Stereospecific Pseudoproline Ligation of N-Terminal Serine, Threonine, or Cysteine-Containing Unprotected Peptides

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Abstract: This paper describes an orthogonal and stereospecific method for ligating free peptide segments to form a monosubstituted pseudoproline bond with a hydroxymethyl moiety at the C2 carbon. The pseudoproline ligation, comprising both the oxaproline and thiaproline ligations, initially involves an imine capture of a peptidyl glycoaldehyde ester with an N-terminal cysteine, serine, or threonine peptide segment and then two spontaneous cyclization reactions. The thiazolidine or oxazolidine ester formed in the first cyclization undergoes an *O,N*-acyl transfer to form an pseudoproline bond. The thiaproline ligation can be carried out exclusively with unprotected peptide segments in both aqueous and nonaqueous pyridine−acetic acid conditions. However, the oxaproline ligation is best performed in a nonaqueous pyridine−acetic acid mixture with unprotected peptide segments except for those containing N-terminal nucleophilic amino acids such as Cys, His, and Trp. Pseudoproline ligation is not only regioselective but also stereospecific. 2D ^{1}H NMR studies of dipeptide models, *Z*-Xaa-ψPro-OMe, indicate that the newly created C2 stereocenter of the pseudoproline ring affords only an *R*-epimer and the C2-hydroxymethyl-substituted pseudoproline exhibits high preference for *cis* conformation. Three of the model peptides have more than 50% *cis* isomers. Finally, this novel method has been used successfully in ligating two segments of 24 and 35 amino acids under mild conditions to synthesize three analogues of bactenecin 7, an antimicrobial peptide containing 59 amino acid residues.

Introduction

Proline, an imino acid, plays a unique role in the structures and folding pathways of peptides and proteins.[1,2] Structurally, it often serves as a hinge amino acid because of its frequent occurrence in the locale where peptide chains turn and reverse directions. Indeed, Pro has been found with a strong preference in turns occupying the *i*, *i* + *1* (types I and II), *i* + *2* (type IV), or *i* + *3* (type VIII) position.[3−5] Pro-rich sequences also exist as extended helices in tandemly repetitive occurrences as found in collagens and antimicrobial peptides.[6,7] In peptides and peptidomimetics, the effect of Pro is believed to confer bioactive conformation or recognition or to increase bioavailability because of the increased lipophilicity and protease-resistance of the imide bond over the secondary amide bond.[8]

The *cis*−*trans* isomerization of Xaa-Pro bonds (Xaa = any amino acid) in peptides and proteins has been well documented,[9−12] and proline has a relatively high ability to impart the *cis*-isomer. Orengo et al.[13] have shown the occurrence of a *cis* Pro bond in 70 out of 205 Pro-containing proteins in a nonredundant database of 214 protein structures. In bovine pancreatic ribonuclease A, two of the four Xaa-Pro bonds are found to be cisoid.[14] Furthermore, the *cis*−*trans* isomerization of Xaa-Pro bonds in proteins is a slow conformational interconversion process that has been shown to be the rate-limiting step in their refolding pathways.[15,16]

The combined effect of the structural peculiarity of Pro and the *cis*−*trans* isomerization of an Xaa-Pro bond has led the development of various mimetics and analogues that are intended to produce Pro-like reverse turns and gain better control of the *cis*−*trans* ratio.[17−24] Toward this end, pseudoprolines (ψPro) derived from Cys, Ser, and Thr as thiazolidine-4-

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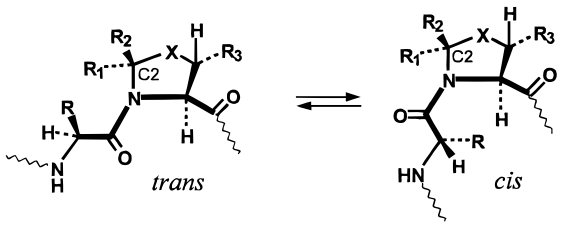

Figure 1. Structures of pseudoprolines (ψPro), 4-Thz and 4-Oxa, derivatized respectively from Cys and Ser/Thr, exhibiting *cis*−*trans* isomerization of the Xaa-ψPro bond. In this study, monosubstituted ψPro with a hydroxymethyl moiety at C2 are denoted as: **a**, thiaproline (*S*Pro), X = S, R_3 = H; **b**, oxaproline (*O*Pro), X = O, R_3 = H; **c**, 5-methyloxaproline (*O*Pro^{Me}), X = O, R_3 = CH_3.

carboxylic acid (4-Thz) and oxazolidine-4-carboxylic acid (4-Oxa) have been developed as Pro surrogates (Figure 1). These surrogates can be accessed by various carbonyl derivatives to afford the corresponding mono- and disubstituted derivatives at the C2 carbon to modulate the *cis*−*trans* isomers of the imidic Xaa-Pro bond.[22−24] Model dipeptides based on Xaa-ψPro showed that monosubstitution at C2 generally stabilizes *cis* conformation while substitution at C4 decreases *cis* isomers. Disubstitution at C2, such as dimethyl-4-Thz, produces >95% *cis* isomer.[22,23]

Thus far, incorporation of an unsubstituted or monosubstituted ψPro has been used in small peptides. The 4-Thz derivatives have been found in the synthesis of small bioactive peptides such as somatostatin,[25] angiotensin II,[26] oxytocin,[27] and various protease inhibitors.[28,29] In contrast, 4-Oxa and its substituted analogues are rarely used[30] because of synthetic difficulties. Although several methods have been developed based on either the acid or metal-catalyzed condensation of aldehydes or ketones to form ψPro,[31−33] these methods are conducted with small protected peptides and generally under conditions which are not suitable for the synthesis of large ψPro-containing peptides.

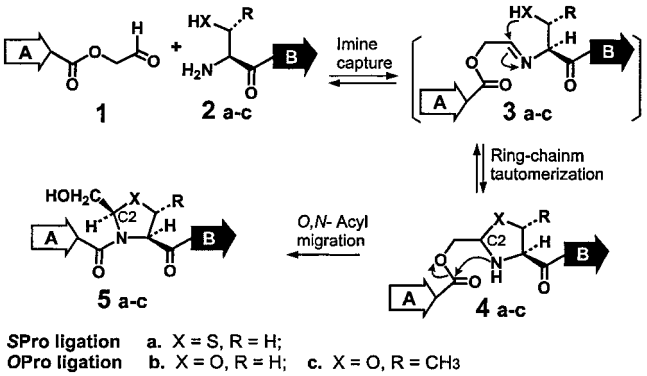

Figure 2. General scheme of ψPro ligation of an acyl segment **1** with an amine segment **2a**−**c** to form a thiaproline bond **5a** and an oxaproline bond **5b** or **5c**.

Over the past six years, we have explored orthogonal ligations of free peptides to form amide bonds in aqueous solutions, which afford products without further need for a deprotection step.[34−40] Thiaproline ligation is perhaps the first example that validates the principle of orthogonal ligation to produce spontaneously an amide bond by ligating two unprotected peptide segments (Figure 2).[34] It utilizes an acyl peptide segment bearing a glycoaldehyde ester (peptideyl-OCH_2CHO) **1** to capture an N-terminal nucleophile (Ntn)-Cys segment **2a**, through an imine **3a** which rapidly tautomerizes to a thiazolidine ester **4a**. The *O*-ester is then rearranged through an *O*,*N*-acyl shift to a monosubstituted thiaproline (*S*Pro) bond **5a** containing a hydroxymethyl moiety at C2 of the thiaproline ring. However, the stereochemistry of the newly created chiral center at C2 has not been determined. The *S*Pro bond formed at the ligation site is a proline mimetic that retains the amide backbone structure.[34,40] We envision that similar chemistry may be feasible for obtaining the analogous oxaproline (*O*Pro) with the Ntn-Ser **2b** or Ntn-Thr **2c** segment, thus providing another pathway to pseudoprolines based on imine capture, ring-chain tautomerization, and *O*,*N*-acyl migration **3**−**5** (Figure 2). However, under similar conditions, oxaproline ligation using Ntn-Ser or Thr segments **2b**,**c** has not been successful in aqueous buffers at pH 4 to 6.

The chemistry to obtain oxazolidine and thiazolidine through a 1,2-dinucleophile of an amino alcohol or amino thiol and a carbonyl is one of the best known reactions[41−44] and ring-chain tautomerization of both thiazolidine and oxazolidine has been extensively studied.[45−48] The capture step in the oxaproline

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ligation is also the formation of an imine. As opposed to the thiaproline ligation, the step from **3b**,**c** to **4b**,**c** is not favored in aqueous conditions, which is in accord with precedents in the literature.[45−48] Oxazolidine ring-chain tautomerization generally prefers the open imine form, and the oxazolidine has been found to be 10^4 times less stable than the analogous thiazolidine.[48] Furthermore, the ring closure of the imine to oxazolidine is a disfavored process in accordance to the Baldwin rule 1,5-endo-trigonal addition.[49] These factors account for the difficulty in achieving oxazolidine ligation in aqueous conditions. However, this difficulty can be viewed as an opportunity for site-specific orthogonal ligation between an Ntn-Cys and an Ntn-Ser or Ntn-Thr peptide to yield a thiaproline or oxaproline bond of multiple segments in a specific order without protecting groups.

The goals of this paper focus on the development of an orthogonal ligation method to afford a pseudoproline, particularly an oxaproline, at the ligation site, the regiospecificity of pseudoproline ligation, the stereochemistry of the newly created C2 carbon with the hydroxymethyl substitution, as well as the application of this newly developed chemistry to two-segment ligation to yield large peptides.

Results

Synthesis of Acyl and Amine Peptide Segments. Unprotected peptide building blocks consisting of an acyl segment bearing a glycoaldehyde at its C terminus and an amine segment carrying the Ntn-amino acid such as Ser, Thr, or Cys were prepared by stepwise solid-phase method.

