Tandem Ligation of Unprotected Peptides through Thiaprolyl and Cysteinyl Bonds in Water†

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Abstract: Tandem ligation for the synthesis and modification of proteins entails forming two or more regiospecific amide bonds of multiple free peptide segments without a protecting-group scheme. We here describe a semi-orthogonal strategy for ligating three unprotected peptide segments, two of which contain N-terminal (NT) cysteine, to form in tandem two amide bonds, an Xaa-SPro (thiaproline), and then an Xaa-Cys. This strategy exploits the strong preference of an NT-cysteinyl peptide under acidic conditions to undergo selectively an SPro-imine ligation rather than a Cys-thioester ligation. Operationally, it was performed in the N → C direction, first by an imine ligation at pH < 3 to afford an Xaa-thiazolidine ester bond between a peptide containing a carboxyl terminal (CT)-glycoaldehyde ester and a second peptide containing both an NT-Cys and a CT-thioester. The newly created *O*-ester-linked segment with a CT-thioester was then ligated to another NT-cysteinyl peptide through thioester ligation at pH > 7 to form an Xaa-Cys bond. Concurrently, this basic condition also catalyzed the *O*,*N*-acyl migration of an Xaa-thiazolidine ester to the Xaa-SPro bond at the first ligation site to complete the tandem three-segment ligation. Both ligation reactions were performed in aqueous buffered solvents. The effectiveness of this three-segment ligation strategy was tested in six peptides ranging from 19 to 70 amino acids, including thiaproline → proline analogues of somatostatins and two CC-chemokines. The thiaproline replacements in these peptides and proteins did not result in altered biological activity. By eliminating the protecting-group scheme and coupling reagents, tandem ligation of multiple free peptide segments in aqueous solutions enhances the scope of protein synthesis and may provide a useful approach for combinatorial segment synthesis.

Introduction

Chemoselective and orthogonal methods for ligating free peptides, proteins, and biopolymers in water have recently attracted considerable attention because of their wide applicability in both chemistry and biology. A major advantage of these methods is that they are not burdened by the use of a protection scheme or a coupling reagent. As a result, they can employ building blocks derived from chemical, natural, or biosynthetic sources to form macromolecules of different shapes or sizes in aqueous solutions. Indeed, successful applications of ligation methods have been demonstrated in semi- and total syntheses of peptides,[1−3] glycopeptides,[4−6] proteins,[7−11] and dendrimers.[12,13] An additional advantage is their mild reaction conditions, mostly performed in aqueous solutions that permit ligation chemistry to modify living systems such as cell surfaces and phage-display libraries.[14,15]

Chemoselective ligation methods, widely popular in protein conjugation chemistry, generally afford a nonamide bond such as a thioether or an oxime at the ligation site. They can be achieved by appropriately placing a mutually reactive nucleophile−electrophile pair on each peptide segment. The more demanding and recently developed orthogonal ligation strategy[16−19] affords a specific amide bond, usually a backbone

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†**Abbreviations:** Standard abbreviations are used for the amino acids and protecting groups [IUPAC−IUB Commission for Biochemical Nomenclature (1985) *J. Biol. Chem.* **1985**, *260*, 14]. Other abbreviations are as follows: NT, N-terminal; CT, carboxyl terminal; SPro, thiaproline; Sm-28, somatostatin-28; N-segment, amine segment; M-segment, middle segment; C-segment, carboxyl segment; Boc, *t*-butoxycarbonyl; Fmoc, fluorenylmethoxycarbonyl; RP-HPLC, reverse phase-high performance liquid chromatography; MALDI-MS, matrix assisted laser desorption ionization mass spectrometry; TFA, trifluoroacetic acid; HF, hydrofluoric acid; MBHA, methylbenzhydrylamine; MIP, macrophage inflammatory protein; TCEP, tris(carboxyethyl)phosphine; MPA, 3-mercaptopropionic acid; DMSO, dimethyl sulfoxide; S-*t*Bu, sulfenyl-*tert*-butyl; Acm, Acetamidomethyl; BOP, benzotrizol-1-yl-oxy-tris(dimethylamino)phosphonium-hexafluorophosphate; DCM, dichloromethane; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DIC, *N*,*N*-diisopropylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; DMF, dimethylformamide; HOBt, *N*-hydroxybenzotriazole.

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peptide bond formed by a regiospecific coupling reaction between an α-amino terminus of one peptide chain and an α-ester terminus of another peptide chain. In this regard, the orthogonal ligation strategy may provide a chemical model for an abiotic approach to peptide synthesis leading to specific formation of functional biopolymers under aqueous conditions without the assistance of known cellular and molecular machinery.

Thus far, the abiotic concept for amide-bond ligation of two unprotected peptide segments largely exploits the principle of a proximity-driven acyl transfer reaction through a cascade sequence of chemoselective capture and acyl migration.[18,19,22−26] An early approach that has elegantly illustrated this principle for peptide synthesis is the "Thiol Capture" developed by Kemp and his co-worker.[20,21] The Thiol Capture, with the aid of a rigid tricyclic template, is regiospecific to an amino-terminal (NT)-Cys and a carboxyl terminal (CT)-ester in the presence of other amines, side-chain nucleophiles, and electrophiles. Subsequent works have shown that NT-Cys-containing peptides with a super nucleophilic thiol side chain can undergo orthogonal ligations without the use of a template. Indeed, NT-Cys-containing peptides have been exploited for two orthogonal ligation strategies that include imine ligation yielding an Xaa-ψPro (pseudoproline)[24] and thioester ligation giving an Xaa-Cys bond.[18,19] Over the past 5 years, other methods based on NT-Cys mimetics[16,26,27] have also extended ligation methods to include peptides containing NT-amino acids other than NT-Cys.

With an expanded repertoire for ligating amide bonds, a tandem ligation scheme of coupling multiple segments to form two or more regiospecific amide bonds without a protection scheme would be desirable to increase the flexibility of the orthogonal ligation for forming complex peptides and proteins.[28−30] Such a strategy would be different from a tandem three-segment ligation that combines chemoselective and orthogonal ligation methods to afford macromolecules with one or two nonamide linkages at both ligation sites.[25,28,29] Recently, we reported a successful method of tandem ligation of three unprotected peptide segments in tandem to form two pseudoproline bonds by imine ligation involving the orthogonality of NT-Cys peptide and an NT-Ser/Thr peptide without a protecting-group scheme between each ligation step.[27] In this paper, we describe the development of a semi-orthogonal tandem ligation strategy by employing imine and thioester ligation methods based on the chemoselectivity of NT-Cys peptides toward two CT-electrophiles to form, in tandem, an Xaa-SPro (thiaproline) and an Xaa-Cys bond at the ligation sites. We also optimize conditions to prepare six SPro → Pro peptides including somatostatin-28 (Sm-28) and two proteins, human macrophage inflammatory proteins MIP-1α and MIP-1β. Since thiaproline is a proline surrogate, we show here that the biological activity is not altered by the proline → thiaproline substitutions in these three bioactive peptides.

