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Chemical Ligation Approach To Form a Peptide Bond between Unprotected Peptide Segments. Concept and Model Study

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Abstract: We describe a novel approach to the chemical ligation of peptide segments with no protecting groups and no activation of the C^{α}-carboxyl group. The key reaction of this approach is based on the intramolecular *O,N*-acyl transfer reaction to achieve high effective molarity so that two closely neighbored functional groups can react efficiently and selectively with each other. The specificity of this approach is contributed by the facile ring formation between the carboxyl component bearing an ester glycolaldehyde and the amino component bearing a 1,2-amino thiol group such as a N-terminal cysteine residue. The feasibility of this scheme was verified by model studies on small compounds and the synthesis of a pentadecapeptide.

Introduction

The formation of a peptide bond by chemical synthesis involves the activation of the α-carboxy group of the first component, which subsequently couples to the α-amine of the second component. This scheme usually requires a protecting-group strategy for the other functionalities in both peptide components. The development and application of protecting groups have become an integral and essential part in stepwise or segment synthesis whether the side chains are globally, partially, or minimally protected.[1-5] However, the usual peptide-bond-forming process has its intrinsic limitations when applied to the coupling between large peptide segments because of the low reactant concentrations and entropy barrier imposed by high molecular masses, especially when side-chain protection is extensively used. Nevertheless, if one can increase the effective local concentration of the amine nucleophile and carboxyl electrophile by bringing two fragments closely together, their proximity would overcome the entropy barrier to allow efficient formation of a peptide bond. In fact, this proximity principle has found its successful applications in protein semisynthesis by either enzymatic or chemical methods,[6-8] where the self-association of the two fragments of a natural protein holds the reacting carboxyl and amino ends together through noncovalent interactions. However, for most large peptide segments where complementarity does not exist,

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particularly when there are protecting groups on the side chains, it is unlikely that such approaches could be of general use. Nevertheless, the features in these approaches of achieving high effective molarity[9] by bringing two reactive groups together and the use of free fragments are highly desirable. A more general way would be to use a covalent linkage to bring two components together through a specific reaction of high efficiency. The prior thiol capture strategy developed by D. S. Kemp et al.[10] is an ingenious design for this purpose. With similar objectives, we have now developed a new chemical ligation method that does not require protecting groups or activation of the C^{α}-component in the conventional sense. The present report describes the basic idea behind this new method and the verification of its feasibility using simple model compounds and peptides.

Results and Discussion

General Concept. A central feature of our approach is to overcome the entropy barrier of an intermolecular reaction between two large molecules by bringing two unprotected peptide segments together through a covalent linkage to effect an intramolecular *O,N*-acyl transfer reaction to form the amide bond. To achieve this goal, a highly specific reaction is required. The reaction of an alkyl aldehyde with a weak base fulfills this requirement and would exclude stronger bases such as side-chain amines and guanidino groups present in an unprotected peptide segment from the reaction by protonation under acidic conditions. The 1,2-substituted moiety of mercapto amine of Cys or hydroxyl amine of Thr/Ser can be qualified as a weak base, because it has the ability to form a stable five-membered-ring thiazolidine or oxazolidine with an alkyl aldehyde,[11,12] while the reaction of an aldehyde with other amines would form Schiff bases which are reversible and unstable. Thus, the unusual property of the N-terminal Cys or Thr/Ser is utilized as the specific recognition motif of the amino component segment in our approach. The specificity of this reaction also makes side-chain protection unnecessary. The alkyl aldehyde moiety such as glycolaldehyde can be introduced to the *C*-carboxyl group of a peptide segment through an ester linkage. The ester bond which links the glycoaldehyde to the C^{α}-carboxyl group is designed so that it is in close proximity with the Cys or Thr/Ser amine to allow an *O,N*-acyl transfer reaction through a favorable five-membered-ring transition state to form a stable amide bond between the two segments (Figure 1). In this paper, we explore the utility of the thiazolidine ring formation only, and the oxazolidine ring formation and other aspects of our work will be reported elsewhere.[13]