Several methods for preparing the acyl segment bearing a C-terminal glycoaldehyde have been developed, including an indirect method using an $n + 1$ strategy that either chemically or enzymatically couples a single amino acid derivative as an acetal with an unprotected peptide ester or thioester.[34,40] A more convenient method for obtaining the peptidyl glycoaldehyde **8** from a peptide glyceryl ester precursor **7** has also been developed using an acetal resin **6** (Figure 3).[50] This method requires Fmoc/tBu chemistry because the acetal is unstable to a strong acid such as anhydrous TFA which is used for deprotection in the tBoc/Bzl strategy for peptide synthesis. After the TFA-mediated cleavage of the peptidyl ester from the acetal resin **6**, the unprotected glyceryl ester segment **7** carrying a 1,2-diol moiety was then transformed into aldehyde **8** by periodate oxidation under aqueous conditions at pH 2 to 7. This two-step conversion scheme has the advantage of avoiding exposure of the sensitive aldehyde moiety to anhydrous TFA and the subsequent purification steps. All acyl segments **8a**−**d** bearing a glycoaldehyde were prepared by the two-step method.

The amine segments **10a**−**l**, including those containing Ser **10a**, Thr **10b**, and Cys **10c** at the N termini, were achieved by tBoc/Bzl chemistry to afford completely free peptides after cleavage by HF from the *p*-methylbenzhydrylamine resin **9** (Figure 3). All unprotected building blocks, the acyl segments **8a**−**d** and amine segments **10a**−**l**, were purified by HPLC and confirmed by TOF-MALDI mass spectrometry and amino acid analysis.

Conditions for Oxaproline Ligation. Previously, we found that oxaproline ligation could be achieved in nearly anhydrous

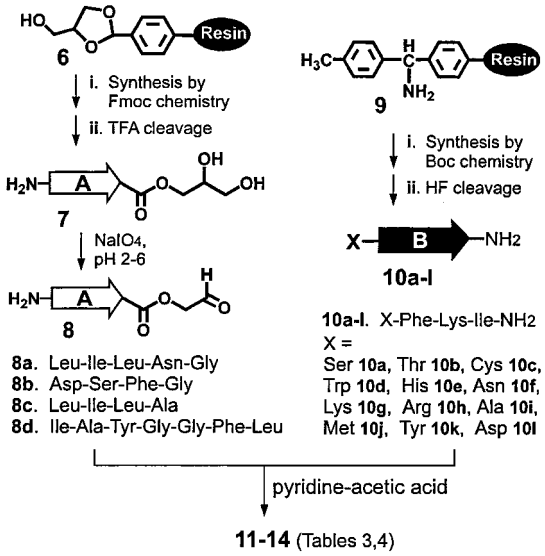

Figure 3. Synthetic scheme of peptide glycoaldehyde esters **8a**−**d** from an acetal resin support **6** (left) and amine segments **10a**−**l** from an MBHA resin **9** (right).

conditions of 90% DMSO and 10% aqueous buffers but at a very slow rate.[51] Since unprotected peptide building blocks were used for the oxaproline ligation, more desirable and compatible polar solvents such as pyridine, acetic acid, and low-molecular-weight alcohol were studied. Similar to the analogous thiaproline ligation, the initial step of oxaproline ligation is known to be imine formation, which is both acid- and base-catalyzed, with the optimal pH in the range of 4 to 6.[34] Pyridine−acetic acid mixtures would provide the desired pH range and are known to be excellent solvent combinations for dissolving unprotected peptide segments. Furthermore, both solvents are volatile and can be removed by lyophilization. Based on this rationale, we explored the rates of oxaproline ligation between Leu-Ile-Leu-Asn-Gly-OCH_2CHO **8a** and Ser-Phe-Lys-Ile-amide **10a** in seven solutions ranging from 0 to 100% pyridine in acetic acid (data not shown). A mixture of pyridine−acetic acid at a 1:1 molar ratio was found to be suitable to mediate oxaproline ligation in 78% yield after 45 h at 20 °C. Under similar conditions, other mixtures with a 2:3, 2:1, 1:2 and 4:1 molar ratio of pyridine−acetic acid also resulted in acceptable yields of 74, 71, 69 and 62%, respectively. However, pyridine alone gave only 49% yield, while no observable product was found with acetic acid alone. The ligation product **11a** containing an oxaproline with a hydroxymethyl substitution at C2 carbon was identified by both chemical and spectroscopic methods and will be discussed later in detail.

We then studied this pyridine−acetic acid mixture (1:1, mol/mol) under various conditions including temperature and cosolvent mixture to accelerate the ligation between **8a** and **10a** (Table 1). Elevating the temperature from 20 °C to 40 °C accelerated the reaction about 2−3-fold. Polar cosolvents such as TFE accelerated the reaction 2−3-fold and > 70% of ligation was achieved at 6.5 h in 50% TFE and 50% pyridine−acetic acid (1:1, mol/mol). Aprotic polar solvents such as DMF slightly retarded the ligation rate as compared to pyridine−acetic acid (1:1, mol/mol) alone, while the aqueous environment of 50% H_2O was strongly inhibitory.

We also investigated the effects of other amine bases in acetic acid on the ligation between Asp-Ser-Phe-Gly-OCH_2CHO **8b**

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Table 1. Solvent Effect on the Oxaproline Ligation between **8a** and **10a**[a]

	yield (%)			
solvent	2.5 h	6.5 h	20 h	45 h
pyridine/HOAc	42.1	50.5	69.2	75.6
TFE	59.8	76.6	83.1	83.7
DMF	10.5	28.4	49.6	55.9
H_2O	8.7	7.6	3.1	2.1

[a] 50% solvent in 50% pyridine−HOAc (1:1, mol/mol). TFE, trifluoroethanol; DMF, *N*,*N*-dimethylformide; HOAc, acetic acid.

Table 2. Effect of Base on the Oxaproline Ligation between Asp-Ser-Phe-Gly-OCH_2CHO, **8b**, and Ser-Phe-Lys-Ile-amide, **10a**, in 50% TFE and 50% Base/Acetic Acid (1:1, mol/mol)[a]

		yield (%)		
base	pK_a	6 h	10.5 h	24 h
pyridine	5.25	63.8	70.6	71.8
DMAP		66.8	67.4	69.8
imidazole	6.95	79.4	82.2	83.6
NMIm	6.95	80.4	85.2	88.4
DIEA	10.60	38.1	49.5	66.1
piperidine	11.32	8.9	15.4	16.8

[a] DMAP, 4-(dimethylamino)pyridine; NMIm, *N*-methyl imidazole; DIEA, *N*,*N*-diiospropylethylamine.

Table 3. Regiospecificity of N-Terminal Amino Acid in the Ligation between the Acyl Segment L-I-L-N-G-OCH_2CHO **8a** and Different Amine Segments X-F-K-I-amide **10a**−**k** in Pyridine−Acetic Acid (1:1, mol/mol)

8a + 10a-l ⟶ H-**L-I-L-N-G-**[ψ**P**]-**F-K-I-**NH_2 **11a-f**

[ψ**P**] in product[a]	segment **X** =	yield (%) 36 h	relative rate
[***O*P**] **11a**	S **10a**	79.6	1
[***O*P**^{Me}] **11b**	T **10b**	74.2	0.95
[***S*P**] **11c**	C **10c**	95.3	>1000[b]
11d	W **10d**	77.9	1.2
11e	H **10e**	73.3	1.1
11f	N **10f**	44.4	0.3
	K **10g**	<1	
	R **10h**	<1	
	A **10i**	<1	
	M **10j**	<1	
	D **10k**	<1	
	Y **10l**	<1	

[a] [***O*P**], 2-hydroxymethyl-oxaproline; [***O*P**^{Me}], 2-hydroxymethyl, 5-methyl-oxaproline; [***S*P**], 2-hydroxymethyl-thiaproline; two isomeric products for **11d** and three isomeric products for **11e** and one of **11f** were obtained; for their possible structures, see ref 51. [b] Rate for thiazolidine ester formation (t_R = 31.9 min), but the rate for amide product (t_R = 29.9 min) through *O*,*N*-acyl transfer is 0.9 relative to **11a**.

and **10a** (Table 2). Weak bases such as imidazole, *N*-methylimidazole (NMIm) or 4-(dimethylamino)pyridine (DMAP) in a 1:1 molar ratio with acetic acid gave results similar to pyridine, while strong bases such as the tertiary and secondary alkylamine were not effective. These results are consistent with the importance of using a proper solvent mixture to achieve imine capture for oxaproline ligation.

Regiospecificity, Orthogonality, and Steric Factors. To determine the regiospecificity of oxaproline ligation in the pyridine−acetic acid mixture, the acyl segment **8a** bearing an glycoaldehyde ester was chosen to react with various amine segments **10a**−**l** bearing either Ntn- or non-Ntn-amino acids to form **11a**−**l** (Table 3). In the amine segments, an internal lysine residue is present to serve as a side-chain amine to test the regiospecifity of the ψPro ligation. No ligation products were observed with non Ntn-amine segments **10i**−**l** such as Ala **10i**, Met **10j**, and Tyr **10k** as well as with those Ntn-amine segments bearing acidic side chains such as Asp **10l**. Ntn-Lys **10g** and Ntn-Arg **10h** were also found to be excluded from the ligation reaction, probably because of the low pK_a of the pyridine−acetic acid condition and the protonated state of the strongly basic side chains of lysine and arginine. However, ligation reactions were observed with those Ntn-segments **10a**−**f**. Rate analysis showed that ligation occurred almost spontaneously with Ntn-Cys segment **10c**, rapidly with those Ntn-amino acid segments such as Ser **10a**, Thr **10b**, Trp **10d**, and His **10e**, but very slowly with Asn **10f**.

The Ntn-Ser **10a**, Ntn-Thr **10b**, or Ntn-Asn **10f** segment provided a predominant single ligation product. However, Ntn-Trp **10d** segment gave two, while Ntn-His **10e** yielded three major isomeric products. The stereochemistries of these isomeric products from **10d**,**e** have not been characterized at this time. However, these results also show that imine ligation is fairly general and Ntn amino acids containing other weak base nucleophiles could participate in ligation similar to Ntn-Cys, -Ser, and -Thr.