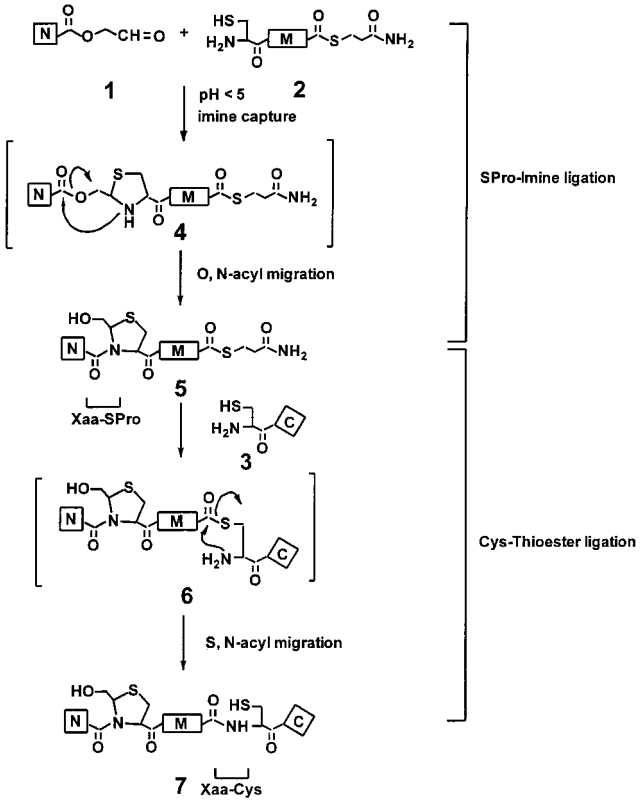

Figure 1. A tandem ligation scheme of SPro-imine and Cys-thioester ligation.

Results and Discussion

Strategy for Tandem Peptide Ligation. The tandem three-segment ligation strategy was performed in the N → C direction (Figure 1) on three unprotected segments to form first an Xaa-SPro (thiaproline as an Xaa-Pro bond surrogate) and then an Xaa-Cys bond. For convenience, these three segments were also designated in the N → C direction as the amine segment (N-segment) **1** containing a C-terminal glycoaldehyde ester, the middle segment (M-segment) **2** containing both a CT-thioester and an NT-cysteine, and the carboxyl segment (C-segment) **3** also containing an NT-cysteine.

SPro-imine ligation of the N- and M-segments proceeds through an imine capture of the CT-glycoaldehyde ester. A thiazolidine ester intermediate **4** is formed, and then an *O*,*N*-acyl transfer takes place to form a C2-hydroxymethyl thiaproline **5** at the ligation site through a favorable five-membered ring intermediate (Figure 1). Recently, we have confirmed that the imine ligation with an NT-Cys (L-configuration) segment is stereospecific and affords an *R*-epimer at the newly created stereocenter at the C2 carbon of the SPro at the ligation site.[27]

Intermolecular Cys-thioester ligation of the NM-segment **5** containing a CT-thioester with the C-segment **3** containing an NT-Cys proceeds through a covalent and branched thioester **6** that undergoes a spontaneous *S*,*N*-acyl migration through a five-membered ring to form an Xaa-Cys bond in the three-segment product **7**. Several methods for the Xaa-Cys bond formation, including the use of a CT-thiocarboxylic with an NT-Cys to

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form a covalent perthioester, have also been reported.[30,31] However, the intramolecular thioester ligation of a peptide such as the M-segment **2** containing both an NT-Cys and a CT-thioester is equally facile and has been extensively exploited for preparing cyclic peptides.[32−36] Thus, the inter- and intramolecular ligation of the M-segment poses a challenge in our synthetic scheme.

Previously, we have shown that inter- and intramolecular thioester-cysteine ligations are best performed at basic conditions (pH > 7),[19,32] whereas SPro-imine ligation prefers acidic conditions at pH < 5.[37,38] In acidic conditions, the thiol−thioester exchange between an NT-Cys and the CT-thioester is slow, while the imine capture between an aldehyde and an NT-Cys is fast. This differential reactivity of an NT-Cys peptide under acidic and basic conditions toward two different CT-esters provides the basis for the three-segment ligation involving two NT-Cys to form two amide bonds in tandem.

Segment Preparation. All segments were prepared by a stepwise solid-phase method. Both N- and M-segments containing CT-esters required specialized resin supports for their syntheses. C-segments, however, required only an NT-Cys and a free carboxylic acid or carboxamide at the CT and were generated either by Boc- or Fmoc chemistry on commercially available resins. All peptide segments were purified by HPLC and characterized by MALDI-MS.

N-segments containing a CT-glycoaldehyde ester **1** were prepared by Fmoc-chemistry on an acetal resin **8**.[38] TFA-mediated cleavage of the assembled peptide esters anchored on the resin supports afforded the unprotected N-segment glyceryl ester. These masked CT-glycoaldehyde esters have the advantage of being purified and stored for future use without side reactions. Prior to the imine ligation, the 1,2-diol glyceryl moiety was then transformed to aldehyde **1** by periodate oxidation under aqueous conditions.

M-segments containing both an NT-Cys and a CT-thioester were synthesized on a thioester resin **10**[39,40] with a bifunctional removal linker of the 3-mercaptopropionic acid coupled to the MBHA resin. This 3-mercaptopropionyl−MBHA resin was then used to assemble the desired sequence. Because thioesters are labile to piperidine treatment in Fmoc chemistry, M-segments **2** were prepared by Boc chemistry and released from the resin supports and protecting groups by HF. Recently, several groups have independently reported the use of a safety-catch resin or a new deblocking reagent to prepare thioester peptides by Fmoc chemistry.[41−43]

Potential Synthetic Problems. Three groups of synthetic problems were considered during the development of the thiaproline-cysteine tandem ligation scheme. The first involved conditions to first ligate N-and M-segments through SPro-imine ligation and then to attach the C-segment through Cys-thioester ligation. A major concern of this ligation step is that the M-segment **2** containing an NT-Cys can react competitively with the CT-glycoaldehyde ester of the N-segment **1** or the CT-thioester of the M-segment. As previously discussed, another concern is that the M-segment containing both an NT-Cys and a CT-thioester is prone to end-to-end cyclization to yield byproduct **12** that terminates the tandem ligation scheme. In addition, these CT-esters are sensitive to aqueous hydrolysis, yielding byproducts **11** and **13** (Figure 2). These problems were examined in the model peptides. The second group of problems involved oxidation-sensitive residues such as NT-Ser, NT-Thr and Met present in N-segments and the presence of Cys in the M-segments also containing CT-thioesters. These problems were examined in the syntheses of the thiaproline analogues of somatostatin-28. The third group of problems, involving site selection, the presence of cysteine in the N-segments, and the influence of conformation in the ligation reactions, was examined in the syntheses of the thiaproline analogues of macrophage inflammatory proteins, MIP-1α and MIP-1β.

