similar synthetic strategy using a StBu protecting group for cysteine and an aldehyde precursor was used for the synthesis of end-to-end cyclized peptides (Figure 4). However, a C-terminal glycoaldehyde is required to obtain an end-to-end cyclic peptide with an all-amide backbone. In our strategy, the peptide-glycoaldehyde is obtained from a glyceric ester precursor **5a**−**e** derived from a novel peptide-resin following TFA cleavage.

The diol of the glyceric esters **5a**−**e** were converted to the glycoaldehyde **6a**−**e** by oxidation with $NaIO_4$ for 30 min at pH 5. The rate of the diol oxidation increased as the pH decreased below 5. Thus, at pH 2.0 the oxidation was complete within 1 min. At pH 6−7 the rate of oxidation was slow, and at pH 7.0 was complete only after 2 h. It should be noted that when the oxidation was performed below pH 5, Met was oxidized[18] to Met(O), even in the presence of large excesses of methionine as a scavenger. This side reaction could be avoided at neutral pH. Again, purification of the aldehyde by RP-HPLC was required to remove the generated formaldehyde. Removal of the StBu group from the cysteine **7a**−**e** and concomitant cyclization were performed at pH 5.5 to give a cyclic peptide with a thiazolidine linkage **8a**−**e**. Thiazolidine formation was complete within 1−6 h for cyclic peptides containing 5, 6, 12, and 26 amino acid residues (Table 1).

The intramolecular O to N-acyl transfer to the cyclic peptides **9a**−**e** with an amide backbone occurred after the pH of the

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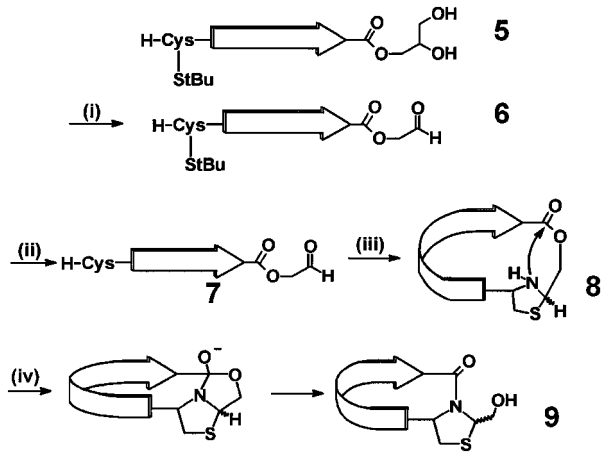

Figure 4. End to end cyclization by thiazolidine formation followed by an intramolecular acyl transfer: (i) $NaIO_4$, pH 5; (ii) BuP_3, pH 5.5; (iii) pH 5.9. Peptide sequence **a** CGRAG; **b** CGRAFG; **c** CGRAFVTIG; **d** CGRAFVTIGKIG; **e** CNNNTRKRIRIQRGPGRAFVTIGKIG; numbers refers to different molecules forms. Thus, the compound number **6d** represents the peptide sequence **d** containing the structure of compound **6**.

buffer was adjusted to 5.9 but required more than 20 h for completion at 22 °C (Figure 5). Increasing the temperature to 52 °C increased the reaction rate 3−5-fold. This O to N-acyl transfer reaction passes through a highly strained tricyclic intermediate when undergoing ring contraction. The thiazolidine formation introduces a chiral center at the C-5 position of the thiazolidine ring. This is at a ring junction of the tricyclic intermediate, and consequently the intramolecular acyl transfer proceeds at a different rate for each of the two diastereomers (Table 1).

To show that cyclization was concentration independent, the cyclization of **6d**, a 12-residue peptide (see Figure 4 for explanation), was performed at 0.6, 6, and 20 mM concentrations at pH 5.8 in the presence of 10−20% isopropyl alcohol. The cyclic thiazolidine formation to **8d** was complete within 0.5 h. The greatly accelerated rate of cyclization of **6d** compared to **2** could be attributed to the different chemical characteristics of the phosphines used, the higher pH, the presence of the organic solvent, or the favorable conformation of the precursor. Again, no polymerization was observed.