It should be noted that there are two α-amines and an ϵ-amine in the reactants of **8a** and **10a**−**l**. Thus, a number of products could be obtained including oligomers, cyclized products, or cyclodimerizations through intramolecular ligation of **8a**, and side-chain acylation of ϵ-amine of lysine. On the basis of the combined use of HPLC and MS analysis, no detectable side products stemming from oligmerization or cyclization of **8a** were found. End group analysis was used to determine the regiospecificity between α- and ϵ-amines in **11a**−**c**. The results revealed that the ligation occurred exclusively on the N-terminal α-amines of **10a**−**c**. These results suggest that the reaction of pseudoproline ligation is highly regiospecific with an Ntn-amino acid. However, minor side products, usually <10%, were detected. These include hydrolysis of the glycolaldehyde **8a** to the corresponding to free carboxylic acid and transesterification to the TFE ester when TFE was used as a cosolvent.

Competitive experiments were employed to establish the orthogonality between thiaproline ligation and oxaproline ligation. Under aqueous conditions at pH 4 to 6, thiaproline ligation product *Z*-Ala-*S*Pro-OMe **17f** was exclusively obtained between *Z*-Ala-OCH_2CHO **15a** and Cys-OMe **16c** in the presence of Ser-OMe **16a** and Thr-OMe **16b**. Similarly, when an acyl segment **15a** and three amine segments **16a**−**c** were mixed in an equimolar ratio in pyridine−acetic acid (1:1, mol/mol), only the thiaproline ligation between **15a** and **16c** to afford **17f** was observed. However, in the absence of **17c**, the oxaproline ligation between **15a** and **16a**,**b** went smoothly in pyridine−acetic acid. Under the condition of aqueous buffers or pyridine−acetic acid mixture (1:1, mol/mol), competitive experiments using unprotected peptide segments Leu-Ile-Leu-Asn-Gly-OCH_2CHO **8a**, Ser-Phe-Lys-Ile-NH_2 **10a**, Thr-Phe-Lys-Ile-NH_2 **10b**, and Cys-Phe-Lys-Ile-NH_2 **10c** gave similar results. These results are consistent with the literature[48,51] that the formation of thiazolidine **4a** is >1000-fold faster than that of oxazolidine **4b**,**c**. Thus, thiaproline and oxazoproline ligations are semi-orthogonal, i.e., thiaproline ligation can be performed in the presence of Ntn-Ser/Thr or other N-terminal amino acid-containing peptide segments under either aqueous conditions or pyridine−acetic acid mixture (1:1, mol/mol). However, regioselective oxaproline ligation can be carried out in the pyridine−acetic acid conditions in the absence of Ntn-Cys, -His, and -Trp peptide segments.

Finally, the steric influence of the C-terminal amino acids of the acyl segments on oxaproline ligation was also studied (Table

Table 4. Effect of C-Terminal Amino Acids of Different Acyl Segments **8a**−**d** on the Oxaproline Ligation with Amine Segments **10a** and **10b** in 50% TFE and 50% Pyridine−Acetic Acid (1:1, mol/mol) for 30 h

product[a]		ligating segments	yield (%)[b]	relative rate
LILN**G**-[***O*P**]-FKI	**11a**	**8a** + **10a**	80.4	1
LILN**G**-[***O*P**^{Me}]-FKI	**11b**	**8a** + **10b**	76.2	0.95
DSF**G**-[***O*P**]-FKI	**12a**	**8b** + **10a**	83.7	1
DSF**G**-[***O*P**^{Me}]-FKI	**12b**	**8b** + **10b**	81.1	0.95
LIL**A**-[***O*P**]-FKI	**13a**	**8c** + **10a**	66.9	0.8
LIL**A**-[***O*P**^{Me}]-FKI	**13b**	**8c** + **10b**	65.3	0.75
IAYGGF**L**-[***O*P**]-FKI	**14a**	**8d** + **10a**	54.8	0.55
IAYGGF**L**-[***O*P**^{Me}]-FKI	**14b**	**8d** + **10b**	51.9	0.50

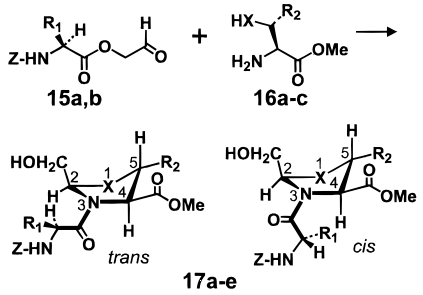
[a] C-terminal amino acids of the acyl segments **8a**−**d** are bold and underlined; [***O*P**] and [***O*P**^{Me}] see Table 3. [b] Yield was calculated from HPLC.

4). We have found previously significant steric effect of the C-terminal (CT) amino acids in thiaproline ligation that undergoes a 5,5-bicyclic intermediate during *O*,*N*-acyl migration.[34] The acyl segments **8a** and **8b** with nonhindered CT-Gly gave higher yields than **8c** with CT-Ala and **8d** with CT-Leu. Furthermore, kinetic study showed that the ligation rate of Ser-OMe **16a** with *Z*-Val-OCH_2CHO **15b** was found to be 6-fold slower than that with *Z*-Ala-OCH_2CHO **15a**, and 10-fold slower than that with *Z*-Gly-OCH_2CHO **15c**.

Product Characterization and the Absence of Ester Intermediates in Oxaproline Ligation. A striking difference was observed between the oxaproline and thiaproline ligations. In thiaproline ligation, two stable *R*,*S* epimers of the thiazolidine esters **4a** were observed. The two esters of *R*,*S* epimers at the C2 stereocenter then underwent *O*,*N*-acyl migration to form a single amide product **5a** (detected by HPLC). In oxaproline ligation, no oxazolidine esters **4b**,**c** as stable intermediates were detected by HPLC in various pyridine−acetic acid ratios (1:0 to 0:1, mol/mol, data not shown). Chemical and spectroscopic analyses were employed to determine the ligation products, since the isomeric ester and amide were unable to be distinguished by MS analysis.

Esters are generally susceptible to aminolysis by concentrated hydroxyamine or basic hydrolysis, while amides are usually stable under the same conditions. Treatment of oxaproline ligation products **11a**,**b** and **12a**,**b** and thiaproline ligation product **11c** with 1 M hydroxylamine at pH 9 or 0.1 M LiOH for 20 h failed to give the aminolysis or hydrolysis products. In contrast, thiazolidine ester (**4a** in Figure 2) intermediate of **11c** gave the expected aminolysis and hydrolysis products. More importantly, no ester bond peak in the range 1720−1800 cm^{-1} was observed from the FT-IR spectra of the ψPro-containing peptides **11a**−**c** and **12a**,**b**. In addition, treatment of *Z*-Ala-ψPro-OMe **17a**−**e** (see Table 5), which were ligation products of **15a**,**b** and **16a**−**c**, under different aqueous alkaline buffers at pH 9.1 to 10.8 for 72 h, afforded only the hydrolyzed product of the methyl ester. Finally, **17a**−**e** were confirmed to be amide rather than ester products by ^{1}H NMR studies. For example, the 1D ^{1}H NMR of the thiazolidine ester intermediate of **17e** showed a peak at 8.12 ppm indicating the azomethyl proton of the open imine form resulting from the tautomerization of the thiazolidine.[46,48] None of the oxaproline ligation products **17a**−**d** or the amide bond product **17e** of thiaproline ligation showed any peak at the region 8.0−9.0 ppm in their 1D ^{1}H NMR spectra. Thus, these results confirmed that only the amide products from oxaproline ligation were obtained. Furthermore, only the *R* epimer at C2 was found.

Table 5. Stereochemistry of Model Dipeptides *Z*-Xaa-ψPro-OMe **17a**−**e** [a]



compd	structure	X	R_1	R_2	C2	*cis*/*trans*
17a	Z−A-[***O*P**]-OMe	O	Me	H	R	68:32
17b	Z−V-[***O*P**]-OMe	O	^{i}Pr	H	R	56:44
17c	Z−A-[***O*P**^{Me}]-OMe	O	Me	Me	R	54:46
17d	Z−V-[***O*P**^{Me}]-OMe	O	^{i}Pr	Me	R	43:57
17e	Z−V-[***S*P**]-OMe	S	^{i}Pr	H	R	40:60

[a] **15a**, R_1 = Me; **15b**, R_1 = ^{i}Pr; **16a**, X = O, R_2 = H; **16b**, X = O, R_2 = Me; **16c**, X = S, R_2 = H; [***O*P**], [***O*P**^{Me}] and [***S*P**] see Table 3.

Racemization was a concern because of the prolonged period for pseudoproline ligation. N^{α}-(2,4-Dinitro-5-fluorophenyl)-L-valinamide (Marfey's reagent)[52] was used for evaluating the racemization because it can distinguish the D,L-diastereomers in the selected pseudoproline ligation products **17a**,**c**. HPLC analysis showed that **17a** gave 0.3% D-Ala and 3.9% D-Ser, and **17c** gave 0.4% D-Ala and 5.4% D-Thr. The control experiments in which L-amino acids were subjected to acid-hydrolytic conditions and derivatiztion by Marfey's reagent similar to **17a**,**c** showed that Ala, Ser, and Thr gave 0.4, 5.3, and 5.8% D-diastereomers, respectively. These results suggest that oxaproline ligation in the pyridine−acetic acid mixture (1:1, mol/mol) does not cause significant racemization.