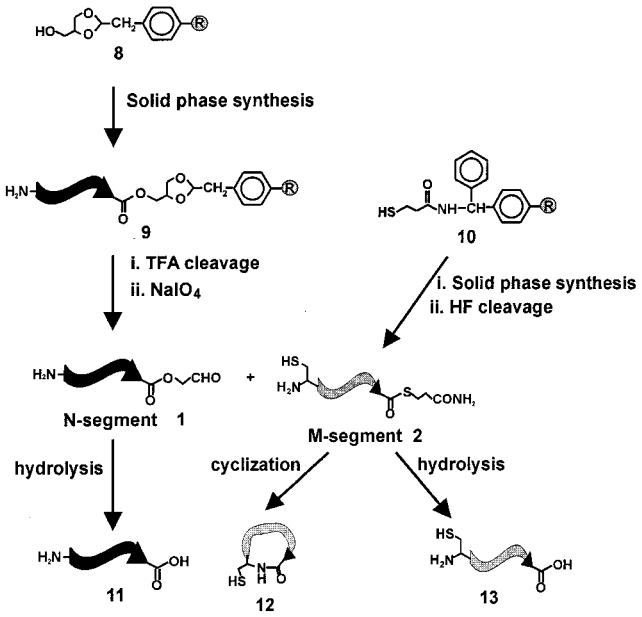

Figure 2. Possible side reactions during the ligation between N- and M-segments.

1. pH Optimization and Minimization of Side Reactions for Tandem Imine and Thioester Ligations. M-segments with 5 to 10 amino acids are prone to end-to-end cyclization and provide suitable models for the feasibility study of the SPro-Cys tandem ligation scheme. The rates and pH effects on imine ligation were examined using an eight-residue N-segment GL8-CHO **1a** (t_R = 12.7 min, MH 839) to ligate a five-residue M-segment CG5-SR **2a** (t_R = 5.9 min, MH 670). When ligations were performed between pH 5 to 7, >80% of the M-segment **2a** underwent the side reaction of end-to-end backbone lactamization to afford the cyclic CG5 **12a** (t_R = 7.9 min, MH 565) within 3 h (Table 1). Significant hydrolysis of the unreacted glycoaldehyde ester **1a** to the free carboxylic **11a** (t_R = 13.5 min, MH 797) was also observed at this pH range. At pH 4, the cyclization rate significantly decreased, but hydrolysis of **2a** to **13a** (hydrolyzed product **13a**, t_R = 6.3 min, MH 573) was still observable. In contrast, when the ligation was performed at pH 3 or lower, both side reactions attributed to cyclization of the M-segment **2a** and hydrolysis of the N-segment **1a** significantly decreased. However, lowering the reaction pH also markedly retarded the *O*,*N*-acyl migration of

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Table 1. Effect of pH on Side Reaction in Ligation between GL8-OCH_2CHO **1a** and CG5SR **2a**[a]

N-segment		M-segment (%)			
pH	aldehyde hydrolysis (%)[b]	thioester remaining[c]	thioester hydrolysis[c]	cyclized product[c]	NM-ligated product[c]
7	>99	<1	<1	>99	<1
6	>99	5.3	5.2	89.5	<1
5	25.5	3.3	7.8	79.4	9.3
4	27.8	41.1	2.3	11.1	45.3
3	<1	63.5	<1	<1	36.0

[a] N- and M- segments were reacted for 3 h. [b] (%) based on N-segment. [c] (%) based on M-segment.

Table 2. Yields and Analytical Data of Thiazolidine Thioester in the Thiaproline Ligation between N- and M-Segments

		ligated NM-segment thioester			
N-segment-OCH_2CHO	M-segment-thioester	NM-thioester	HPLC (min)[a]	yield[b] (%)	MH^+ (calcd)
GL8[c] **1a**	CG5 **2a**	[Thz[9]]GG13 **4a**	14.9	79.6	1490.1 (1490.0)
FK9 **1b**	CG5 **2a**	[Thz[10]]FG14 **4ba**	17.9	80.0	1779.9 (1781.9)
FK9 **1b**	CG7 **2c**	[Thz[10]]FG16 **4bc**	13.0	69.5	2016.8 (2018.1)
SA9 **1c**	CG5 **2a**	[Thz[10]]SG14 **4ca**	17.7	70.7	1555.9 (1556.7)
SA9 **1c**	CG7 **2c**	[Thz[10]]SG16 **4c**	13.4	86.4	1792.5 (1791.8)
AK37 **1d**	CV13 **2d**	[Thz[38]]AV50 **4d**	22.8	91.9	5901.9 (5901.3)
AL20 **1e**	CL14 **2e**	[Thz[21]]AL34 **4e**	22.3	93.0	4100.0 (4099.0)

[a] t_R in min. [b] Yields were based on the limited M-segments and calculated from area ratio corresponding to peaks of M- and NM-segments. [c] Letters indicate the first and last amino acids and numbers indicate the numbers of amino acids in the sequences. GL8 = GIAYGGFL, FK9 = FETSSQC(Acm)SK, SA9 = SANSNPAMA, AK37 = ASLAADTPTAC(Acm)C(S-*t*Bu)FSYTSRQIPQNFIADY-FETSSQ-C(Acm)SK, AL20 = APMGSDPPTACCFSYTARKL, CG5 = CKLYG, CG7 = CRERKAG, CV13 = CGVIFLTKRSRQV, CL14 = CRNFVVDYYETSSL, Thz = $-OCH_2$-thiazolidine.

the thiazolidine ester **4a** to the Xaa-SPro bond and required >100 h for completion. Once formed, the thiazolidine ester **4** is relatively stable, and we envisioned that the *O*- to *N*-acyl migration could be mediated concurrently during or even after the second ligation.[19,26,32,38]

Table 2 shows the ligation yields between N- and M-segments. The imine capture forms a new stereocenter at the C2-position of the thiazolidine ring, and the thiazolidine-ester products eluted as doublets in HPLC profiles. NMR study showed that the *O*,*N*-acyl migration of the C2-*R* epimer proceeds faster than the C2-*S* epimer.[27] The unreacted C2-*S* epimer likely undergoes an equilibration to a mixture of *R* and *S* epimers through ring-chain tautomerization to favor the C2-*R* epimer as major product after *O*,*N*-acyl migration under basic conditions.