Ring Formation. To demonstrate the feasibility of this scheme, model studies using simple compounds were performed (Figure 2). The glycolaldehyde group was introduced to a Z-Gly as a protected methyl acetal by esterification of the corresponding Z-GlyO-Cs^+ salt with bromoacetaldehyde dimethyl acetal. Deprotection of the acetal was easily achieved by brief treatment with CF_3COOH in 15 min. The free aldehyde was then allowed to react with mercaptoethylamine or other cysteine derivatives to form various thiazolidine derivatives. The reactions between the aldehyde and the β-mercapto amines were fast and completed in 15 min at pH 5–6 and <5 min under neutral or basic pH. The course of the reaction was conveniently monitored by RP HPLC and the product identified by NMR and MS. The ability of this reaction to be performed in acidic conditions is highly desirable because it avoids side reactions of aldehyde with other nucleophiles present in peptides. These features make this reaction very useful for the purpose of bringing two molecules together, and it can also be considered as a direct conjugation method for the preparation of protein conjugates.

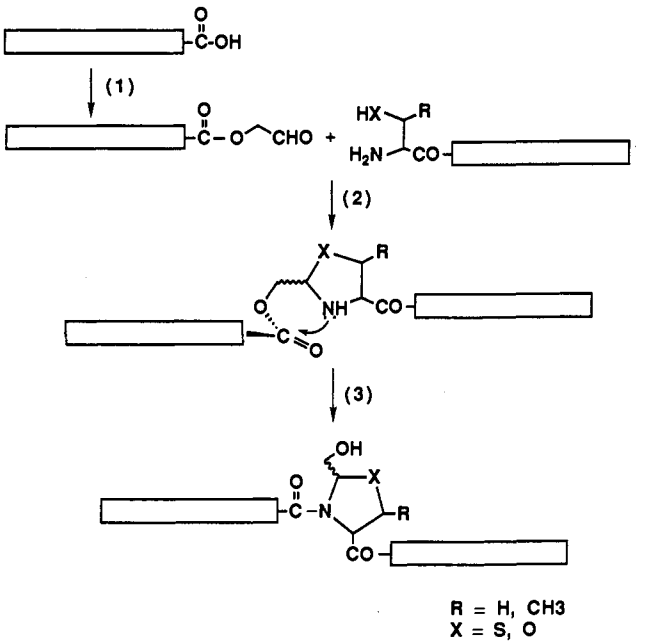

Figure 1. A general scheme of peptide segment ligation strategy: (1) aldehyde introduction; (2) ring formation; (3) *O,N*-acyl rearrangement.

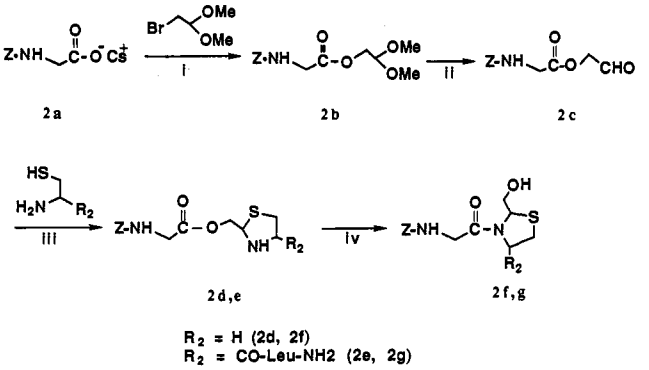

Figure 2. A model study of the chemical ligation strategy: (i) DMF, 60 °C, 24 h; (ii) 30% TFA in CH_2Cl_2 (2–5% H_2O); (iii) H_2O/CH_3CN, pH 2–5; (iv) pH 4–9.

***O*- to *N*-Acyl Transfer Reaction.** The subsequent intramolecular acyl transfer is a key reaction in our approach and can be effected by adjusting the pH of the reaction after the ring formation. The *O,N*-acyl rearrangement is a well-studied reaction, with the five-membered-ring transition state as the most favorable.[14,15] The secondary amine in the thiazolidine ring is a weak base. As a result, the acyl transfer reaction also occurred at acidic pH (Table 1). While the ring formation was fast, the rearrangement was generally slower and constitutes the rate-determining step of our approach. The rate of the rearrangement depended on the steric and electronic environment of the amino and carboxyl components. For the unhindered mercaptoethylamine, the rate increased with the increased pH. With cysteine derivatives such as Cys-Leu-NH_2, the optimal pH was found to be 4–5, which gave the fastest rate. The reaction was very clean, and no significant side reaction was observed. The optimal rate