Stereochemistry at C2 Carbon of ψPro Ring and *cis*−*trans* Conformational Ratio of Xaa-ψPro Bond. To determine the stereochemistry of the newly created C2-stereocenter of oxaproline and the *cis*−*trans* conformational preference of the oxaproline containing peptides, five dipeptides **17a**−**e** were examined by the 2D ^{1}H NMR studies at 20 °C. For each sample, two sets of independent NMR data were obtained based on the assignment of the 2D (DQF) COSY, indicating only two *cis*−*trans* isomers with the *R* epimer at the C2 carbon was found in each compound. Table 5 summaries the C2 stereochemistry of the oxaproline ring and the *cis*−*trans* ratios of the Xaa-ψPro bonds in compounds **17a**−**e**.

The C2 stereochemistry of the oxaproline ring was determined by the NOE cross-peak between the C2−H and C4−H of the oxaproline ring.[22,23,53−56] In **17a**−**e**, a comparatively strong NOE cross-peak between the C2−H and C4−H was observed in both *cis* and *trans* isomers. In contrast, no such a cross-peak between

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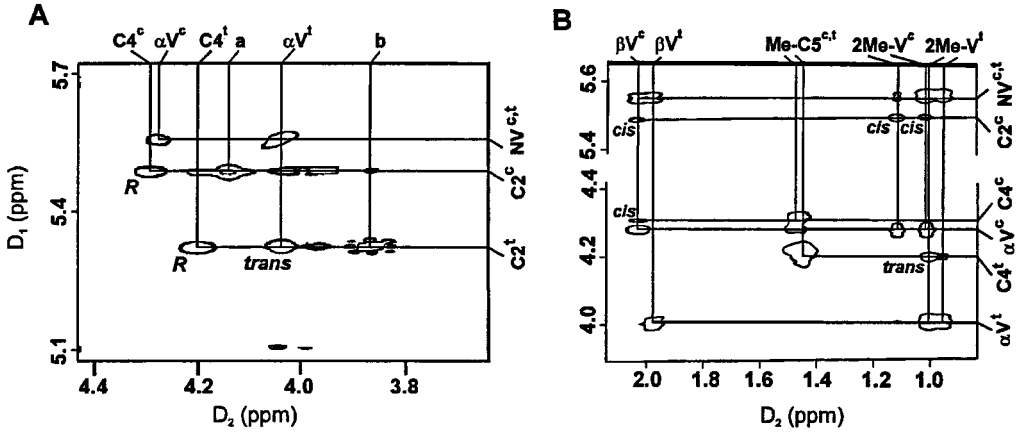

Figure 4. Expanded regions of the 2D 1H NMR NOESY spectrum of **17d** showing the assignment of C2 stereocenter and the *cis* and *trans* isomers. The NOE cross-peaks are labeled with *R*, *cis*, and *trans* for the C2 *R*-epimer, *cis* and *trans* conformers, respectively. **a** and **b** in Figure 4A indicate the NOE cross-peaks between C2 and C2-hydroxymethyl protons.

C4−H and C2-hydroxymethyl protons was found. These results indicate that the C2 stereocenter is an *R* rather than an *S* epimer. Other observable NOE cross-peaks also support that C2 centers in **17a**−**e** are *R* epimers. These include the NOE cross-peaks between the C2-hydroxymethyl protons and the *axial* C5−H but no NOE peaks between the C5−H and C2−H.

The *cis*−*trans* isomer ratios of the Xaa-ψPro bond was measured by conventional NOE experiments.[54,55] A typical pattern[56] of the αH_i−C2−H_{i+1} (i = Xaa, $i + 1 = \psi$Pro) NOE cross-peak was observed in **17a**−**e** (Figure 4). This pattern, i.e., the αH_i−δH_{i+1} in a proline containing peptide, is characteristic of the *trans* Xaa-ψPro bond.[22−24,56] The *cis* isomer in each compound is characterized by the NOE cross-peak between the βH_i and C4−H_{i+1} (Figure 4).[23,56] In **17b**, **17d**, and **17e**, NOE cross-peaks between C2−H_{i+1} and βH−Val or CH_3−Val were also observed to confirm the assignment of *cis* isomers (Figure 4). In addition, the NOE cross-peaks between the αH_i and C2$_{i+1}$-hydroxymethyl protons were observed in *cis* isomers. However, the *cis*−*trans* ratios vary depending on the side chains around the pseudoproline bonds.[22−24] For example, Val at the *i* position has an unfavorable effect on *cis* conformation because of the steric hindrance between its large side chain and C4−H_{i+1}.

Synthesis of Bactenecin 7 Analogues by Two-Segment Ligation. Bac 7 is a highly cationic antimicrobial peptide isolated from the large granules of bovine neutrophils.[57] It contains 59 amino acid residues with molecular weight about 7 kD, characterized by a high content of proline (47%) and arginine (29%) and three tandem repeats of a tetradecamer.[58] This exceptional Arg-rich peptide can also provide a test for oxaproline ligation in pyridine−acetic acid because the guanidine side chain is susceptible to side reaction with aldehydes. Furthermore, it is known that proline-rich sequences such as Bac 7 are difficult to obtain by stepwise synthesis due to the propensity of the proline residue to cleave off as a cyclic dipeptide. To obtain synthetic analogues for studying the mechanism of antimicrobial activity of Bac 7 which is not known yet, we undertook the synthesis of Bac 7 analogues **20a**, **20b**, and **20c** by a two-segment ligation strategy.

The ligation site was at Pro25 where the Gly-Pro sequence offers the least steric hindrance (Figure 5). A 24-amino acid-containing acyl segment **18** with a glycoaldehyde ester at the C terminus was synthesized on an acetal resin **6** as described

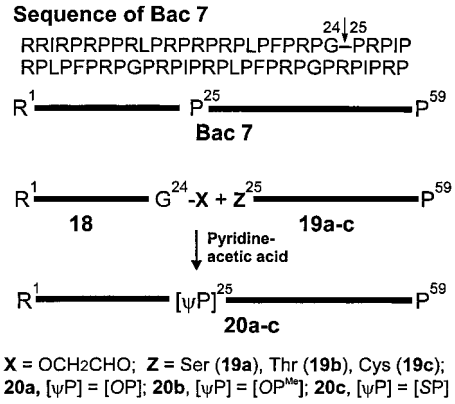

Figure 5. Orthogonal ligation of Bac 7 analogues **20a**, **20b**, and **20c** at Gly^{24}-Pro^{25} indicated by an arrow between acyl segment **18** and amine segments **19a**, **19b**, and **19c**.

in Figure 3. Three 35-amino acid-containing-segments with Ntn-Ser 19a, -Thr 19b, and -Cys 19c were synthesized by tBoc/Bzl chemistry. Peptide segments **18**, **19a**, **19b**, and **19c** were purified by HPLC and identified by TOF-MALDI MS and amino acid analysis. The purified peptide segments were readily soluble in the pyridine−acetic acid mixture suggesting that this condition is a suitable solvent. The oxaproline ligations were carried out in pyridine−acetic acid mixtures (1:1, mol/mol), and in each case, the amine segment **19a** or **19b** was used in slight excess. The ligation reaction was relatively slow and required >35 h for completion. However, HPLC monitoring showed that the reaction proceeded cleanly and predominantly gave a single product together with the unreacted starting materials and hydrolyzed acyl segment **18** as observable byproducts (Figure 6A, B). The thiaproline ligations between **18** and **19c** were performed in both pyridine−acetic acid (1:1, mol/mol) and aqueous conditions with the acyl segment **18** slight excess. In contrast to oxaproline ligation, the HPLC patterns of thiaproline ligations (Figure 6C, D) were complicated by the presence of the *R*,*S*-ester intermediates **20d**,**e** which converted to a single amide product **20c** after 20 h in aqueous buffer at pH 6.2 or after 40 h in more acidic condition of pyridine−acetic acid (1:1, mol/mol).

The ligation sites of **20a**−**c** were shown to be amide bond due to the absence of the ester peaks at 1710−1780 cm^{-1} in their FT-IR spectroscopies. They were further confirmed by the treatment with 1.0 M H_2NOH at pH 9.1 and 0.1 M LiOH for 10 h. Under these conditions, no hydrolysis or aminolysis product was detected by HPLC. Furthermore, no evidence of

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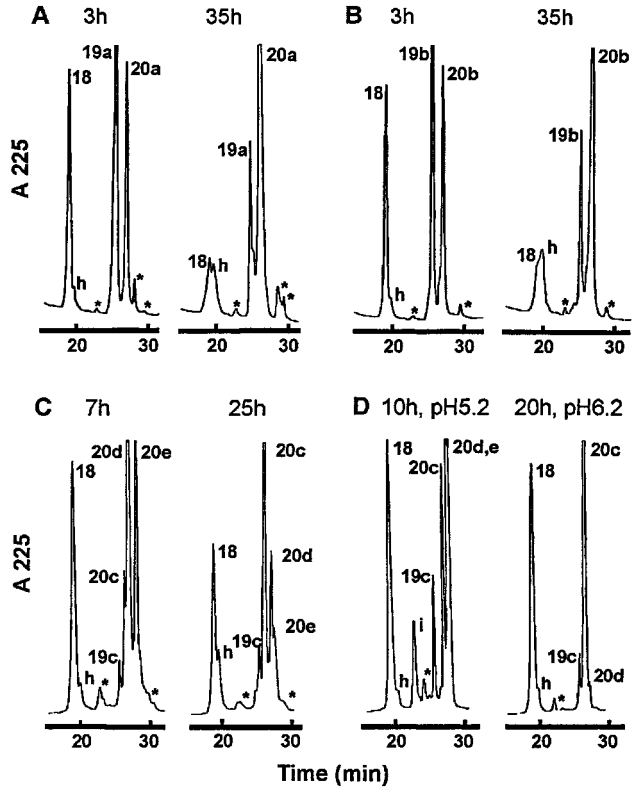

Figure 6. HPLC profiles of oxaproline and thiaproline ligations. A and B, oxaproline ligations of **18** with **19a** and **19b** in pyridine−acetic acid (1:1, mol/mol) to form **20a** and **20b**. C, thiaproline ligation between **18** and **19c** in pyridine−acetic acid (1:1, mol/mol). First, two intermediates as thiazolidine esters **20d**,**e** were formed, then **20d**,**e** transferred to the amide bond product **20c** under the same condition. D, thiaproline ligation between **18** and **19c** in aqueous buffers, first at pH 5.2 for 10 h to form thiazolidine esters **20d**,**e**, and then the *O*,*N*-acyl transfer was carried out at pH 6.6 for 20 h to form the amide bond product **20c**. *, impurities from pyridine. **h**, hydrolysis product of **18**. **i**, possible imine intermediate (**3c** in Figure 2).