NM-segment and C-segment proceeded with thioester ligation smoothly at pH 7.2 phosphate buffer and in the presence of excess TCEP and 3-mercaptopropionic acid (MPA). Table 3 shows the yields of model peptide and analogues. MPA, a small thiol, is a useful additive because it is a water-soluble reducing agent and does not have a strong stench. More importantly, it maintains the NT-cysteinyl thiol in the reduced form critical for the thiol capture step.[19] However, it also promotes thioester hydrolysis. This disadvantage may not be significant for small- or middle-size peptides in the range of 30−50 residues because the ligation is normally completed within 3 h. To minimize side reactions, we also used other thiol catalysts such as thiophenol[44] and 2-mercaptoethanesulfonic acid[45] in the tandem ligation for the synthesis of the CC-chemokines.

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Table 3. Yields of Cys-Thioester Ligation between NM- and C-Segments

		NMC-ligated product			
NM-segment thioester	C-segment	ligated product	HPLC (min)[a]	yield[b] (%)	MH^+ (calcd)
[Thz[9]]GG13 **4a**	CY6[c] **3a**	[SPro[9]]GY19 **7a**	25.9	82.0	2172.6 (2173.0)
[Thz[10]]SG14 **4ca**	CC12 **3b**	[SPro[10]]SC26 **7cb**	25.5	69.0	2962.6 (2963.4)
[Thz[10]]FG14 **4ba**	CC12 **3b**	[SPro[10]]FC26 **7bb**	24.7	69.0	3188.5 (3187.6)
[Thz[10]]FG16 **4bc**	CC12 **3b**	[SPro[10]]FC28 **7b**	22.8	72.4	3423.9 (3424.7)
[Thz[10]]SG16 **4c**	CC12 **3c**	[SPro[10]]Sm-28 **7c**	23.5	80.6	3199.2 (3199.0)
[Thz[38]]AV50 **4d**	CA20 **3d**	Cys[11,35] (Acm) [SPro[38]]MIP-1α **7d**	24.2	66.3	8066.8 (8065.8)
[Thz[21]]AL34 **4e**	CN35 **3e**	[SPro[21]]MIP-1β **7e**	22.6	73.6	7869.7 (7868.0)

[a] t_R in min. [b] Yields were based on NM-segment thioesters and calculated from area ratio corresponding to peaks of NM-segment and NMC-ligated product. [c] CY6 = CNSFRY, CC12 = CKNFFWKT-FTSC, CA20 = CADPSEEWVQKYVSDLELSA, CN35 = CSQPAV-VFQTKRSKQVCADPSESWVQEYVYDLELN.

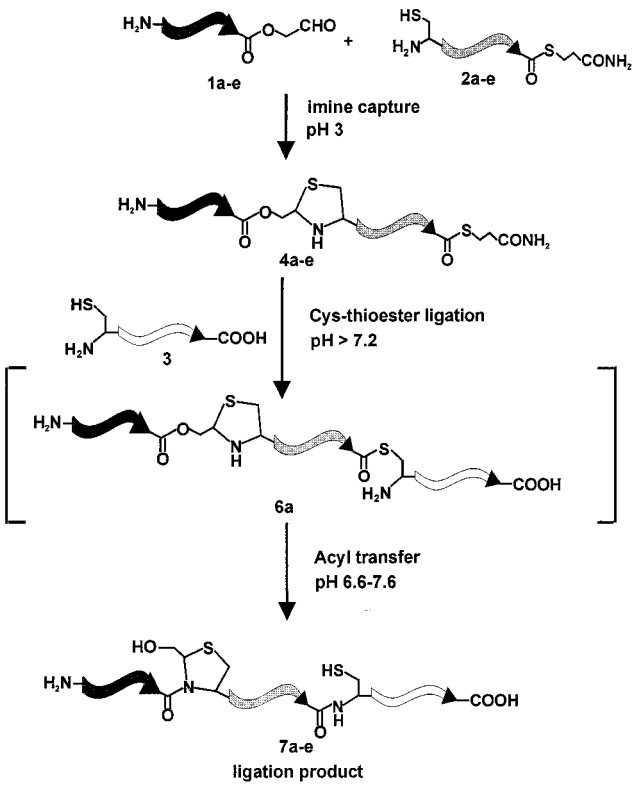

Figure 3. A scheme of three-segment tandem ligation mediated by a diester intermediate.

In thioester ligation, the thiol capture is a rate-limiting step, while the *S*,*N*-acyl migration occurs spontaneously to generate a Xaa-Cys bond at the ligation site. In contrast, the slow *O*,*N*-acyl migration at the N-to-M-segment ligation site becomes the rate-determining step of this three-segment tandem ligation reaction. Because this acyl migration was often not complete even when the thioester ligation was completed in <12 h, the thioester ligation was prolonged >24 h. For the model peptides, the *O*,*N*-acyl migrations were observed from changes in the HPLC profile.

Tandem Ligation Using the Diester Intermediate. On the basis of the above results, an optimized condition for the thiaproline-thioester ligation in tandem was used for all subsequent syntheses (Figure 3). (1) The first SPro-imine ligation of N- and M-segments (**1** and **2**) was performed at pH 3. Under such a condition, the protonated nature of the NT-cysteinyl thiol inhibits thioester ligation but permits imine capture of the CT-glycoaldehyde ester and its tautomerization to a stable thiazolidine ester **4**. At pH 3, the *O*,*N*-acyl migration to form an Xaa-

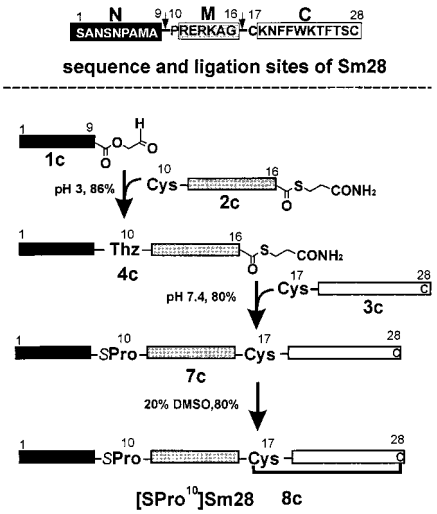

Figure 4. A tandem ligation synthetic scheme of [SPro^{10}]Sm 28.

SPro bond is slow and incomplete but can be accomplished during steps 2 and 3 when they are performed at pH 7 to 8. (2) The second step is the Cys-thioester ligation of NM- and C-segments that produces NMC-branch esters **6a** (Figure 3). It can proceed, after adjusting the pH to >7.2 with solid $NaHCO_3$, either directly by adding a C-segment to the reaction mixture (one-pot reaction) or to the purified NM segment. Yields of the Cys-thioester ligation are shown in Table 3. (3) The third step is the disulfide formation mediated by adding 10−20% DMSO by volume to the reaction medium at pH 6−8 of step 2 to complete the three-segment ligation in tandem.