Development of New Resins To Yield a C-Terminal Peptide Glyceric Ester. The benzaldehyde-polystyrene resin **11** developed by Frechet et al.[23] was used in our experiment to link an amino acid glyceric ester to the resin (Figure 6). Two variations were developed to prepare the glyceric ester resin **13**, both starting from the benzaldehyde-resin **11** prepared by oxidizing a chloromethyl-polystyrene resin with DMSO/$NaHCO_3$. In the first method the preformed Fmoc-Gly-glyceric ester **10** was attached to the resin **11** by refluxing with a catalytic amount of *p*-toluenesulfonic acid in dioxane. Using this method the yield was in range of 43%, and the final loading of resin **13** was 0.25 mmol/g, as determined by elemental analysis and by quantitative analysis of the Fmoc protecting group released from the resin support with piperidine. The unreacted aldehyde was presumably converted to the dipiperidine adduct during the Fmoc cleavage step.[24] With this resin, excess of HOBt was used in the coupling step to account for the presence of the adduct.

In the second method a more general approach was adopted in which the benzylic cyclic acetal **12** was first formed on the resin by refluxing the benzaldehyde resin **11** with glycerol and a catalytic amount of *p*-toluenesulfonic acid in DME. The reaction was complete in 24 h as monitored by the disappearance of the IR absorption of the aldehyde carbonyl. Fmoc-Gly-OH was then coupled to the glyceric acetal-resin **12** via the symmetrical anhydride in the presence of 0.05 equiv of DMAP. Using this method, the final loading of the resin **13** was 0.5 mmol/g, a >95% yield with about 1% of the double insertion of glycerine[25] to give the Fmoc-Gly-Gly-glyceric ester. No evidence of unreacted aldehyde was detected by FT-IR. Treatment of the Fmoc-Gly-resin **13** with 90% TFA and a combination of scavengers (thioanisole, water, anisole) gave the Fmoc-Gly-glyceric ester **10**, indicating that the glyceric-ester bond was generally stable to the TFA cleavage conditions. No formation of Fmoc-Gly-OH was observed after 1 h, but after 2.5 and 12 h, 2%, and 20% hydrolysis had occurred, respectively.

In general, the first method of linking the preformed glyceric ester of an Fmoc-amino acid to the benzaldehyde resin is unambiguous and avoids the possible racemization and double insertion side reactions present in the second method.

Discussion

There are four aspects of our work that should be emphasized. First, we have developed the use of thiazolidine ring formation as a general method to give cyclic peptides from unprotected peptide precursors. This method is general because the cysteine and the masked aldehyde can be placed anywhere on the peptide chain. We have shown that very efficient end-to-side chain cyclization is ensured when they are placed at the N-terminus and on the side chain of lysine. Varying the placement of these groups on the peptide side chains or at the N-terminus would yield other types of cyclic peptides. For example, placement of both groups on the side chains of lysine residues would give side chain-to-side chain cyclic peptides.

Secondly, the thiazolidine ring formation is also exploited to form an end-to-end cyclic peptide with an all-amide bond backbone by utilizing a novel ring contraction via a tricyclic intramolecular rearrangement. In general, cyclizations by amide or lactam formation are not accessible from unprotected linear peptide precursors due to the lack of regioselectivity at relative sites. For the cyclization to occur by the formation of a covalent amide bond between the N-terminal amino acid and either the C-terminus or a side chain moiety, all other functional groups in the peptide have to be protected. However, protecting groups can be avoided if the appropriate N and C termini are in close proximity and the amide bond is formed by entropic activation.[26] The intramolecular O to N acyl transfer proceeds via a ring contraction through a tricyclic intermediate. The rate of rearrangement is dependent on the number of amino acids in the ring. The cyclic pentapeptides required heating to 52 °C to rearrange to the desired amide product in 30% yield after 100 h. Generally, the rate of the O, N acyl transfer reaction increased with ring size, and with the 26-mer peptide, the rearrangement was complete within 30 h.