Table 6. Summary of the Synthesis of Bac 7 Analogues **20a**, **20b**, and **20c**

product	segments	ligation site [ψ**P**]	yield (%)
20a	**19a** + **18**	[***O*P**][25]	82.4
20b	**19b** + **18**	[***O*P**^{Me}][25]	83.0
20c	**19c** + **18**	[***S*P**][25]	84.1

Arg modification by the glycoaldehyde as a branched peptide was found. Both the oxaproline and thiaproline ligations gave excellent yields ranging from 82 to 85% after 40 h (Table 6).

Furthermore, to examine the Pro surrogates derived from pseudoproline ligation on the antimicrobial activity of Bac 7, Bac 7 and three analogues **20a**−**c** were assayed against a Gram positive (Staphylococcus aureus) and a Gram negative (Escherichia coli) species using the radial diffusion method.[59] The results (Table 7) showed very close activities between Bac 7 and its three analogues **20a**−**c**. The detailed results of structure−activity studies will be published elsewhere.

Discussion

The 1,2-amino alcohol of an N-terminal serine and threonine as well as the 1,2-amino thiol of an N-terminal cysteine represent unique combinations of dinucleophiles which are not found elsewhere in a free peptide. Furthermore, they are known to react selectively with aldehydes under very mild conditions to afford oxazolidines or thiazolidines, a criterion important for the orthogonal ligation of free peptide segments.[34] This paper confirms that the Ntn-Cys and Ntn-Ser/-Thr can be exploited successfully for regiospecific oxaproline and thiaproline ligation to afford monosubstituted ψPro at the ligation site using unprotected peptides.

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Table 7. Comparison of Antimicrobial Activity of Bac 7 and Three ψPro Analogues **20a**−**c** from Pseudoproline Ligation

	MIC (μM)[a]	
peptide	*E. coli*	*S. aureus*
Bac 7	0.24	0.23
20a	0.31	0.29
20b	0.23	0.22
20c	0.27	0.28

[a] MIC, minimal inhibition concentration, obtained by radial diffusion assay with underlay gel containing 1% agarose in 10 mM phosphate buffer.

Although the thiaproline ligation proceeds smoothly in aqueous solutions at pH 4 to 6, no reaction is observed for oxazolidine ligation of Ntn-Ser or Ntn-Thr peptides under the same conditions. In contrast, oxaproline ligation requires the use of nearly anhydrous conditions.[51] We have found that pyridine−acetic acid mixtures for oxaproline ligation can achieve reasonable rates, minimize side reactions and solubilize free peptide segments.

Thiaproline and oxaproline ligations are semi-orthogonal, i.e., the former ligation of an Ntn-Cys peptide can be performed in the presence of an Ntn-Ser or Ntn-Thr peptide. Such orthogonality may be useful for assembling multiple free peptide segments based on the regioselectivity of different Ntn-amino acids without the need for a protection scheme.

Thiaproline ligation in pyridine−acetic acid conditions or aqueous buffers proceeds in a two-step-reaction scheme with the isolated *R*,*S* epimers of the thiazolidine esters as stable intermediates (Figures 2 and 5). In the first step, imine capture and ring-chain tautomerization are fast. The subsequent step of *O*,*N*-acyl transfer of the thiazolidine esters to form the amide *S*Pro bonds is the slow step which can proceed smoothly under acidic conditions because of the low pK_a (6.24) of thiazolidine.[41] This step is also pH dependent and is significantly accelerated in more basic conditions as shown in the thiaproline ligation of **18** and **19c**. However, in oxaproline ligation no oxazolidine ester intermediate was detected by HPLC. A plausible explanation is that the oxazolidine ester is unstable, $>10^4$ fold less stable than its analogous thiazolidine ester,[48] and transfers into the stable amide *O*Pro bond or equilibrates back to the starting materials.

In pseudoproline ligation, the stereochemistry of the newly created stereocenter at C2 of the *O*Pro ring is a concern. Using *Z*-Xaa-ψPro(R)-OMe **17a**−**e** as models, only one epimer (>95%) was detected by HPLC and NMR. In each compound, the single C2 epimer of the oxaproline was assigned an *R* configuration by 2D NOESY (Table 5). Thus, the oxaproline ligation under our described conditions is not only regiospecific but also stereospecific and does not produce diastereomers during synthesis.

Two plausible mechanisms can be advanced to account for the stereospecific nature of the oxazolidine ligation. First, the oxazolidine formation is stereospecific, which results in only the C2-*R* epimer. This single epimer undergoes the *O*,*N*-acyl migration to give diastereomeric selectivity to the ligation reaction. The second mechanism is that the oxazolidine formation is not stereospecific but the *O*,*N*-acyl migration is ste-

reospecific because the C2-*R* epimer proceeds at a faster rate than the C2-*S* epimer. The unreacted C2-*S* epimer undergoes an equilibration to a mixture of *R*,*S* epimers through oxazolidine ring-chain tautomerization. Our experiments as well as literature precedents generally support the second proposed mechanism.

Condensation reactions between a nonchiral aldehyde component and chiral 1,2-amino alcohols or 1,2-amino thiols such as Ser, Thr, or Cys generally produce a mixture of epimers at the C2 atom in N-unsubstituted oxazolidine or thiazolidine derivatives. However, upon acylation or methoxylcarbonylation, a single C2 diastereomer *cis* to the C4-configuration of Ser, Thr, or Cys has been found to be the only observed product.[54,55,60] It has been reported that Cys derivatives react with benzaldehydes and *p*-tolualdehyde to produce a mixture of C2 thiazolidine epimers. Upon acetylation by acetic anhydride in pyridine at 25 °C, however, only a single diastereomer is obtained.[55] To account for this observation, it is proposed that the epimerization of the C2 atom occurs at the Schiff base intermediate and the acetylation of the *R*-(C2)-epimer is fast compared with the *S*-(C2) epimer. A similar mechanism for stereospecific control can be invoked for both oxaproline and thiaproline ligations, i.e., the first step of ester formation produces a mixture of epimers at C2 and the *O*,*N*-acyl migration of the *R*-epimer is fast compared with the *S*-epimer.

The pseudoprolines obtained in our syntheses are monosubstituted with a hydroxymethyl side chain at the C2 carbon. The C2-substitution enhances the *cis* isomer content of the Xaa-ψPro bonds. Monosubstitution at C2 with a methyl group increases the *cis* isomer to 38% compared with 18% obtained from the unsubstituted ψPro surrogates derived from Cys or Ser.[23,24] However, substitution at the C5 decreases the *cis* isomer,[22] as shown in our study, from 68% in **17a** to 60% in **17c** and 56% in **17b** to 43% in **17d**. The trend showing that the monosubstituted ψPro in our series ranges from 40% to 68% is consistent with the literature.[22−24] The slight increase in our observed result could be due to the nature of the C2 substitution which may form a hydrogen bond in the *cis* Xaa-ψPro isomer in $CDCl_3$ used in the NMR experiments.

In general, we have found that replacing Pro with *S*Pro and *O*Pro based on ligation scheme does not result in significant changes in biological activity, as demonstrated in the synthesis of three ψPro analogues **20a**−**c** which exhibit identical antibacterial activities as the native peptide Bac7. Similar results have been observed in the synthesis of HIV-1 protease *S*Pro analogues.[40]

The C2-hydroxymethyl *O*Pro and *S*Pro derivatives are also amino acid chimeras of proline and γ-hydroxyl amino acid. These types of amino acids have recently been explored by Marshall et al. in the synthesis of peptide hormone mimetics of the "bioactive form" of angiotensin II.[17] Furthermore, the use of different acyl segments bearing other aldehydes and ketones may yield other substituted ψPro at the ligation site to control the *cis*−*trans* isomers. Although we have focused our attention to peptide synthesis, various applications through non-peptides or peptide mimetics suitable for combinatorial library syntheses can also be envisioned. In conclusion, pseudoproline ligation provides a convenient approach to the synthesis of a ψPro-containing peptide using a 1,2-dinucleophile with a glycoaldehyde ester under mild conditions and holds promise for other applications for the synthesis of peptide mimetics using the orthogonal approach.

(60) Seebach, D.; Aebi, J. D. *Tetrahdron Lett.* **1984**, *25*, 2545−2548.

Experimental Section

General. 1D ^{1}H NMR was obtained on Bruker AC 400 instrument. Analytical HPLC was run on a Shimadzu 10A system using Vydac C18 column (4.6 × 250 mm, 5 μm) with a flow rate of 1.0 mL/min, monitored at 225 nm. Preparative HPLC was performed on a Waters 600 equipment with a Vydac C18 column (22 × 250 mm). All HPLC was carried out with reversed phase linear gradient of buffer A, 0.05% TFA in H_2O, and buffer B, 60% CH_3CN in H_2O with 0.04% TFA. Low-resolution mass spectra (LRMS) were obtained by MALDI-TOF method on a PerSeptive Biosystems Voyager Elite 2 instrument and high-resolution mass spectrometries (HRMS) were determined by ESI Fourier transform ion cyclotron resonance method (ESI-FT-ICR)[61] on a PerSeptive Biosystems Mariner instrument. FT-IR spectra were recorded on an ATI Mattson Genesis Series FTIR spectrometer with the sample filmed on CaF_2 surface.