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Table 1. Rates of *O*- to *N*-Acyl Transfer Reactions from Peptide Ester to Amide

compound	$t_{1/2}$ (h) pH 6	pH 7	pH 8	pH 9
Z-Gly-HMThz **2d** → **2f**	21.5	8.8	3.4	2.8

at acidic pH is probably due to the electron-withdrawing effect of the carboxamide group, which decreases the basicity of the thiazolidine amine. The steric effect imposed by the large amino acid Leu considerably decreased the reaction rate ($t_{1/2}$ = 20 h at pH 4) compared to the unsubstituted mercaptoethylamine. The steric effect of the C-component on the rearrangement is also relevant. Gly/Ala being the unhindered amino acid is the preferred choice because more hindered amino acids such as Val decrease the rate by >10-fold (data not shown).

One reason for the inefficiency of large segment condensation by conventional methods is the modest nucleophilicity of the amino group which is exacerbated by compensation with excessively increasing the electrophilicity of the activated carboxyl group that requires extensive protection and is prone to side reactions. Thus, the acyl transfer reaction to form a peptide bond differs from the conventional coupling of peptide segments in four aspects. (1) The thiazolidinyl-2-methyl ester prior to rearrangement is similar to other alkyl esters and is not activated when comparing to the usual active esters or other activated forms of the carboxyl group used in peptide synthesis. We have observed that intermolecular aminolysis of this ester does not occur in the presence of other primary amines in a usual organic solvent such as DMF. (2) The absence of overactivation which usually leads to oxazolinone formation[16] also eliminates racemization, which is a major concern in the conventional segment coupling. (3) Due to the favorable proximity of the ester carbonyl and the N^{α}-amine, the intramolecular acyl transfer reaction overcomes the entropic barrier in the usual biomolecular peptide-bond-forming process. (4) The *N*-acyl thiazolidine compound is an acyl–proline-like structure and can be considered as a substitution for the Pro residue in a natural peptide sequence. Such thiazolidine product is susceptible to electrophiles, and it is possible to cleave the thiazolidine ring to release the free cysteine residue. Similar thiazolidine structure has been used as a temporary protection of the cysteine residue in peptide synthesis.[17] Current work is now in progress to accomplish its conversion by a mild reagent. However, before the chemistry for the reversion of thiazolidine to cysteine is completely demonstrated, one should consider using this approach for ligation at a –Gly/Ala-Pro– bond and using –Gly/Ala-Thz– as a surrogate.

Synthesis of a Pentadecapeptide. The overall scheme of our ligation approach to use unprotected peptide segments was demonstrated by the synthesis of a pentadecapeptide using two segments of six and nine amino acid residues (Figure 3). To introduce the glycolaldehyde moiety onto the C-terminus of an unprotected peptide, enzymatic synthesis[18] was found to be suitable. An unprotected octapeptide ester was first synthesized using a newly prepared resin. The glycolaldehyde component, alanine dimethoxyethyl ester prepared from its Z-protected form by hydrogenolysis, was incorporated onto the C-terminus of the octapeptide through the trypsin-catalyzed, kinetically controlled aminolysis[19] of the peptide ester bond, usually conducted in a solution containing a high content of a water-miscible organic solvent such as DMF/H_2O (60:40, v/v). Such an enzymatic reaction was efficient and favorable because the acetal-protected aldehyde component is small and was used in large excess to force the reaction to completion in a short period. As expected, the enzymatic synthesis was fast and completed in 15 min. After purification, the obtained nonapeptide with an acetal-protected aldehyde ester at its C-terminus was treated with TFA to release the aldehyde function. We found that neat TFA with 5% of H_2O was suitable to give a quantitative reaction in 5 min. Afterwards, TFA was removed rapidly to prevent possible acidolytic side reactions. The free aldehyde was more hydrophilic than the acetal form, which allowed the deprotection reaction to be monitored (Figure 4). The peptide aldehyde gave a characteristic broad peak in HPLC, due to the reversible hydration of the aldehyde group. The ring formation reaction containing both components was performed at pH 4 in highly dilute aqueous solution at about 5 mM. The acidic condition was useful to inhibit the oxidation of the cysteine thiol group to disulfide. This reaction proceeded cleanly without any observable side reactions and was completed in about 3 h. The *O*- to *N*-acyl transfer reaction was effected by adjusting the pH to 5, which was also the optimal pH for such a rearrangement. It is interesting to note that a new stereogenic carbon on the thiazolidine ring gave rise to two diastereomers, which, in theory, could be separable in HPLC. In the present case, the two diastereomers were separated only at the ester stage (Figure 4).