Thirdly, based on our model peptides in acidic aqueous conditions the cyclization by thiazolidine formation is efficiently

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Table 1. Conditions and Yields of End-to-End Cyclization and Intramolecular O to N-Acyl Transfer

sequence		no. of amino acids	time for 95% completion (h) ester (22 °C)	amide 22 °C	amide 52 °C	yield[a] %
CGRAG	**5a**	5	0.1		>100[b]	25
CGRAFG	**5b**	6	0.1	100	30	90
CGRAFVTIG	**5c**	9	24[c]	100	[d]	70
CGRAFVTIGKIG	**5d**	12	0.5	100(20)[e]	20(0.1)[e]	96
CNNNTRKRIRIQRGPGRAFVTIGKIG	**5e**	26	6	36(10)[e]	14[f]	75

[a] Yield of the final product based on HPLC analysis for the reaction at 22 °C. Cyclization were effected by Bu_3P except otherwise stated. [b] 30% completion in 100 h. [c] Cyclization was effected by TCEP at pH 5.5. [d] Decompose. [e] Rate of rearrangement of two isomers. The faster rate is in parentheses. [f] 35% of hydrolyzed product was found.

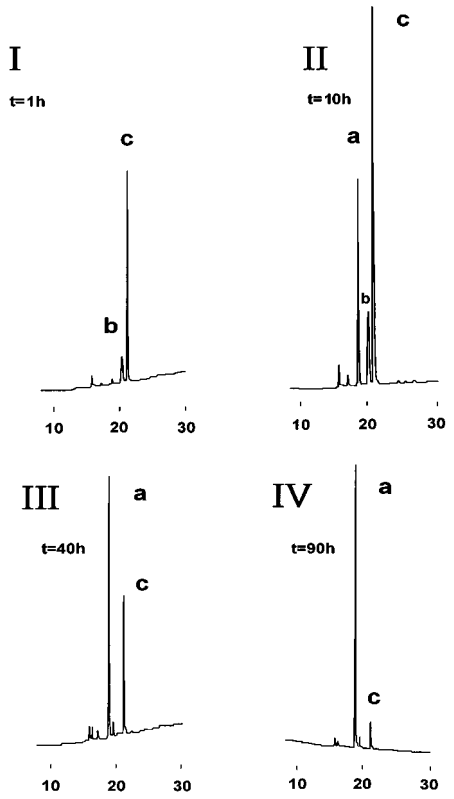

Figure 5. RP-HPLC analysis of the end-to-end cyclization of CGRAFVTIGKIG **6d**. Panel I, cyclization after 1 h; peaks **b** and **c** are diastereomers of **8** (see Figure 4). Panel II, O,N-acyl transfer after 10 h; peak **a** desired cyclic peptide **9d**. Panel III, O,N-acyl transfer after 40 h. Panel IV, O,N-acyl transfer after 90 h. See Experimental Section for HPLC conditions.

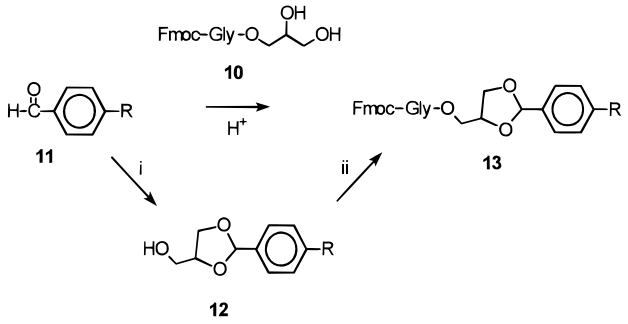

Figure 6. Synthesis of the acetal resin: (i) glycerol and (ii) Fmoc-Gly, DCC/DMAP.