2D NMR Spectroscopy. Spectra were recorded at 500.13 MHz using Bruker DRX 500 instrument. Chemical shifts were calibrated by the residual $CHCl_3$ signal as reference at 7.26 ppm. Phase sensitive NOESY spectra used for resonance assignments were recorded using the TPPI procedure with mixing time of 400 ms. In the d_1 dimension, 1024 real data points were collected using 16 acquisitions per FID with a 1.5 s relaxation delay; 2048 real data points were obtained in the d_2 dimension. Scalar connectivities were recorded from 2D double quantum filtration (DQF) COSY experiments. Complete proton resonance assignments were fulfilled with the (DQF) COSY experiments, in some cases, with the aid of ^{1}H−^{13}C HSQC experiments. All NMR data were transferred to Indigo II workstation and processed using FELIX software.

Solid-Phase Peptide Synthesis. The amine peptide segments **10a**−**l**, **19a**, **19b,** and **19c** were synthesized on resin using Boc/Bzl and HBTU/HOBt strategy.[62] The peptides were cleaved from the resin by anhydrous HF−anisole (95:5, v/v), and then purified by HPLC. The amino acid analysis and MS gave the desired results. **10a**, Ser-Phe-Lys-Ile-NH_2, t_R = 11.2 min (20−60% B), HRMS *m*/*e* 493.3147 (M + H^+, $C_{24}H_{41}N_6O_5$ requires 493.3138); **10b**, Thr-Phe-Lys-Ile-NH_2, t_R = 13.4 min, HRMS *m*/*z* 507.3291 (M + H^+, $C_{25}H_{43}N_6O_5$ requires 507.3295); **10c**, Cys-Phe-Lys-Ile-NH_2, t_R = 13.1 min, HRMS *m*/*z* 509.2923 (M + H^+, $C_{24}H_{41}N_6O_4S$ requires 509.2910); **10d**, Trp-Phe-Lys-Ile-NH_2, t_R = 21.2 min, HRMS *m*/*z* 592.3628 (M + H^+, $C_{32}H_{46}N_7O_4$ requires 592.3611); **10e**, His-Phe-Lys-Ile-NH_2, t_R = 11.5 min, HRMS *m*/*z* 543.3429 (M + H^+, $C_{27}H_{43}N_8O_4$ requires 543.3407); **10f**, Asn-Phe-Lys-Ile-NH_2, t_R = 11.3 min, HRMS *m*/*z* 520.3260 (M + H^+, $C_{25}H_{42}N_7O_5$ requires 520.3247); **10g**, Lys-Phe-Lys-Ile-NH_2, t_R = 11.1 min, HRMS *m*/*z* 534.3768 (M + H^+, $C_{27}H_{48}N_7O_4$ requires 534.3774); **10h**, Arg-Phe-Lys-Ile-NH_2, t_R = 11.9 min, HRMS *m*/*z* 562.3822 (M + H^+, $C_{27}H_{48}N_9O_4$ requires 562.3829); **10i**, Ala-Phe-Lys-Ile-NH_2, t_R = 14.4 min, HRMS *m*/*z* 477.3191 (M + H^+, $C_{24}H_{41}N_6O_4$ requires 477.3189); **10j**, Met-Phe-Lys-Ile-NH_2, t_R = 17.2 min, HRMS *m*/*z* 537.3236 (M + H^+, $C_{26}H_{45}N_6O_4S$ requires 537.3223); **10k**, Tyr-Phe-Lys-Ile-NH_2, t_R = 15.6 min, HRMS *m*/*z* 569.3463 (M + H^+, $C_{30}H_{45}N_6O_5$ requires 569.3451); **10*l***, Asp-Phe-Lys-Ile-NH_2, t_R = 12.5 min, HRMS *m*/*z* 521.3070 (M + H^+, $C_{25}H_{41}N_6O_6$ requires 521.3088); **19a**, Ser-Arg-Pro-Ile-Pro-Arg-Pro-Leu-Pro-Phe-Pro-Arg-Pro-Gly-Pro-Arg-Pro-Ile-Pro-Arg-Pro-Leu-Pro-Phe-Pro-Arg-Pro-Gly-Pro-Arg-Pro-Ile-Pro-Arg-Pro-OH, t_R = 22.4 min, LRMS *m*/*z* 3980.71 (M + H^+, 3980.83 calcd for $C_{188}H_{302}N_{59}O_{37}$); **19b**, Thr-Arg-Pro-Ile-Pro-Arg-Pro-Leu-Pro-Phe-Pro-Arg-Pro-Gly-Pro-Arg-Pro-Ile-Pro-Arg-Pro-Leu-Pro-Phe-Pro-Arg-Pro-Gly-Pro-Arg-Pro-Ile-Pro-Arg-Pro-OH, t_R = 22.5 min, LRMS *m*/*z* 3994.49 (M + H^+, 3994.86 calcd for $C_{189}H_{304}N_{59}O_{37}$); **19c**, Cys-Arg-Pro-Ile-Pro-Arg-Pro-Leu-Pro-Phe-Pro-Arg-Pro-Gly-Pro-Arg-Pro-Ile-Pro-Arg-Pro-Leu-Pro-Phe-Pro-Arg-Pro-Gly-Pro-Arg-Pro-Ile-Pro-Arg-Pro-OH, t_R = 22.9 min, LRMS *m*/*z* 3995.27 (M + H^+, 3996.90 calcd for $C_{188}H_{302}N_{59}O_{36}S$). Peptide glycoaldehydes **8a**−**d** were synthesized on the acetal resin **6** using Fmoc/tBu strategy (Figure 3). Sequential coupling was accomplished by the HBTU/HOBt method[62] with 2.5 equiv of amino acids, and the Fmoc group was deprotected by 20% piperidine in DMF. Final cleavage of peptides from the resin

(61) Marsh, A. J.; Henrickson, C. L.; Jackson, G. S. *Mass Spectrom. Rev.* **1998**, *17*, 1−35.

(62) Dourtoglou, V.; Gross, B. *Synthesis* **1984**, 572−574.

was performed with TFA−glycerol−anisole−thioanisole (90:4:3:3, 40 mL/g resin) for 3 h. The residue was dissolved in buffer B and lyophilized to dryness after removal of the TFA and resin. The produced peptidyl glyceryl esters **7a-d** were oxidized by sodium periodate (4−6 equiv) in aqueous buffers at pH 2 to 6 for 10 min. Preparative HPLC gave the purified peptide glycoaldehydes **8a**−**d** (50−70% yield) which were characterized by amino acid analysis and MS. **8a**, Leu-Ile-Leu-Asn-Gly-OCH_2CHO, t_R = 12.8 min (20−60% B), HRMS *m/z* 571.3466 (M + H^+, $C_{26}H_{47}N_6O_8$ requires 571.3455); **8b**, Asp-Ser-Phe-Gly-OCH_2-CHO, t_R = 12.6 min, HRMS *m/z* 467.1788 (M + H^+, $C_{20}H_{27}N_4O_9$ requires 467.1778); **8c**, Leu-Ile-Leu-Ala-OCH_2CHO, t_R = 15.7 min, HRMS *m/z* 471.3169 (M + H^+, $C_{23}H_{43}N_4O_6$ requires 471.3182); **8d**, Ile-Ala-Tyr-Gly-Gly-Phe-Leu-OCH_2CHO, t_R = 25.5 min, HRMS *m/z* 782.4091 (M + H^+, $C_{39}H_{56}N_7O_{10}$ requires 782.4089). Similarly, acyl segment **18**, Arg-Arg-Ile-Arg-Pro-Arg-Pro-Pro-Arg-Leu-Pro-Arg-Pro-Arg-Pro-Arg-Pro-Leu-Pro-Phe-Pro-Arg-Pro-Gly-OCH_2CHO, was prepared in 74.4% yields, t_R = 17.4 min, LRMS *m/z* 2980.18 (M + H^+, 2980.60 calcd for $C_{135}H_{227}N_{51}O_{26}$).

Optimization of Ligation Condition. a. Pyridine−Acetic Acid Ratios. A mixture of peptide glycoaldehyde **8a** (7.2 mg, 12.7 μmol) and peptide **10a** (6.3 mg, 12.8 μmol) was divided into seven equal portions. Pyridine−acetic acid (300 μL) was added to each part to bring the final concentration to 6.0×10^{-3} M with pyridine−acetic acid molar ratios of 1:0, 4:1, 2:1, 1:1, 2:3, 1:2, and 0:1, respectively. The reactions were followed by HPLC.

b. Solvents. Peptide glycoaldehyde **8a** (3.8 mg, 6 μmol) and peptide **10a** (2.96 mg, 6.0 μmol) were dissolved in 600 μL of pyridine−acetic acid (1:1, mol/mol), divided into four equal portions, and 150 μL of TFE, DMF, H_2O, and pyridine−acetic acid (1:1,mol/mol) were added to each. The reaction was followed by HPLC, and the results were shown in Table 1.

c. Bases. Peptide glycoaldehyde **8b** (5.6 mg, 12μmol) and peptide **10a** (5.9 mg, 12 μL) were dissolved in 600 μL of TFE and 120 μL of acetic acid. After being divided into six equal portions, TFE and a different base were added to bring the final concentration to 6×10^{-3} M with an acetic acid−base molar ratios of 1:1. The reaction was followed by HPLC and summarized in Table 2.