2. Synthesis of Somatostatin-28 Analogues: Susceptibility of NT-Ser to Oxidation and the Presence of Cysteine in the M-Segments. Somatostatin-28 (Sm-28) contains 28 amino acid residues and is widely distributed in central nervous system and digestive tract tissues.[46] [SPro^{10}]Sm-28 with a thiaproline → proline10 replacement was prepared by three-segment tandem ligation to validate its application for a medium-length peptide containing disulfides (Figure 4). The ligation sites were based on the occurrence of Pro and Cys, respectively, at positions 10 and 17 of the Sm-28 sequence.

Sm-28 was also selected because it contains an NT-Ser that presents both a potential opportunity and synthetic problem. Recently, imine ligation has been successfully extended to ligating segments with NT-Ser or Thr to form an oxaproline at the ligation sites.[27] Thus, the presence of NT-Ser at the N-segment is desirable for developing a four-segment tandem ligation using oxaproline ligation. However, NT-Ser at the N-segment is susceptible to oxidization during the $NaIO_4$ conversion of the CT-glyceric to its glycoaldehyde ester for imine ligation. At pH 7, oxidation of NT-Ser to an α-oxo moiety was 1000-fold faster than the CT-1,2-diol. At pH < 6, our model compound showed a reverse selectivity of diol over the 1,2-amino alcohol of NT-Ser oxidation with $NaIO_4$. Selectivity increased with an increase of acidity, which probably can be attributed to the increased protonated states of the NT-Ser. At pH 3, the CT-glyceryl ester was completely oxidized to aldehyde within 3 min without significant oxidation of NT-Ser. Thus, for N-segments containing NT-Ser and NT-Thr, selective conversion of CT-glyceryl ester to its corresponding aldehyde can be achieved at acidic pH. Under this condition the methionine residue in the N-segment SA-9 was oxidized to Met-(O).[47] To avoid the Met oxidation, we fine-tuned the oxidation condition and found that the optimal pH was around pH 6. At pH 6.2, treatment of the N-segment SA9-glycerylester with 5 equiv of $NaIO_4$ in 0.5 h afforded the desired CT-aldehyde with 80% yield without affecting Met. Longer oxidation time produced a significant amount of Met(O) and an α-oxo side product from NT-Ser oxidation.

Imine capture between N- and M-segments (**1** and **2**) and thioester ligation between NM-and C-segments (**4** and **3**) proceeded smoothly under the conditions used in model peptides. In the presence of excess thioester **4**, the free cysteinyl thiol produced under the ligation condition can react again with the NM-segment thioester **4** yielding an *N*,*S*-diacyl byproduct. To limit the formation of this diacyl byproduct, a slight excess of C-segment **3** was used. After ligation, a small thiol MPA was added to the reaction mixture to regenerate product **7** from the *N*,*S*-diacyl byproduct.[16,19]

After completing the tandem ligation, the crude product was treated with 20% DMSO in an aqueous solvent buffered at pH 6.2 for 24 h for the disulfide formation between Cys^{17} and Cys^{28}. As discussed previously, the *O*,*N*-acyl migration of the thiazolidine ester at the N-to-M-segment ligation site was slow, and therefore the period for disulfide oxidation was prolonged to permit completion of the *O*,*N*-acyl migration. Progress of the ligation reaction as monitored by HPLC showed that the thiaproline bond was indeed complete after the DMSO oxidation. Treatment with 1 M NH_2OH at pH 9.0 for 1 h further confirmed that the thiaproline ligation site was an amide bond because the thiazolidine ester bond was susceptible to base treatment and would yield two segments.[24,38]

In the synthesis of Sm-28 analogues, three short segments of <10 residues are used. Since these segments can be synthesized rapidly by solid-phase methods, the SPro-Cys tandem ligation scheme may be useful for combinatorial segment synthesis. At present, combinatorial peptide synthesis by a stepwise method is not reliable for peptides >20 residues due to unpredictable side reactions that make decoding impurities difficult. The three-segment ligation of the somatostatin model with purified segments provides unambiguous products and, more importantly, can be performed entirely in aqueous conditions.

3. Syntheses of Human MIP-1α and MIP-1*β*: Site Selection, Presence of Cysteines in the N-Segments, and Conformational Influence. MIP-1*β* and MIP-1α are members of the CC-chemokine proteins of 8−10 kD.[48−50] These chemokines are primarily involved in the chemoattractant potential of monocytes and lymphocytes and have been implicated in a variety of clinically important inflammatory diseases, and more recently as inhibitors of HIV pathogenesis. Structurally, these two chemokines display significant sequence homology that permits comparison of ligation sites at different Pro and Cys residues to test the conformational influence of the tandem ligation reactions. A synthetic challenge is the presence of two adjacent Cys at the N-terminal segments, a sequence motif of CC-chemokines, that requires manipulations to avoid side reactions during the oxidative transformation to the CT-glycoaldehyde ester for the SPro-imine ligation between the N- and M-segments.

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MIP-1α. The 70-residue MIP-1α contains Pro at positions 21 and 38, either of which is a suitable choice as an imine ligation site to afford a SPro → Pro analogue. Ligation at Pro[38] would require an N-segment with a CT-Lys[37]-glycoaldehyde ester. The participation of this CT-lysyl side-chain amine through either intramolecular lactam or Schiff base formation may interfere in the imine ligation. To resolve this potential side reaction, we selected Lys[37]-Pro[38] as the imine ligation site and Cys[52] for the thioester ligation. Thus, three segments of 37, 13, and 20 residues corresponding to the N-, M-, and C-segments (**1d**, **2d**, and **3d**, respectively), with the N-segment **1d** being the longest of the three, were prepared for the tandem ligation via thiaproline (37 + 13 aa-segments) and thioester (50 + 20 aa-segments) for the synthesis of [SPro[38]]MIP-1α **7d**.

MIP-1*β*. The 69-residue MIP-1*β* also contains Pro at positions 21 and 38. To prepare [SPro[21]]MIP-*β*, Pro[21] and Cys[35] were selected as ligation sites and required the syntheses of three segments **1e**, **2e**, and **3e** containing 20, 14, and 35 amino acid residues of which the C-segment **3e** was the longest. While the Cys-thioester ligation of MIP-1α consisted of two segments of 50 and 20 residues, the NM- and C-segments (**4e** and **3e)**, ligated in MIP-1*β* were roughly equal, containing 34 and 35 residues, respectively.