accomplished in relatively high concentration. The cyclization yields are high and are based on the HPLC analysis. Furthermore, analyses of crude product mixtures do not show evidence of polymeric products. We can offer two reasons for such a high efficiency. First it may be due to "ring-chain" tautomerism of the amino-aldehyde peptide precursor. To avoid an intermolecular reaction, the conventional approaches of cyclization to form a lactam use a highly activated acyl moiety and usually require high dilution methods, typically in the micromolar range.[2,3] In our scheme, the acyl moiety is weakly activated as a glycoaldehyde ester amenable to the amine-aldehyde ring-chain tautomerization, which has many precedents in the literature.[11,12]

In the "ring-chain" tautomerism, the initial step is the addition of a weak base at one end of the chain to a multiple polar bond to such an aldehyde on the other end. In our case, it is likely that the thiol nucleophile is involved in the first step and this reaction is reversible under aqueous conditions.[27] The reversibility of the reaction is further favored by the *tert*-butylthiol liberated during the cysteine deprotection. However, the second step, which involves the thiazolidine formation and elimination of a water molecule to form a nonisomeric heterocycle, is slow and rate determined, but it drives the reaction to completion to favor the cyclic thiazolidine rather than polymeric products. We have used concentrations as high as 20 mM, which is 1000-fold higher than the conventional approach, and there were no detectable polymerized products. Thus, cyclization via "ring-chain" tautomerism of an amine-aldehyde precursor may provide the explanation for the high yield of our observed results and the avoidance of polymerization.

Second, it is possible that our linear peptides, which are derived from the looped region of the V_3 sequence, are conformationally predisposed to form cyclic structures. The macrocyclization tendency of peptides has been discussed by Mutter.[28] He found that the presence of Gly, Pro, or D-amino acid in the peptide sequence favors macrocyclization. Thus, it is possible that the concentration-independent nature of thiazolidine cyclization is a direct result of the conformation predisposition of the linear peptide precursor. However, it remains to be determined whether thiazolidine cyclization is concentration independent in other peptides. Nevertheless, our results show that our approach to cyclization using weakly activated reactive moieties and "ring-chain" tautomerism is different from the conventional cycloamidation approaches using highly activated acyl moieties and may significantly reduce the risk of random polymerization.

The apparent concentration-independent nature of our cyclization of unprotected peptides is noteworthy as an approach to the self-assembly of cyclic peptide dendrimers.[5] Such a self-assembly process, must be error free since many open branches are simultaneously cyclized to form the desired dendritic cyclized structures. This is a difficult process to achieve by conventional approaches, and thiazolidine cyclization through ring-chain tautomerization may provide a useful solution.

Finally, for end-to-end cyclic peptides, the presence of a thiazolidine ring provides opportunities for the inclusion of

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chimeric structural features and novel chemical modifications. In our previous study, we have found that the thiazolidine ring as a thiaproline analog is stable in aqueous solution from pH 3 to 9.[29] However, the thiaproline contains a new chiral center at the hydroxy methyl at the C-5 position which results in a mixture of two diastereoisomers in the final product. Since proline often occurs at reverse turns, the thiaproline side chain substituent is unlikely to perturb the conformation of an all-amide backbone, and a thiaproline analog is likely to adopt a similar conformation to a natural proline-containing peptide. Recently, we have shown that replacement of thiazolidine for Proline-39 in three analogues of HIV-1 protease does not affect their folding, dimerization, and biological activities.[30] Furthermore, such a thiaproline analog could be considered a chimeric amino acid with the structural constraint of a proline and the 5-hydroxyl methyl substituent of a hydroxylic amino acid.

Chimeric proline analogs have recently found successful applications in the rational design of peptide hormones. Kolodzriej et al.[31] have found that replacement of a 3 or 4 alkylthiaproline for the Met residue of a cholecystokinin (CCK) tetrapeptide yields a highly potent and selective CCK-B agonist, possibly because of the local constraint imposed by the proline analog that stabilizes a reverse turn, and because of the presence of the alkyl side chain mimicking the Met residue. In addition, there are a wealth of synthetic routes derived from penicillin chemistry for the transformation of the thiazolidine ring into other functional groups. These synthetic routes will be useful for transforming the thiazolidine ring into other unusual amino acids for analog studies. Furthermore, we anticipate that these thiazolidine ligation methods are not only applicable to the intramolecular cyclization of peptide segments but can also be used as a method to provide conformational constraints for a cyclic peptide library.