It is important to stress two other aspects of the ring formation and *O,N*-acyl rearrangement in the synthesis of the 15-mer peptide. (1) It is essentially a one-pot reaction. Both reactions are effected by pH changes, and both components can be presented together. (2) The side-chain functional groups were not protected in both peptides, including the ϵ-amine of Lys and the β-carboxyl group of Asp, which must be protected in a conventional segment coupling. In addition, there is no need to protect the N^{α}-amines even in the enzymatic introduction of the masked glycolaldehyde component because a large excess of nearly 100-fold of this amino component is used. This was already demonstrated by the published work in protein semisynthesis,[8,16b,20] and has been further confirmed by the new examples of ligating totally unprotected peptides in our laboratory (unpublished data). Other approaches of ligating unprotected protein fragments together using thiol and hydrazino groups usually lead to nonpeptide bonds.[21] Our approach goes one step further to give an amide bond.

To summarize, our proposed ligation approach provides high selectivity of coupling unprotected peptide segments and consists of three steps: (1) aldehyde introduction, (2) ring formation, and (3) *O*- to *N*-acyl transfer. All these steps eliminate the necessity of protecting groups and allow ligation in aqueous solution. The mild conditions used in the ring formation and *O,N*-acyl transfer steps prevent many side reactions, as seen in the conventional segment coupling strategy. The use of acyl rearrangement to form amide bonds preceded by a specific capture step to bring two peptides together provides a promising strategy for ligating large peptide segments, including protein domains to form proteins with unusual architectures.

Experimental Section

General. H NMR was obtained at 300 MHz on a Bruker AC 300 spectrometer. Thin-layer chromatography was performed on 250-μm layers of Whatman silica gel (fluorescence UV_{254}) plates coated on aluminum. Trypsin (bovine pancreas) was purchased from Sigma and used without further purification. Analytical HPLC was run on a Shimadzu system with a Vydac column (0.46 × 25 cm, C18 reverse phase, 5 μm) at a flow rate of 1.5 mL/min, detected at 225 nm. All HPLC was performed by linear gradients of two buffers: A, 5% CH_3CN in H_2O (0.045% TFA); B, 60% CH_3CN in H_2O (0.039% TFA). Mass

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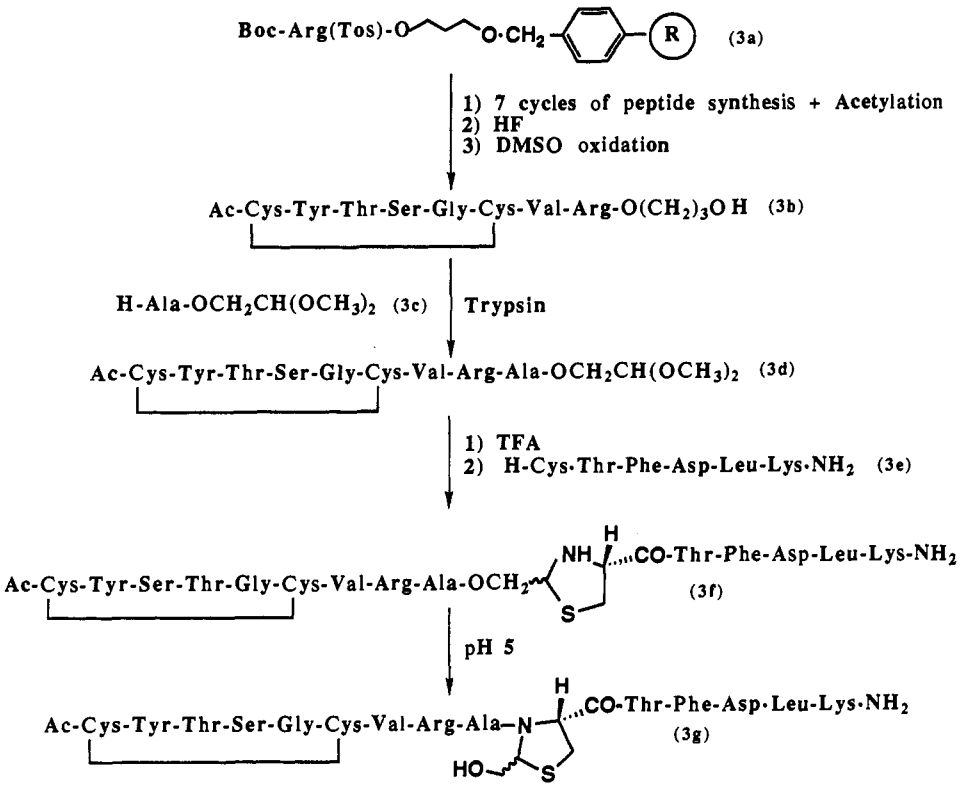