Regiospecific Ligation. a. Competitive Reaction. *Z*-Alanine glycoaldehyde **15a** (100 mg, 0.40 mmol) was dissolved in 3 mL of TFE and 3 mL of pyridine−acetic acid (1:1, mol/mol), and then H-Ser-OMe HCl **16a** (65 mg, 0.42 mmol), H-Thr-OMe HCl **17b** (70 mg, 0.41 mmol), and H-Cys-OMe HCl **16c** (70 mg, 0.41 mmol) were added. The reaction was monitored by HPLC. First, the ester bond product *Z*-Ala-OCH_2-Thz-COOMe (t_R = 27.7 min, 20−70% B, 30 min) formed between **15a** and **16c** was observed. Then, the ester bond product rearranged into the amide product *Z*-Ala-*S*Pro-OMe **17f** (t_R = 20.5 min, 20−70% B, 30 min) with half time $t_{1/2}$ = 17 h.

b. Regiospecificy of N-Terminal Amino Acid. Peptide glycoaldehyde **8a** (8.6 mg, 15 μmol) was dissolved in 600 μL of acetic acid and divided into 10 equal portions. To each portion, an N-terminal segment (**10a**−**l**, 1.5 μmol) in 240 μL of pyridine was added. The mixture was shaken for 45 h at room temperature, and the reaction was followed by HPLC. The ligation products were confirmed by chemical analysis and MS, and the yields were shown Table 3. **11a**, t_R = 27.6 min (20−50% B in 30 min, then 50−90% B in 15 min) LRMS *m/z* 1045.0 (M + H^+, 1045.3 calcd for $C_{50}H_{84}N_{12}O_{12}$); **11b**, t_R = 28.2 min, LRMS *m/z* 1058.7 (M + H^+, 1059.3 calcd for $C_{51}H_{86}N_{12}O_{12}$); **11c**, t_R = 29.9 min, LRMS *m/z* 1060.8 (M + H^+, 1061.4 calcd for $C_{50}H_{84}N_{12}O_{11}S$); **11d**, t_R = 32.6, 33.6 min, LRMS *m/z* 1143.9 (M + H^+, 1144.3 calcd for $C_{58}H_{89}N_{13}O_{11}$); **11e**, t_R = 23.3, 24,7, 33.6 min, LRMS *m/z* 1095.9 (M + H^+, 1096.4 calcd for $C_{53}H_{87}N_{14}O_{11}$);. **11f**, t_R = 27.5 min, LRMS *m/z* 1071.8 (M + H^+, 1072.3 calcd for $C_{51}H_{86}N_{13}O_{12}$);

Steric Effect of C-Terminal Amino Acids at the Acyl Segments. Peptide glycoaldehyde **8a** (0.95 mg, 1.5 μmol) and Ntn-serine peptide **10a** (0.74 mg, 1.5 μmol) were dissolved in a solution of 150 μL TFE, 90μL of pyridine and 60μL of acetic acid. The ligation reaction was carried out at room temperature for 45 h, and the process was monitored by HPLC. Ligations between **8b**−**d** and **10a,b** were performed under the same conditions. The results are shown in Table 4. The ligation products were confirmed by chemical analysis and MS, and the yields are shown Table 4. **11a**, t_R = 27.6 min (20−50% B in 30 min, then 50−90% B in 15 min) LRMS *m/z* 1045.0 (M + H^+, 1045.3 calcd for $C_{50}H_{84}N_{12}O_{12}$); **11b**, t_R = 28.2 min, LRMS *m/z* 1058.7 (M + H^+, 1059.3 calcd for $C_{51}H_{86}N_{12}O_{12}$); **12a**, t_R = 23.0 min, HRMS *m/z* 941.4758 (M + H^+, $C_{44}H_{65}N_{10}O_{13}$ requires 941.4732); **12b**, t_R = 23.2 min, HRMS *m/z* 955.4878 (M + H^+, $C_{45}H_{67}N_{10}O_{13}$ requires 955.4889); **13a**, t_R = 25.9 min, HRMS *m/z* 945.6127 (M + H^+, $C_{47}H_{81}N_{10}O_{10}$ requires 945.6136); **13b**, t_R = 26.4 min, HRMS *m/z* 945.6127 (M + H^+, $C_{47}H_{81}N_{10}O_{10}$ requires 945.6136); **14a**, t_R = 34.6 min, LRMS *m/z* 1256.0 (M + H^+, 1256.5 calcd for $C_{63}H_{93}N_{13}O_{14}$); **14b**, t_R = 35.8 min, LRMS *m/z* 1270.0 (M + H^+, 1270.5 calcd for $C_{63}H_{93}N_{13}O_{14}$).

Model Dipeptide Synthesis for Stereochemistry Study. *Z*-Alanine glycoaldehyde (**15a**, 53 mg, 0.20 mmol) synthesized according to an earlier described procedure[51] was dissolved in 2 mL of TFE and 2 mL of pyridine−acetic acid (1:1,mol/mol), and then H-Ser-OMe (**16a**, 65 mg, 0.42 mmol) was added. The mixture was stirred at room temperature for 36 h. The product *Z*-Ala-*O*Pro-OMe (**17a**) was characterized by HPLC, MS, and ^{1}H NMR. Similarly, *Z*-Val-*O*Pro-OMe (**17b)** from *Z*-Val-OCH_2CHO **15b** and **16a**, *Z*-Ala-*O*Pro^{Me}-OMe (**17c)** from **15a** and Thr-OMe **16b**, *Z*-Val-*O*Pro^{Me}-OMe (**17d)** from **15b** and **16b** and *Z*-Val-*S*Pro-OMe **17e** from **10b** and Cys-OMe **16c** were obtained.

17a: t_R = 17.4 min (20−70% B, 30 min), yield 89.3% based on **15a**. HRMS *m/z* 367.1512 (M + H^+, $C_{17}H_{23}N_2O_7$ requires 367.1505). ^{1}H NMR (500 MHz, 298 K, $CDCl_3$, 10 mg/mL):

C2, *R*; *cis* (68%, by NH-Ala): 7.31−7.36 (m, 5H, C_6H_5-*Z*), 5.60 (d, 1H, *J* = 7.5, NH−Ala), 5.49 (m, 1H, H−C2), 5.02−5.14 (m, 2H, CH_2-*Z*), 4.52 (t, 1H, *J* = 7, H−C4), 4.43 (t, 1H, *J* = 7, *axial* H−C5), 4.33 (t, 1H, *J* = 7, *equatorial* H−C5), 4.31 (m, 1H, αH−Ala), 3.86 (dd, 1H, *J* = 3.5, *J* = 9, CH_2−C2), 3.82 (dd, 1H, *J* = 3.5, *J* = 9, CH_2−C2), 3.76 (s, 3H, OMe), 1.37 (d, 3H, *J* = 7, CH_3−Ala). **NOE:** H(C4)−H(C2), R; H(C4)−×−CH_2(C2), R; CH_3(Ala)−H(C4), *R-cis*; αH(Ala)−CH_2(C2), *R-cis*; CH_3(Ala)−H(C2), *R-cis*; *axial* H(C5)−H(C2) and *equatorial* H(C5)−×− H(C2), one epimer.

C2, *R*; *trans* (32%): 7.31−7.36 (m, 5H, C_6H_5-Z), 5.49 (m, 1H, H−C2), 5.39 (d, 1H, *J* = 7.5, NH−Ala), 5.02−5.14 (m, 2H, CH_2-Z), 4.68 (d, 1H, *J* = 5.5, H−C4), 4.50 (m, 1H, αH−Ala), 4.38 (dd, 1H, *J* = 7, *J* = 9.5, *axial* H−C5), 4.15 (dd, 1H, *J* = 7.5, *J* = 9.5, *equatorial* H−C5), 3.97 (dd, 1H, *J* = 4, *J* = 13, CH_2−C2), 3.89 (dd, 1H, *J* = 4.5, *J* = 13, CH_2−C2), 3.75 (s, 3H, OMe), 1.41 (d, 3H, *J* = 7, CH_3−Ala). **NOE:** H(C4)−H(C2), R; αH(Ala)−CH(C2), *R-trans*; *axial* H(C5)−H(C2) and *equatorial* H(C5)−×−H(C2), one epimer.

17b: t_R = 17.7 min (35−80% B, 30 min), yield 77.3% based on **15b**. HRMS *m/z* 395.1827 (M + H^+, $C_{19}H_{27}N_2O_7$ requires 395.1818). ^{1}H NMR (500 MHz, 298 K, $CDCl_3$, 10 mg/mL):

C2, *R*, *cis* (56%, by CH_3−Val): 7.36−7.29 (m, 5H, C_6H_5-*Z*), 5.55−5.50 (m, 1H, NH−Val), 5.55−5.50 (m, 1H,H−C2), 5.01−5.12 (m, 2H, CH_2-*Z*), 4.43 (t, 1H, *J* = 7, H−C4), 4.41 (d, 2H, *J* = 7.5, H−C5), 4.28 (d, 2H, *J* = 8.5, H−C5), 4.09 (dd, 1H, *J* = 7, *J* = 9, αH−Val), 4.01 (dd, 2H, *J* = 3.5, *J* = 13, CH_2−C2), 3.90 (dd, 2H, *J* = 3.5, *J* = 13, CH_2−C2), 3.68 (s, 3H, OMe), 2.17−2.03 (m, 1H, *β*H−Val), 1.12 (d, 6H, *J* = 7, $2CH_3$−Val), 1.02 (d, 6H, *J* = 7, $2CH_3$−Val). **NOE:** H(C4)−H(C2), R; *β*H(Val)−H(C4), *R-cis*; αH(Val)−CH_2(C2), *R-cis*; *axial* H(C5)−H(C2) and *equatorial* H(C5)−×−H(C2), one epimer;

C2, *R*; *trans* (44%): 7.36−7.29 (m, 5H, C_6H_5-*Z*), 5.55−5.50 (m, 1H, NH−Val), 5.55−5.50 (m, 1H, H−C2), 5.01−5.12 (m, 2H, CH_2-*Z*), 4.64 (t, 1H, *J* = 9, H−C4), 4.62 (d, 2H, *J* = 7.5, H−C5), 4.17 (t, 1H, *J* = 7.5, αH−Val), 4.12 (dd, 2H, *J* = 1.5, *J* = 9, H−C5), 3.82 (dd, 2H, *J* = 4, *J* = 18, CH_2−C2), 3.75 (s, 3H, OMe), 1.99−1.92 (m, 1H, *β*H−Val), 0.97 (d, 6H, *J* = 6.5, $2CH_3$−Val), 0.93 (d, 6H, *J* = 6.5, $2CH_3$−Val). **NOE:** H(C4)−H(C2), R; αH(Val)−H(C2), *R-trans*; CH_3-(Val)−*axial* H(C5), *R-trans*; *axial* H(C5)−H(C2) and *equatorial* H(C5)−×−H(C2), one epimer.