Experimental Section

Materials and Methods. Analytical RP-HPLC (Shimadzu Inc.) was performed on Vydac C_{18} reverse phase columns (25 × 0.46 cm i.d.) with a flow rate of 1 mL/min, monitored at 225 nm. Peptides were purified using Waters equipment on a Vydac C_{18} reverse phase column (25 × 1 cm i.d.) with a flow rate of 5 mL/min, monitoring at 225 nm. Eluent used were (A) 0.046% TFA in water, and (B) 0.039% TFA in 60% acetonitrile. Gradients were (1) 1 min isocratic 10%B, 30 min 10−100%B (Figure 3); (2) 30 min 20−70% B (Figure 5); (3) 30 min 5−50% B. Laser desorption mass spectra were obtained in positive ion mode on a Kratos MALDI-MS III instrument. FT-IR spectra were obtained from KBr discs. Elemental analysis was performed by Atlantic Microlab Inc. Trypsin was obtained from Sigma (T1005, DPCC treated).

Peptides were all synthesized using standard Fmoc/tBu chemistry using the stepwise solid phase method. Couplings were accomplished using BOP/DIEA in DMF, unless otherwise specified. Yields were determined by peak integration from the RP-HPLC trace. For the end-to-end cyclization the yields refer to the cyclization and acyl transfer steps only. The absence of polymer formation was established by two lines of evidence. First MS analysis of crude products after cyclization and acyl transfer showed no peaks corresponding to dimer or trimer. Second, there was absence of unexplained peaks from the HPLC trace. The major products after cyclization were shown to be isomers by MS and amino acid analysis and were presumed to be diastereoisomers.

Synthesis of Peptide 2. (H-Cys(StBu)GlyIleGlyProGlyArgAlaPheGlyLys(Ser)*β*-Ala-OH). Dde-Lys(Fmoc)-OH was coupled to a *β*-alanyl-*p*-(benzoyloxy)benzyl alcohol resin (*β*-alanyl-Wang resin). The Fmoc group was removed by treatment with 1% DBU in DMF, and Boc-Ser(tBu)-OH was coupled to the Lys side chain. The Dde group was then removed by treatment with 1% hydrazine hydrate in DMF, and the rest of the peptide assembled according to the standard Fmoc/tBu protocol. After the final removal of the N-terminal Fmoc group the peptide was cleaved from the resin using CF_3COOH/TIS/thioanisole/H_2O (92.5/2.5/2.5/2.5) for 1 h. The resin was removed by filtration, and the filtrate concentrated *in vacuo*. Following precipitation by dry ether the peptide was taken up in 5% aqueous acetic acid and then lyophilized. The crude peptide was purified by semipreparative RP-HPLC and then lyophilized to give the title compound in 89% overall yield. Anal. MALDI-MS M^+ (found) = 1308.2 (Calcd for $C_{56}H_{94}N_{17}O_{15}S_2$, 1308.724).

End-to-Side Chain Cyclization of 2. In the sequential strategy, the amino terminal Ser was oxidized first and the removal of the StBu protecting group and cyclization were carried out concomitantly.

(1) Oxidation. To a solution of **2** (1 mg, 0.76 *μ*mol) in sodium phosphate buffer (0.01 M, pH 6.8, 1 mL) was added sodium periodate (0.33 mg, 2 equiv) in water (3.5 *μ*L). The mixture was shaken for 2 min and then injected onto a semipreparative RP-HPLC column and purified. The product was lyophilized to give **3** in quantitative yield. Anal. MALDI-MS M^+ (found) = 1277.7 (Calcd for $C_{55}H_{89}N_{16}O_{15}S_2$, 1277.613).