Figure 3. Synthesis of a model pentadecapeptide by the chemical ligation approach.

spectrometry was obtained with the FAB method. Amino acid analysis of each peptide was performed on the hydrolyzate by 5.7 N HCl for 24 h at 110 °C.

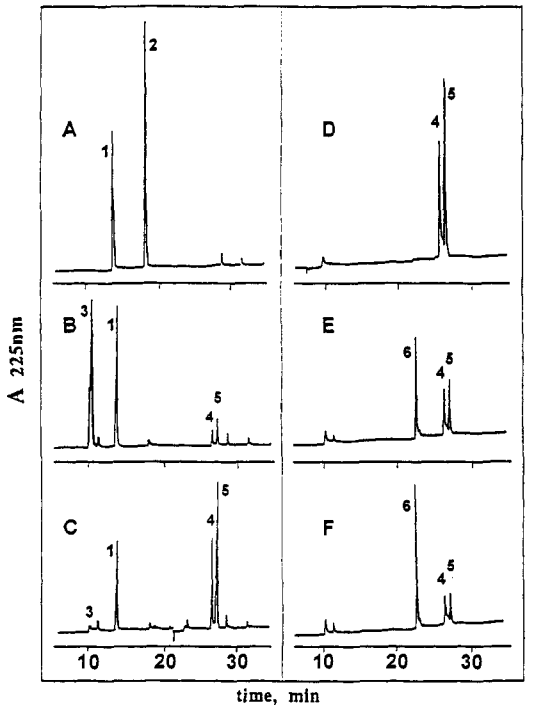

Figure 4. Progress of the synthesis of the model 15-residue peptide (15-mer): (A) 6- and 9-mer (as acetal); (B) TFA treatment of the 9-mer to give free aldehyde in the presence of the 6-mer; (C) reaction of the 6-mer with the 9-mer aldehyde after 3 h at pH 4; (D) the purified 15-mer in ester form; (E) *O,N*-transfer at pH 5 after 20 h and (F) 2 days; peak 1, amino component of the 6-mer **3e** in excess; peak 2, carboxyl component of the 9-mer in acetal form **3d**; peak 3, the 9-mer in aldehyde form; peaks 4 and 5, diastereomers of the 15-mer in the ester form **3f**; and peak 6, the *O,N*-acyl rearranged product of the 15-mer **3g**. See Experimental Section for HPLC conditions.