Conformation Influence in the Tandem Ligation of MIP-1α and MIP-1*β*. The SPro-imine ligation of N-segments **1d** and **1e** of MIP-1α and MIP-1*β* with their corresponding M-segments proceeded smoothly to form the NM-segments at pH 3.0. Turbidity in the reaction solutions was observed, and 33% acetonitrile by volume was added to the aqueous solution to improve the solubility of the N-segments. However, denaturants were not required in these thiaproline ligation steps. Furthermore, the presence of Lys[37] at the CT-glycoaldehyde ester did not pose a synthetic problem, probably due to reversibility of the intramolecular Schiff base formation under the aqueous acidic condition in which the SPro-imine ligation was performed.

An unpredictable factor in ligation chemistry for protein synthesis is the conformational influence of large peptide segments on the ligation rate observed in the three-segment tandem ligation of CC-chemokines MIP-1α and MIP-1*β*. Although we found that the SPro-imine ligation of the N-and M-segments proceeded smoothly under aqueous conditions, the second ligation of the resulting larger NM segment **4d**, **4e** with the C-segment **3d**, **3e** had to be performed under strongly denatured conditions.

Various runs of thioester ligation of both NM-segments **4d**, **4e** with their corresponding C-segments **3d**, **3e** under aqueous conditions in pH 7.6 phosphate buffer with additional organic cosolvents and reducing agents such as TCEP and MPA were unsuccessful. In all cases, the expected ligated products were not observed from the HPLC profile after 3 h. After 24 h, most of the thioesters of the NM-segments were hydrolyzed to their corresponding carboxylic acids, while their C-segments formed disulfide dimers. Thus, a denatured condition using 8 M guanidine HCl at pH 7.6 was used for ligating NM- and C-segments of MIP-1α and MIP-1*β*. Thiophenol (1 vol %) was added to maintain the reaction mixture under this reducing condition and also as an additional denaturing reagent.[18] Under this denatured condition, thioester ligations between NM- and C-segments of the thiaproline analogues of MIP-1α and MIP-1*β* were obtained in satisfactory yields (Figure 7, Table 3).

The difficulty in achieving the thioester ligation may due to the conformational resistance of these two segments. Various structural determinations have shown that MIP-1α and MIP-1*β* contain three *β* strands from the N-terminal to the middle of the molecule and then an α-helix beginning with Cys[52] at the C-terminus.[49] Furthermore, these CC-chemokines have a strong propensity to form dimers. Thus, a folded or partially folded NM-segment or dimerization of the Cys[52]-segment may hamper the Cys-thioester ligation. Our observation that under nondenaturing conditions the Cys[52]-segment **3d** or **3e** rapidly formed a covalent dimer provides experimental support for this explanation.

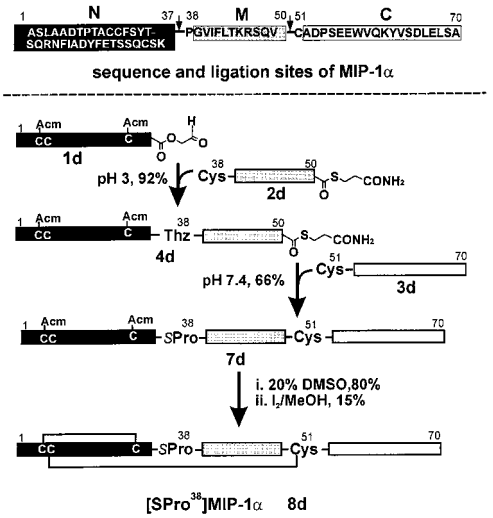

Figure 5. A tandem ligation synthetic scheme of [SPro[38]]MIP-1α.

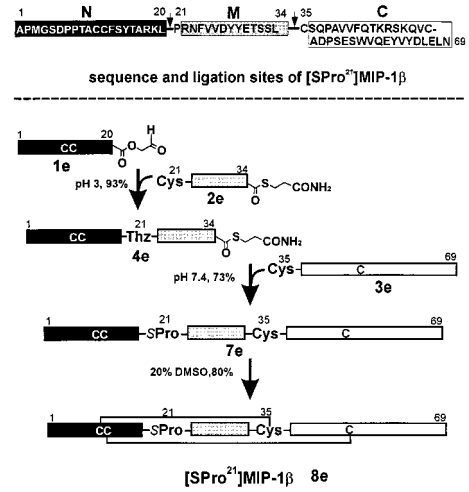

Figure 6. A tandem ligation synthetic scheme of [SPro[21]]MIP-1*β*.

Disulfide Formation. Both MIP-1α and MIP-1*β* contain four cysteine residues with two adjacent cysteines (Cys[11] and Cys[12]) proximally located at the N terminus (Figures 5 and 6). During the $NaIO_4$ treatment for forming CT-aldehyde, the random disulfide formation of these two free Cys in the N-segment posed a problem that necessitated a temporary protection scheme. We pursued two methods that employed sulfenyl-*tert*-butyl (S-*t*Bu) and Acm-protecting groups. In MIP-1*β*, only the S-*t*Bu-protecting groups were used, including both cysteines of the N-segment. The disulfide bonds of the S-*t*Bu-cysteinyl residues offered protection during the $NaIO_4$ oxidation, but they were removed during the thioester ligation by the presence of small thiols in the reaction mixture. Since two of the three geometric disulfide isomers were favored to form under the DMSO-mediated oxidation, we were able to obtain the desired isomer after sequential RP-HPLC purification in relatively low

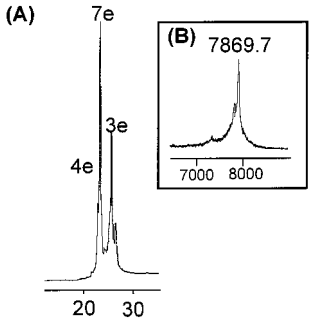

Figure 7. (A) HPLC profile of ligation between NM-segment **4e** and C-segment **3e** to form [SPro^{21}]MIP-1β **7e** and (B) MS profiles of [SPro^{21}]MIP-1β **7e**.

yield without optimization of the oxidative refolding of the ligated MIP-1β product.

To avoid the random oxidation method for disulfide formation, we used an orthogonal thiol-protecting strategy of S-*t*Bu and Acm for Cys^{12,51} and Cys^{11,35} in the synthesis of MIP-1α, respectively. The S-*t*Bu-protecting group at Cys^{12} was removed during the thioester ligation of the MN + C segments. DMSO-mediated disulfide formation of the ligated MNC segments generated the Cys^{12,51} disulfide pair. The Cys^{11,35} disulfide was formed by treatment with I_2/MeOH. This two-step oxidation forms a single product with the desired disulfide linkages. The stability of the S-*t*Bu-protecting groups during the oxidation conditions for CT-aldehyde formation of N-segments of MIP-1α and MIP-1β was also studied. At pH 6.2 with 5 equiv of $NaIO_4$, the S-*t*Bu group was found to be stable, and the desired CT-aldehyde was formed in a 70% yield. At pH $<$ 4.2 or with prolonged oxidation over 30 min, a complex mixture was obtained with observation of the CT-ester diol being overoxidized and with decomposition of the S-*t*Bu groups to form various undetermined byproducts.