(2) Cyclization. To a solution of **3** (1.2 mg, 0.94 *μ*mol) in sodium acetate buffer (0.01M, pH 4.2, 1 mL) was added tris(2-carboxyethyl)-phosphine (2.3 mg, 9.3 *μ*mol, 10 equiv) in the same buffer (23 *μ*L). The mixture was shaken, and the aliquot was removed every 12 h for analysis by RP-HPLC (gradient 1). After 72 h the reaction was 90% complete, and the mixture was injected onto a semipreparative RP-HPLC and purified. The collected product was then lyophilized to give cyclic peptide **4** in 61% overall yield. Anal. MALDI-MS M^+ (found) = 1170.6 (Calcd for $C_{51}H_{78}N_{16}O_{14}S$, 1170.560).

Characterization of End-to-Side Chain Cyclic Peptides. (1) Enzymatic Digestion of 2. To a solution of **2** (0.5 mg, 0.35 *μ*mmol) in Tris buffer (0.01 M, pH 7.8, 1 mL) was added trypsin (5 *μ*g, w/w 0.01 equiv) in the same buffer (5 *μ*L). The mixture was shaken for 15 min, then injected onto a semipreparative RP-HPLC, and purified. Two products were isolated with retention times of 10.9 and 18.1 min. MALDI-MS showed these to be the C-terminal and the N-terminal fragments, respectively. Anal. MALDI-MS M^+ (found) = 579.8 (Calcd for $C_{26}H_{41}N_7O_8$, 579.302), C-terminal fragment; M^+ (found) = 746.3 (Calcd for $C_{30}H_{54}N_{10}O_8S_2$ 746.357), N-terminal fragment.

(2) Enzymic Digestion of 4. Using the same conditions as above, one product was isolated with a retention time of 16.7 min. MALDI-MS confirmed the peptide as the linear thiazolidine. Anal. MALDI-MS M^+ (found) = 1188.6 (Calcd for $C_{51}H_{81}N_{16}O_{15}S$, 1188.57).

Preparation of Benzaldehyde Resin 11. To a suspension of chloromethylated polystyrene(1% divinylbenzene)-copolymer (0.76 mmol/g, 0.5 g) in DMSO (3 mL) was added sodium bicarbonate (0.2 g). After stirring for 7 h at 155 °C the resin was washed with water, methanol, DMF, DCM, and diethyl ether and then dried *in vacuo*. FT-IR analysis showed the typical carbonyl absorption at 1700.5 cm^{-1}, and microanalysis showed that the resin contained no chlorine. The resin **11** was then treated with a large excess of hydroxylamine hydrochloride in pyridine for 6 h at 95 °C, then washed, and dried *in vacuo*. FT-IR analysis showed no carbonyl absorbance at 1700.5 cm^{-1} and microanalysis showed 0.82% nitrogen, which indicated a yield of 77% and a loading of the aldehyde at 0.59 mmol/g.

Preparation of Fmoc-Gly Glyceric Ester 10. Fmoc-Gly-OH (10 mmol, 2.97 g) was dissolved in DMF/DCM (1:2, v/v 30 mL) with DCC-(11 mmol, 2.37 g)/HOBt (11 mmol, 1.68 g). After stirring for 20 min, the insoluble DCU was filtered off, and the solution was added to glycerol (5.5 g, 60 mmol) in DMF (20 mL). After stirring overnight, TLC analysis showed no Fmoc-Gly-OH was present. The mixture was reduced to an oil *in vacuo* and was taken up in ethyl acetate, filtered, washed with sodium carbonate solution (3%), and then with saturated NaCl solution. After drying over sodium sulfate the solution was concentrated *in vacuo*, and the product was crystallized from n-hexane/ethyl acetate (yield 3.2 g, 85%). Anal. FAB-MS [M + H^+] (found) = 372 (Calcd for $C_{20}H_{21}NO_6$, 371.4).