Synthesis of Z-Glycine 2,2'-Dimethoxyethyl Ester (2b).[12] Z-Glycine (4.2 g, ~20 mmol) was dissolved in 100 mL of methanol, and 10 mL of H_2O was added. The solution was neutralized with a 0.5 M solution of Cs_2CO_3 in water (about 20 mL) and then evaporated *in vacuo* to dryness. The residue was redissolved in DMF and evaporated. This was repeated several times (alternatively, lyophilize) to remove all the water. The dried residue was then dissolved in 100 mL of anhydrous DMF, and bromoacetaldehyde dimethyl acetal (40 mmol) was added. The mixture was stirred at 65–70 °C for 2 days. After evaporation of all volatile material, the residue was dissolved in 150 mL of ethyl acetate and washed successively with 0.25 M Na_2CO_3, saturated NaCl solution, and water. The organic phase was dried over Na_2SO_4, and evaporated to give a pale yellowish oil (~5 g, yield 85%). The product showed a single spot in TLC and was used without further purification: R_f = 0.5, ethyl acetate: hexanes = 1:1; t_R = 24.0 min (15%–55% B in 40 min). MS: $[M + H]^+$ 298. ^1H NMR ($CDCl_3$/TMS_{int}): δ 7.33 ppm (s, 5H), 5.29 (bt, 1H, J = 5.7 Hz), 5.13 (s, 2H), 4.56 (t, 1H, J = 5.3 Hz), 4.18 (d, 2H, J = 5.3 Hz), 4.03 (d, 2H, J = 5.7 Hz), 3.39 (s, 6H).

Synthesis of Z-Glycine Glycolaldehyde (2c). 2a (300 mg, ~1 mmol) was dissolved in 20 mL of CH_2Cl_2, and 10 mL of TFA (containing 6% H_2O) was added; the mixture was kept at room temperature for about 20 min. The solution was then evaporated *in vacuo* to give an oil that was used immediately for the next step: R_f = 0.17, ethyl acetate:hexanes = 1:1; t_R = 9.5 min (15%–55% B in 40 min). ^1H NMR ($CDCl_3$): δ 9.6 (s, 1H), 7.35 (s, 5H), 5.28 (bt, 1H, J = 6.4 Hz), 5.14 (s, 2H), 4.76 (s, 2H), 4.14 (d, 2H, J = 5.8 Hz).

Synthesis of Z-Glycine Thiazolidinyl-2-methyl Ester·TFA (2d). Aldehyde **2c** and 2-aminoethanethiol hydrochloride (1.2 mmol) were dissolved in 10 mL of H_2O and 5 mL of CH_3CN. The pH of the solution was adjusted to 4 with an acetate buffer. The reaction was completed within 1 h, as shown in TLC and RP HPLC. After removal of the solvent the product was isolated by RP HPLC to give 360 mg of **2d** (yield 82% from **2c**): R_f = 0.43, $CHCl_3$:MeOH:AcOH (CMA) = 90:8:2; t_R = 10.3 min (15%–45% B in 30 min). MS: $[M + H]^+$ 311. ^1H NMR ($CDCl_3$): δ 9.22 (2H), 7.33 (s, 5H), 5.83 (bt, 1H), 5.09 (2H), 4.95 (t, 1H, J = 5.5 Hz), 4.39 (d, 2H, J = 5.5 Hz), 3.97 (d, 2H), 3.70–3.44 (m, 2H), 3.21–3.04 (m, 2H).

Synthesis of Z-Gly-OCH$_2$-Thz-Leu-NH$_2$·Tfa (2e). The same procedure as that for **2d** was used: yield 80% from **2c**; R_f = 0.49, CMA = 90:8:2, t_R = 15.6, 16.2 min (25%–45% B in 20 min); $[M + H]^+$ 467. ^1H NMR (mixture of two diastereomers) ($CDCl_3$): δ 7.99, 7.80 (2d, 1H), 7.35 (s, 5H), 6.77, 6.72 (2s, 2H), 6.27 (bs, 2H), 5.62, 5.49 (2t, 1H), 5.12 (s, 2H),

4.86–4.81 (m, 1H), 4.43 (m, 2H), 4.38–4.29 (m, 1H), 4.26–4.23 (m, 1H), 4.01 (t, 2H), 3.35–3.22 (m, 2H), 1.50–1.68 (m, 2H + 1H), 0.94–0.84 (m, 6H).

Preparation of *N*-(Z-Glycyl)-2-(hydroxymethyl)thiazolidine (2f). Samples of **2d** (0.1 mmol) were dissolved in 4 mL of 0.1 M phosphate buffer and 2 mL of CH_3CN. The pH of the solution was adjusted to values ranging from 6 to 9. The reaction was monitored by HPLC: R_f = 0.48, CMA = 90:8:2; t_R = 14 min (15%–45% B in 30 min). MS: $[M + H]^+$ 311. ^1H NMR ($CDCl_3$): δ 7.35 (s, 5H), 5.80 (bt, 1H), 5.44 (t, 1H), 5.12 (s, 2H), 4.02 (d, 2H), 3.79–3.70 (m, 2H + 2H), 3.44 (bs, 1H), 3.24–2.97 (m, 2H).