Biological Activity. The biological activities of three ligation products, [SPro^{10}]somatostatin **8c**, [SPro^{38}]MIP-1α **9d**, and [SPro^{21}]MIP-1β **8e**, were compared with their native products by radioreceptor binding assay and chemotaxis assay, respectively. Dose-dependent response of radioreceptor assays showed that the activity of the native somatostatin-28 and [SPro^{10}]-somatostatin **8c** were indistinguishable and both completely inhibited specific receptor binding in mouse AtT-20 cells expressing somatostatin receptors at about 10 nM. The migration capacity of [SPro^{38}]MIP-1α **9d** and [SPro^{21}]MIP-1β **8e** counter was compared with the native MIP-1α and MIP-1β on P4-CCR5 cells using a chemotaxis assay (Figure 8). All four compounds showed dose-dependent responses at concentrations of 0.01−1000 ng/mL to P4-CCR5 cells with a maximal response at 1 ng/mL. The chemotactic indexes of MIP-1α and MIP-1β at 1 ng/mL were 3.9 and 2.7, respectively. At the same concentration, the chemotactic indexes of [SPro^{38}]MIP-1α **9d** and [SPro^{21}]MIP-1β **8e** were 3.1 and 2.3 ng/mL, respectively. There was no significant difference ($P < 0.05$) between the thiaproline and native MIP-1α or MIP-1β chemokines.

Conclusions

The development of tandem ligation of three peptides is of both practical and fundamental interest. The ability to ligate multiple peptide segments to form two or more regiospecific amide bonds in aqueous solutions, without the use of a protecting-group scheme and coupling reagents, may provide a useful strategy for convergent, combinatorial, or conformation-assisted ligation for both semi- and total protein syntheses.[16,51,52] A successful demonstration of an abiotic scheme may provide clues to the formation of functional biopolymers under prebiotic conditions.

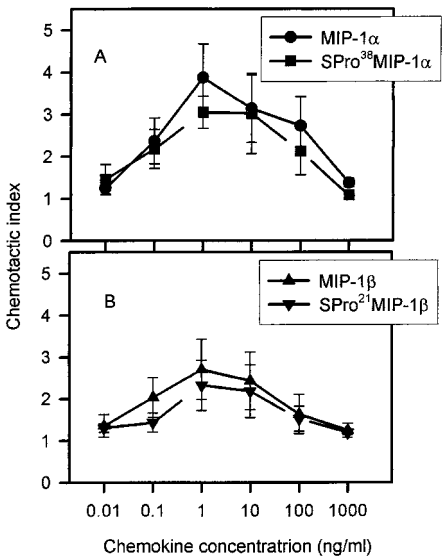

Figure 8. Chemotaxis assays of synthetic and native (A) MIP-1α and (B) MIP-1β. Migration of P4−CCR5 cells at the indicated chemokines concentrations are shown as the mean ± SEM of three individual experiments. The chemotactic index is defined as the number of cells migrating in response to test peptides divided by the number of cells migrating in response to medium alone.

Our report shows the potential of a tandem ligation approach to peptide and protein synthesis by employing two ligation methods, SPro-imine and Cys-thioester ligation, to couple three unprotected peptides to form larger peptides and proteins in aqueous solutions with high efficiency. With the development of recombinant DNA methods, particularly the intein-expressed CT-thioester as building blocks, abiotic peptide synthesis approach through orthogonal and tandem ligation will extend the range and the versatility for protein syntheses. These will include many soluble proteins with interesting biological functions such as growth factors, chemokines, cytokines, and other regulatory proteins.

A concern for our tandem ligation scheme is the formation of an unusual thiaprolyl bond (SPro) instead of a normal prolyl peptide bond in peptides and proteins. Proline is a convenient ligation site because it often occurs at reverse turns, and thiaproline appears to be a good structural surrogate. Thus far, we have found that substitution of proline by ψPro in selected bioactive peptides and proteins, as demonstrated in this work with somatostatin-28 and CC-chemokines, does not result in loss of their bioactivities. These results are consistent with our previous work on antimicrobial peptides and enzymes. For example, replacement of SPro for Pro^{39} in HIV protease analogues does not affect their folding, dimerization, and biological activity.[53] In addition, the 2-hydroxymethyl thiazolidine ring could be considered as a chimeric amino acid, which has found applications in the rational design of peptide hormones.[54]

Recently, another imine ligation, oxaproline ligation, has been developed in our laboratory.[27] Oxazolidine ligation uses Ser or

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Thr instead of Cys as NT-nucleophiles to react with aldehyde to form oxaproline in the ligation site. Since the oxaproline ligation is orthogonal to Cys-thioester ligation, a three- or four-segment tandem ligation strategy can be envisioned which incorporates both ligations. However, the technical aspects of such a strategy need to be studied to arrive at a tandem ligation for multiple segments without a protecting-group scheme.

Experimental Section

Materials and Methods. All solvents of HPLC grade were obtained from VWR Scientific Co. and used without further purification. Chemicals were obtained from Aldrich Chemicals (Milwaukee, MI). Boc-amino acids and MBHA resin were purchased from Bachem, California. Analytical RP-HPLC was performed on a Shimadzu system with a Vydac column (0.46 cm × 25 cm, C_{18}) at a flow rate of 1 mL/min with a linear gradient of 10−100% buffer B (60% acetonitrile in H_2O/0.04% TFA) in buffer A (5% acetonitrile in H_2O/0.045% TFA) over 30 min. Effluent was monitored with UV at 225 nm. A preparative HPLC equipment (Waters Associates) with a Vydac C_{18} reverse phase column was used for peptide purification. Mass spectra were obtained from a Kratos mass spectrometry instrument with positive matrix-assisted laser desorption ionization mode (MALDI-MS).

Synthesis of Peptide-Glycoaldehyde. Fmoc-Gly-OCH_2-cyclic acetal resin was prepared according to the described procedure.[38] After cleavage using 95% TFA, the crude peptide-glyceric ester was dissolved in a phosphate buffer (0.2 M, pH 6.2). To this solution, $NaIO_4$ solution in the same buffer (0.5 mmol/mL) was added. After 30 min, the peptide aldehyde was purified by HPLC. Typically, AK37-CHO **1d** was prepared from AK37-$(OH)_2$ (1 mg in 200 mL of buffer, 0.2 μmol) and 20 μL of $NaIO_4$ solution (1 μmol) in a yield of 72% (yield was based on area ratio of corresponding HPLC peaks, MH^+ calcd 4326.9, found 4327.5).