Preparation of the Cyclic Acetal Resin 12. The benzaldehyde resin **11** (0.59 mmol/g, 1.0 g) was suspended in anhydrous DME (10 mL) with glycerol (1 g) and *p*-toluenesulfonic acid (10 mg). After 24 h at

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90 °C the resin was washed with a 3% solution of sodium bicarbonate and 1,4-dioxane (1:1, v/v), water, dioxane, DMF, methanol, DCM, and diethyl ether and then dried *in vacuo*. FT-IR analysis showed the disappearance of the carbonyl absorbance at 1700.5 cm^{-1}.

Preparation of Fmoc-Gly-O-CH_2-Cyclic Acetal Resin 13. Method A. The aldehyde resin **11** (0.59 mmol/g, 1.0 g) was suspended in 1,4-dioxane (10 mL) and Fmoc-Gly-glyceric ester **10** (1.16 mmol, 0.43 g) and *p*-toluenesulfonic acid (8 mg) were added. After 24 h at 100 °C the resin was washed with a 3% solution of $NaHCO_3$ and 1,4-dioxane (1:1, v/v), water, dioxane, DMF, methanol, DCM, and diethyl ether and then dried *in vacuo*. FT-IR analysis still showed an absorbance at 1700 cm^{-1}. Microanalysis indicated 0.32% of nitrogen found, corresponding to a loading of 0.23 mmol/g. UV absorbance of the cleaved Fmoc (301 nm, $\epsilon = 7840$) indicated a substitution of 0.3 mmol/g. **Method B.** Fmoc-Gly-OH (5.58 mmol, 1.66 g), DCC (3 mmol, 0.62 g), DMAP (0.28 mmol, 35 mg), and HOBt (0.28 mmol, 44 mg) were stirred for 20 min in DMF (10 mL). Insoluble DCU was removed, and the solution was added to a suspension of the glycerol-acetal-resin **12** (1.0 g) in DMF (5 mL). After 18 h, the resin was washed with dioxane, DMF, methanol, DCM, and diethyl ether and then dried *in vacuo*. Microanalysis indicated 0.71% of nitrogen, corresponding to a substitution of 0.5 mmol/g. UV absorbance of the cleaved Fmoc (wavelength 301 nm, $\epsilon = 7840$) indicated a substitution of 0.5 mmol/g. RP-HPLC analysis of the cleavage mixture at 301 nm using gradient 2 (material and methods) indicated that about 1% of Fmoc-Gly-Gly-glyceric ester had formed. Anal: FAB-MS [M + H^+] (found) = 428.9 (Calcd for $C_{22}H_{24}N_2O_7$, 428.4).

End-to-End Cyclization. Solid Phase Synthesis of Peptide Glyceric Esters 5a−e. Peptides glyceric ester **5a** and **5b** were synthesized by the Fmoc chemistry utilizing the cyclic acetal resin **13** prepared with method A and peptides **5c−e** were prepared with method B.

When the resin method A was used, an additional equivalent of HOBt was added to the acylating mixture during coupling to neutralize the covalently linked piperidine adduct. In method B couplings were observed to be slow in DMF. To overcome this problem, couplings were conducted in a mixture 70% DMF 30% DCM (V/V) for 1 h.

(a) Cleavage of the Glyceric Esters from the Resin (5a−e). After removal of the Fmoc protecting group from the N-terminal Cys(StBu), the resin was washed with DCM and then dried *in vacuo*. The resin (1.0 g) was stirred for TFA/water/thioanisole/anisole (91:3:3:3) for 2.5 h. Small scale trial cleavage indicated the best combination of scavengers and hydrolysis time. The peptide was then precipitated with ether, lyophilized, and then purified by preparative RP-HPLC. MALDI-MS M^+ (found) = **5a** 624.3 (Calcd for $C_{23}H_{43}N_8O_8S_2$, 623.7); **5b** 770.8 (Calcd for $C_{32}H_{52}N_9O_9S_2$, 770.9; **5c** 1084.3 (Calcd for $C_{47}H_{80}N_{12}O_{13}S_2$, 1085.3); **5d** 1384.2 (Calcd for $C_{61}H_{105}N_{16}O_{16}S_2$, 1382.7); **5e** 3088 (Calcd for $C_{131}H_{230}N_{47}O_{35}S_2$, 3087.7).