Preparation of Z-Gly-(2-$HOCH_2$)Thz-Leu-NH_2 (2g). The same procedure as that for **2f** was used except that the pH for rearrangement was set to a lower value of 4 with acetate buffer for 2 days: yield 90%; R_f = 0.28, CMA = 90:8:2; t_R = 12.7 min (25%–45% B in 20 min). MS: $[M + H]^+$ 467. ^1H NMR (mixture of two diastereomers) ($CDCl_3$): δ 7.32, 7.30 (2s, 5H), 7.07 (bd, 1H), 6.73, 6.59 (2b, 2H), 6.00 (b, 1H), 5.44 (m, 1H), 5.14, 5.09 (2s, 2H), 4.83, 4.79 (2d, 2H), 4.36 (m, 1H), 4.27–4.20 (m, 1H), 3.72 (m, 2H), 3.66–3.47 (m, 2H), 3.05–3.01 (2bs, 1H), 1.81–1.60 (m, 2H + 1H), 0.95, 0.86 (2d, 6H).

Synthesis of 3b and 3e. Peptides **3b** and **3e** were synthesized by solid-phase peptide synthesis.[2] **3b** was prepared with (hydroxypropionyloxy)-methyl polystyrene resin (1% DVB) at a substitution level of 0.5 mmol/g. The first amino acid (Arg) was loaded on the resin through symmetric anhydride of Boc-Arg(Tos)-OH catalyzed by 0.1 equiv of *N*-methylimidazole in DMF overnight to give **3a** with a substitution of 0.3 mmol/g, as determined by a quantitative ninhydrin test.[22] The residual hydroxy group was blocked by acetylation for 3 h using acetic anhydride in the presence of *N*-methylimidazole. **3e** was prepared using 4-methylbenzhydrylamine (MBHA) resin at a substitution level of 0.75 mmol/g. Both peptides were prepared using Boc/Bzl chemistry. Side-chain protecting groups were Arg(Tos), Asp(OBzl), Cys(4-MeBzl), Lys(2-ClZ), Ser(Bzl), Thr(Bzl), and Tyr (2-BrZ). Each synthetic cycle consisted of (i) deprotection with 50% trifluoroacetic acid/CH_2Cl_2 for 1 and 20 min, (ii) single coupling using 3 equiv of Boc–amino acids and benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) reagent[23] for 30 min in CH_2Cl_2 with in situ neutralization,[24] double coupling (each for 1 h) for Thr and Val. N^{α}-Acetylation for **3b** was performed with acetic anhydride in CH_2Cl_2/DMF (1:1) containing 5% DIEA for 15 min. All couplings were monitored by the ninhydrin test.[25] Final deprotection of side chains and cleavage from the resin were achieved by treatment of 0.5 g of dried protected peptide resin (N^{α}-deprotected for peptide **3e**) with 1 mL of anisole and 10 mL of anhydrous HF at 0 °C for 75 min. HF was removed by evaporation at 0 °C. After washing with cold anhydrous ether to remove anisole, the crude peptide was extracted with 25% CH_3CN/H_2O (1% TFA). The oxidation of **3b** for disulfide formation was performed directly on the extraction solution from HF cleavage with 10% DMSO[26] in a total volume of 250 mL diluted with water (pH adjusted to 5.5 by sodium acetate) overnight. The oxidized **3b** was purified by C_{18} reverse-phase HPLC using a preparative Vydac column. The HPLC fractions were lyophilized. **3e** was used without purification: t_R(**3b**) = 11.3 min, t_R(**3b** reduced) = 10.6 min, t_R(**3e**) = 13.8 min (gradient 10%–45% B in 35 min; this gradient was used for all next steps). Amino acid analysis: **3b**, Arg (1), Gly (1), Ser (1), Thr (1), Tyr (1), Val (1); **3e** Asp (1), Leu (1), Lys (1), Phe (1), Thr (1). MS: **3b**, $(M + H)^+$ 986.4 (calc), 986.2 (found); **3e**, $(M + H)^+$ 725.3 (calc), 725 (found); $(M + 2H)^{2+}/2$, 363.1 (calc), 362 (found).