Thioester Resins. Thioester resins were prepared with a modified Hojo and Aimono's procedure.[39,40] Four equivalents each of 3-mercaptopropionic acid, HOBt, and DIC were sequentially added to a suspension of 4-methylbenzhydrylamine (MBHA) resin in DMF (10 mL/g resin). The mixture was shaken at room temperature until a ninhydrin test of the resin indicated that no free amino sites were present. The resin was washed with DMF, DCM, CH_3OH, DCM, and DMF. The resulting resin was treated with a mixture of 1 equiv each of cysteine methyl ester hydrochloride, triphenylphosphine, and DIEA in DMF/DCM (3:1, v/v) for 2 h. After thorough washing with DMF, DCM, CH_3OH, and DCM and drying in vacuo, the mercaptopropionyl MBHA resin was obtained in quantitative yield (6.3 g from 6.0 g of MBHA resin).

Boc amino acid (4 equiv) preactivated with BOP (4 equiv) and DIEA (6 equiv) for 5 min was added to a suspension of mercaptopropionyl MBHA resin (1 equiv) in DMF (10 mL/g resin). The mixture was shaken at room temperature for 2 h, and free thiol groups were monitored by Ellman's reagent.[55] The resin was washed with DMF, DCM, CH_3OH, and DCM and used for stepwise peptide synthesis**.**

Synthesis of Peptide Thioesters. All thioesters were prepared by solid-phase synthesis starting from Boc amino acid thioester resin. The solid-phase synthesis was carried out on a peptide synthesizer 430A (Applied Biosystems Inc., Foster City, CA) using conventional Boc-chemistry and DCC-HOBt coupling protocol. The side-chain protecting groups of Boc-amino acid used were as follows: Arg(Tos), Asp(OcHex), Cys(4-MeBzl or Acm), Glu(OcHex), His(Dnp), Lys(ClZ), Ser(Bzl), and Thr(Bzl). After peptide chain elongation, the resin was treated with high HF procedure (90% HF). After evaporation of HF, the residual solid was washed with ether. The crude peptide thioesters were extracted with 50% acetic acid, purified by preparative HPLC, and lyophilized. All synthetic peptides were characterized by MALDI-MS.

Imine Ligation between N- and M-Segment. Typically, to the solution of 0.1 μmol N-segment dissolved in 200 μL of 0.2 M acetate buffer pH 3.0, 1.1 equiv of M-segment was added. The ligation mixture was kept at room-temperature overnight. The ligation product was then purified by HPLC, giving a yield of 70−90%. For MIP-1α and MIP-1β, one-third volume of acetonitrile was added to increase the solubility and give better results. Yields and MS data are shown in Table 2.

Thioester Ligation between NM- and C-Segments. To the solution of NM-segment (0.1 μmol) in 100 μL of 0.2 M phosphate buffer (pH 7.2) containing 3 equiv of TCEP was added 1.1 equiv of C-segment. After 3 h at room temperature, 1 μL of MPA was added, and the reaction mixture was agitated for 40 min. The ligation product was then purified by HPLC and lyophilized to dryness. For MIP-1α and MIP-1β, 0.1 μmol of NM-segments **4d**, **4e** and 1.2 equiv of C-segments **3d**, **3e** were dissolved in 200 μL of 8 M guanidine HCl/0.2 M phosphate buffer (50/50, v/v) pH 7.6. Thiophenol (1 vol %) was added. The ligation mixture was shaken overnight (18−24 h). Water (400 μL) then was added ,and the reaction mixture was extracted with 3 × 200 μL of ether to remove the thiophenol. The aqueous phase was evaporated under vacuum to remove ether. The solution was then applied to HPLC to give the ligation product **7d** and **7e**. Yields and MS data are given in Table 3.

Disulfide Bond Formation. The disulfide bond formation was achieved by dissolving 1 mg of three-segment ligated products **7c**, **7d,** or **7e** in 1 mL of phosphate buffer (pH 6.6) with 10−20% in volume of DMSO. The solution was stirred at room temperature for 24 h, and the oxidized product was purified by HPLC. [$SPro^{10}$]Sm28 **8c**: MS 3197.8 (calcd 3197.2), [$Cys^{11,35}$Acm-$SPro^{37}$]MIP-1α **8d**: MS 8064.8 (calcd 8063.8), and [$SPro^{21}$]MIP-1β **8e**; MS 7867.7 (calcd 7866.0). In the two-step disulfide formation of MIP-1α, the solution containing **8d** was adjusted with acetic acid (2 M) to pH 3 and treated with 3 equiv of I_2/MeOH solution to remove Acm and to form [$SPro^{37}$]MIP-1α with 15% overall yield; MH^+ 7974.9 (calcd 7976.0).

Somatostatin Radioligand Binding Assay. The receptor-binding assays for Sm-28 and [$SPro^{10}$]Sm-28 were performed by MDS Panlabs (Bothell, WA). Briefly, AtT-20 cells were grown in DMEM containing 10% fetal calf serum at 37 °C in a humidified atmosphere of 10% CO_2−90% air. For receptor binding assay, cells were incubated with [^{125}I-Tyr^{25}]Sm-14 in the absence or presence of [$SPro^{10}$]somatostatin. Cells were pellected by centrifugation and washed with buffer. The radioactivity of pellects was quantitated in γ-spectrometer.[56]

CC-Chemokine Chemotaxis Assay. The Hela-CD4/LTR-lacZ indicator and CCR5 expression cell line P4-CCR5[57,58] was employed for chemotaxis assay. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Cellgro) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals) and penicillin plus streptomycin as well as hygromycin (1 μg/mL). Cell migration in response to MIP peptides was assessed in a modified 48-well microchemotaxis chamber (Neuro Probe Inc., Cabin John, MD). Serial dilutions (0.01−1000 ng/mL) of $SPro^{38}$MIP-1α and $SPro^{21}$ MIP-1β were compared with two recombinant human chemokines MIP-1α and MIP-1β (R and D Systems) in 35 μL of DME medium loaded in the wells of the lower compartment in duplicates. Medium alone without chemokine was loaded in three wells as control. A suspension of 4 × 10^4 cells in 50 μL of DME medium with 10% FBS without protein was added to the upper compartment which was separated from the lower compartment by a collagen-coated poly(vinylpyrrolidone)-free polycarbonate membrane with 5-μL diameter pores. The chambers were incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C for 4−6 h. The filter was fixed with Diff-Quik Fixative and stained in Diff-Solution I and II (Dade Begring Inc.). The cells that migrated into lower wells were counted under microscopy. Results were expressed as a chemotactic index, which defined as the number of cells migrating in response to test protein divided by the number of cells migrating in response to medium alone. The concentration required for 50% of the maximal response (chemotaxis EC_{50}) was derived from the dose−response curves.

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