(b) Peptide-Glycoaldehyde 6a−e. $NaIO_4$ (30 μmol) in sodium phosphate buffer (0.01 M, pH 5, 300 μL) was added to the peptide-glyceric ester **5d** (12 mg, 7 μmol) dissolved in the same buffer (1 mL). After 30 min RP-HPLC showed the reaction to be 90% complete, and the mixture was then purified by preparative RP-HPLC.

(c) Cyclic Peptide Ester 8a−e. A typical procedure was as follows (**8d**). Isopropyl alcohol/tributylphosphine (10:1 v/v, 10.9 μmol, 30 μL) was added to the purified peptide-glycoaldehyde **6d** (2 mg, 1.18 μmol) in sodium phosphate buffer (0.01 M, pH 5.5, 200 μL). RP-HPLC analysis showed that after 1 h the starting material had formed two major products (Figure 5), which were shown to be the diastereomers of the cyclized peptide **8d**, obtained by preparative RP-HPLC: MALDI-MS M^+ (found) = 1244.9 (peak 1), 1246.0 (peak 2) (Calcd for $C_{56}H_{92}N_{16}O_{14}S$, 1245.5); peak 1 and 2 are diastereomers. Similarly, other end-to-end cyclic peptides (ester) were obtained (Table 1). MALDI-MS M^+ (found) = **8a** 486.7 (Calcd for $C_{18}H_{30}N_8O_6S$, 486.2); **8b** 633.6 (peak 1), 633.3 (peak 2) (Calcd for $C_{27}H_{39}N_9O_7S$, 633.7); **8c** 948 (peak 1 and 2) (Calcd for $C_{42}H_{66}N_{12}O_{11}S$, 947.1); **8e** 2949 (Calcd for $C_{126}H_{217}N_{47}O_{33}S$ 2950.5).

(d) O to N-Acyl Shift To Yield Cyclic Peptide Amide 9d. The lyophilized **8d** was dissolved in sodium phosphate buffer (0.01 M, pH 6). After 20 h at 22 °C one diastereomer had completely rearranged to form the cyclic amide, while the other diastereomer required approximately 100 h for 95% amide formation. At 52 °C the reaction was complete within 24 h. MALDI-MS M^+ (found) = 1245.5 (Calcd for $C_{56}H_{92}N_{16}O_{14}S$, 1245.5). Similarly other end-to-end cyclic peptides (amide) were obtained (Table 1). MALDI-MS M^+ (found) = **9a** 486.3 (calcd for $C_{18}H_{30}N_8O_6S$, 486.2); **9b** 634.5 (Calcd for $C_{27}H_{39}N_9O_7S$, 633.7); **9c** 947 (Calcd for $C_{42}H_{66}N_{12}O_{11}S$, 947.1); **9e** 2952 (Calcd for $C_{126}H_{217}N_{47}O_{33}S$, 2950.5).

Enzymic Digestion of Cyclic Amide Peptide 9d. To a solution of 12-mer cyclic amide peptide (0.5 mg, 0.31 μmol) in Tris buffer (0.01 M pH 7.8, 1 mL) was added trypsin (5 μg, w/w, 0.01 equiv) in the same buffer (5 μL). The mixture was shaken for 15 min, and then an aliquot was taken for analytical RP-HPLC using gradient 3. As expected, two products were isolated with retention times 9.4 and 22.1 min. MALDI-MS showed these to be the expected products Arg-Ala and Lys-Ile bond hydrolysis, respectively. Anal. MALDI-MS M^+ (found) = 548 (IG-SPro-GR seq. Calcd for $C_{21}H_{40}N_8O_7S$ 548.6) for peak at room temperature; 9.4 min, and 735 (AFVTIGK seq., Calcd for $C_{35}H_{58}N_8O_9$ 734.9) for peak at 22.1 min.

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