Preparation of 3c. Z-Ala-$OCH_2CH(OCH_3)_2$ (622 mg, 2 mmol) prepared as **2b** was dissolved in 50 mL of tetrahydrofuran in a Parr apparatus for hydrogenation, and 0.46 g of 10% Pd/C was added. The system was connected to a water aspirator. Vacuum was applied, and the system was refilled with hydrogen. This procedure was repeated twice. Hydrogen was finally filled to a pressure of 60 psi. The system was shaken at 20 °C for 2 h. The catalyst was removed by filtration. The solvent was removed *in vacuo* to give a slightly yellowish oil that showed a single spot in TLC. The product was dissolved in 2 mL of DMF and kept at −70 °C for the next use: R_f = 0.65, $CHCl_3$:MeOH:AcOH = 6:2:1. MS: $[M + H]^+$ 178.

Preparation of 3d. **3b**·TFA salt (5.5 mg, ~5×10^{-3} mmol) was dissolved in 40 μL of 0.25 M Tris·HCl buffer (pH 8.5, 20 mM $CaCl_2$). **3c** solution in DMF (60 μL, 1 M) was added followed by 5 μL of a freshly prepared solution of trypsin (1 mg/10 μL) in 0.25 M Tris (pH 8.5, 20 mM Ca^{2+}). The mixture was stirred for 20 min at room temperature. HPLC showed that all of the starting material (eluted at 10.7 min; gradient, 10%–45% B in 35 min) disappeared, which gave 65% coupling product (estimated by RP HPLC, the product eluted at 18.2 min) and 35% hydrolysis product of the ester handle (eluted at 7.6 min). The product was separated by a semipreparative Vydac column, and the major fractions were lyophilized. Amino acid analysis: Ala (1), Arg (1), Gly (1), Ser (1), Thr (1), Tyr (1), Val (1). MS: **3d** $(M + H)^+$ 1087.4 (calc), 1087 (found); hydrolysis product, $(M + H)^+$ 928.4 (calc), 928 (found).

Preparation of 3f·3e·2TFA salt (4.6 mg, 4.8×10^{-3} mmol) was mixed with the obtained **3d** in a 10-mL flask. Cooled TFA (1 mL) (5% H_2O) was added. The reaction was kept at 20 °C for about 5 min. HPLC monitoring showed that all **3f** (acetal form, eluted at 18.2 min) was transformed to the free aldehyde (eluted at 10.4 min). The TFA reagent was then removed under reduced pressure using a water aspirator and then with an oil pump furnished with a base trap. Acetate buffer (1 mL, 50 mM, pH 4) was added, and the pH was readjusted to about 4 with 0.2 M sodium acetate solution. The reaction between the free aldehyde and **3e** occurred immediately and was completed in 3 h. The product gave two distinct peaks in RP HPLC, which eluted at 26.5 and 27.2 min (Figure 6). Both peaks showed the correct molecular weight in MS: $(M + H)^+$ 1747.7 (calc), 1748 (found). The rearrangement reaction was performed by adjusting the pH to 5; but the slow dimerization of the excess **3e** under this condition gave a dimer product that eluted at 23.4 min, very close to the rearranged product at 23.1 min, which complicated the next purification step. **3f** was then separated from the excess **3e** by a semipreparative HPLC, and the fractions were lyophilized.

Preparation of 3g. The lyophilized **3f** was dissolved in 5 mL of 50 mM acetate buffer (pH 5). The rearranged product showed only one peak at about 23.1 min in HPLC. The $t_{1/2}$ of rearrangement reaction under this condition was about 20 h. The product was purified by semipreparative HPLC after 3 days; the yield estimated (by HPLC) was about 85%. Amino acid analysis: Ala (1), Arg (1), Asp (1), Gly (1), Leu (1), Phe (1), Ser (1), Thr (1), Tyr (1), Val (1). MS: $[M + H]^+$ 1747.7 (calc), 1747 (found).

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