17c: t_R = 21.9 min (20−70% B, 30 min), yield 78.4% based on **15a**. HRMS *m/z* 381.1662 (M + H^+, $C_{18}H_{25}N_2O_7$ requires 381.1652). ^{1}H NMR (500 MHz, 298 K, $CDCl_3$, 10 mg/mL):

C2, *R*, *cis* (60%, by NH−Ala): 7.33−7.31 (m, 5H, C_6H_5-*Z*), 5.57 (d, 1H, *J* = 7 H_Z, NH−Ala), 5.42 (s, 1H, H−C2), 4.53 (d, 1H, *J* = 7 H_Z, αH−Ala), 4.24 (d, 1H, *J* = 11, HC4), 4.18−4.15 (m, 1H, H−C5), 4.11 (d, 2H, *J* = 14, CH_2−C2), 3.95 (d, 2H, *J* = 13, CH_2 −C2), 3.75 (d, 3H, *J* = 7, OMe), 1.47−1.43 (m, 3H, CH_3−C5), 1.37−1.34 (m,

3H, CH_3−Ala). **NOE:** H(C4)−H(C2), *R*; H(C5)−×−H(C2), *R*; αH-(Ala)−CH_2(C2), *R-cis*.

C2, *R*; *trans* (40%): 7.33−7.31 (m, 5H, C_6H_5-*Z*), 5.64 (d, 1H, J = 8, NH−Ala), 5.28 (d, 1H, J = 3, H−C2), 5.13−4.99 (m, 2H, CH_2-*Z*), 4.50 (d, 1H, J = 7, H−C4), 4.35−4.31 (m, 1H, H−C5), 4.23 (d, 1H, J = 13, αH−Ala), 4.05 (d, 2H, J = 13, CH_2−C2), 3.86 (d, 2H, J = 13, CH_2−C2), 3.77 (d, 3H, J = 5.5, OMe), 1.47−1.43 (m, 3H, CH_3−C5), 1.37−1.34 (m, 3H, CH_3−Ala). **NOE:** H(C4)−H(C2), *R*; H(C5)−×−H(C2), *R*; CH_3(Ala)−CH_2(C2) *R-trans*; αH(Ala)−H(C2), *R-trans*.

17d: t_R = 21.9 min (35−80% B, 30 min), yield 73.3% based on **15b**. HRMS *m*/*z* 409.1959 (M + H^+, $C_{20}H_{29}N_2O_7$ requires 409.1975). ^{1}H NMR (500 MHz, 298 K, $CDCl_3$, 10 mg/mL):

C2, *R*; *cis* (43%, by CH_3−Thr): 7.34−7.29 (m, 5H, C_6H_5-*Z*), 5.52 (d, 1H, J = 9, NH−Val), 5.46 (s, 1H, H−C2), 5.12−5.00 (m, 2H, CH_2-*Z*), 4.25 (d, 1H, J = 8.5, H−C4), 4.24 (d, 1H, J = 7, αH−Val), 4.22 (m, 1H, H−C5), 4.08 (dd, 2H, J = 2.5, J = 15, CH_2−C2), 3.87 (dd, 2H, J = 2.5, J = 15, CH_2 −C2), 3.75 (s, 3H, OMe), 2.05−1.98 (m, 1H, βH−Val), 1.42 (d, 3H, J = 6, CH_3−C5), 1.05 (d, 6H, J = 7, 2, CH_3−Val), 0.99 (d, 6H, J = 6.5, 2CH_3−Val). **NOE:** H(C4)−H(C2), *R*; H(C5)−CH_2(C2), *R*; αH(Val)−H(C2), *R-trans*.

C2, *R*, *trans* (57%): 7.34−7.29 (m, 5H, C_6H_5-*Z*), 5.52 (d, 1H, J = 9, NH−Val), 5.29 (d, 1H, J = 5, H−C2), 5.12−5.00 (m, 2H, CH_2-*Z*), 4.16 (d, 1H, J = 9, H−C4), 4.17−4.14 (m, 1H, H−C5), 3.98 (d, 1H, J = 8, αH−Val), 3.95 (d, 2H, J = 13, CH_2−C2), 3.77 (dd, 2H, J = 5, J = 17, CH_2 −C2), 3.71 (s, 3H, OMe), 1.98−1.92 (m, 1H, βH−Val), 1.46 (d, 3H, J = 6, CH_3−C5), 0.98 (d, 6H, J = 7, 2CH_3−Val), 0.92 (d, 6H, J = 7, 2CH_3−Val). **NOE:** H(C4)−H(C2), *R*; H(C5)−CH_2(C2), *R*; βH(Val)−H(C4), *R-cis*; CH_3(Val)−H(C4), *R-cis*.

17e: t_R = 23.4 min (35−80% B, 30 min), yield 83.6% based on **15b**. HRMS *m*/*z* 411.1599 (M + H^+, $C_{19}H_{27}N_2O_6S$ requires 411.1590). ^{1}H NMR (500 MHz, 298 K, $CDCl_3$, 10 mg/mL):

C2, *R*, *cis* (60%, by CH_3−Val): 7.35−7.30 (m, 5H, C_6H_5-*Z*), 5.55 (d, 1H, J = 8.5, NH−Val), 5.42 (t, 1H, J = 9.5, HO−CH_2), 5.19 (t, 1H, J = 12, H−C2), 5.12−5.01 (m, 2H, CH_2-Z), 4.59 (dd, 1H, J = 6.5, J = 15, αH−Val), 3.90 (dd, 2H, J = 6, J = 18, CH_2−C2), 3.73 (s, 3H, OMe), 3.49 (m, 1H, H−C4), 3.08 (d, 2H, J = 13, H−C5), 2.14−2.07 (m, 1H, βH−Val), 1.27 (d, 6H, J = 7, 2CH_3−Val), 0.96 (d, 6H, J = 7, 2CH_3−Val). **NOE:** βH(Val)−H(C2), R-*cis*; CH_3(Val)−H(C2), R-*cis* CH_3(Val)−H(C4), R-*cis*.

C2, *R*; *trans* (40%): 7.35−7.30 (m, 5H, C_6H_5-*Z*), 5.62 (d, 1H, J = 9, NH−Val), 5.42 (t, 1H, J = 9.5, HO−CH_2), 5.12−5.01 (m, 2H, CH_2-*Z*), 4.92 (dd, 1H, J = 6, J = 21, H−C2), 4.16 (dd, 1H, J = 6, J = 15.5, αH−Val), 3.77 (dd, 2H, J = 6, J = 17.5, CH_2 −C2), 3.65 (s, 3H, OCH3), 3.49 (m, 1H, H−C4), 3.34 (d, 2H, H−C5), 1.95−1.89 (m, 1H, βH−Val), 0.95 (d, 6H, J = 6.5, 2CH_3−Val), 0.91 (d, 6H, J = 6.5, 2CH_3−Val). **NOE:** H(C4)−H(C2), *R*; αH(Val)−H(C2), *R-trans*.

Evaluation of Racemization. The peptide samples **17a,c** as well as reference amino aicids, L-Ser, L-Thr, and L-Ala, were treated by the following procedure: (1) hydrolysis in 6 M HCl aqueous solution at 110 °C for 24 h, (2) reaction of about 5 μmol of the sample with 200 μL of 1% N^{α}-(2,4-dinitro-5-fluorophenyl)−L-valinamide in acetone and 40 μL of 1.0 M sodium bicarbonate in H_2O at 40 °C for 1 h, (3) HPLC analysis on a Vydac C18 column (4.6 × 250 mm) using a linear gradient (35−65% B, in 40 min, at 340 nm), (4) comparison with the HPLC of standard single D- or L-amino acid derivative to distinguish the D-,L-diastereomers. The HPLC analysis of reference L-Ser gave 94.7% L-diastereomer (t_R = 16.0 min) and 5.3% D-diastereomer (t_R = 16.8 min, racemization from hydrolysis). Similarly, the results of HPLC showed that reference L-Thr: 94.2% L-diastereomer (t_R = 17.4 min) and 5.8% D-diastereomer (t_R = 17.8 min, racemization from hydrolysis); The reference L-Ala: 99.6% L-diastereomer (t_R = 22.5 min) and 0.4% D- diastereomer (t_R = 24.9 min, racemization from hydrolysis); *Z*-Ala-*O*Pro-OMe **17a**: 96.1% L- and 3.9% D-Ser diastereomers as well as 99.7% L- and 0.3% D-Ala diastereomers; *Z*-Ala-*O*Pro^{Me}-OMe **17c**: 94.6% L- and 5.4% D-Thr diastereomers as well as 99.6% L- and 0.4% D-Ala diastereomers.

Synthesis of Bac 7 Analogues by Two-Segment Ligation. The Bac 7 analogues **20a, 20b**, and **20c** were synthesized by a two-segment ligation strategy between acyl segment **18** and amine segments **19a**−**c**. **18**, 18.0 mg in 3 mL pyridine−acetic acid mixture (1:1, mol/mol) was divided into three equal portions, and then **19a** (8.0 mg), **19b** (8.1 mg), and **19c** (8.2 mg) were added to each portion, respectively. The well dissolved solutions stood at room temperature for 35 h, and the reactions were monitored by HPLC (Figure 6, Table 6). The ligation products were confirmed by chemical analysis and MS. **20a**, t_R = 25.7 min (20−55% B in 30 min), LRMS *m*/*z* 6940.9 (M + H^+, 6942.4 calcd for $C_{323}H_{526}N_{110}O_{62}$); **20b**, t_R = 25.8 min, LRMS *m*/*z* 6955.9 (M + H^+, 6956.4 calcd for $C_{324}H_{528}N_{110}O_{62}$); **20c**, t_R = 26.3 min, LRMS *m*/*z* 6958.1 (M + H^+, 6958.5 calcd for $C_{323}H_{526}N_{110}O_{62}S$).

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