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Methionine Ligation Strategy in the Biomimetic Synthesis of Parathyroid Hormones

Abstract: *In biological systems, both proteolysis and aminolysis of amide bonds produce activated intermediates through acyl transfer reactions either inter- or intramolecularly. Protein splicing is an illustrative example that proceeds through a series of catalyzed acyl transfer reactions and culminates at an O- or S-acyl intermediate. This intermediate leads to an uncatalyzed acyl migration to form an amide bond in the spliced product. A ligation method mimicking the uncatalyzed final steps in protein splicing has been developed utilizing the acyl transfer amide-bond feature for the blockwise coupling of unprotected, free peptide segments at methionine (Met). The latent thiol moiety of Met can be exploited using homocysteine at the α-amino terminal position of a free peptide for transthioesterification with another free peptide containing an α-thioester to give an S-acyl intermediate. A subsequent, proximity-driven S- to N-acyl migration of this acyl intermediate spontaneously rearranges to form a homocysteinyl amide bond. S-methylation with excess* p-*nitrobenezensulfonate yields Met at the ligation site. The methionine ligation is selective and orthogonal, and is usually completed within 4 h when performed at slightly basic pH and under strongly reductive conditions. No side reactions due to acylation were observed with any other α-amines of both peptide segments as seen in the synthesis of parathyroid hormone peptides. Furthermore, cyclic peptide can also be obtained through the same strategy by placing both homocysteine at the amino terminus and the thioester at the carboxyl terminus in an unprotected peptide precursor. These biomimetic ligation strategies hold promise for engineering novel peptides and proteins.* Biopoly 46: 319–327, 1998

Keywords: *methionine ligation; parathyroid hormones; biomimetic ligation; S-methylation*

INTRODUCTION

Recent advances in blockwise orthogonal or chemoselective ligation using unprotected, free peptide segments or proteins have provided a new biotechnological platform for accessing diverse groups of macromolecules. These compounds, including branched peptides,[1] peptide dendrimers,[2,3] cyclic peptides,[4] and proteins and protein conjugates,[5] are usually difficult to obtain directly by recombinant methods or through conventional approaches of chemical synthesis with protecting group schemes.

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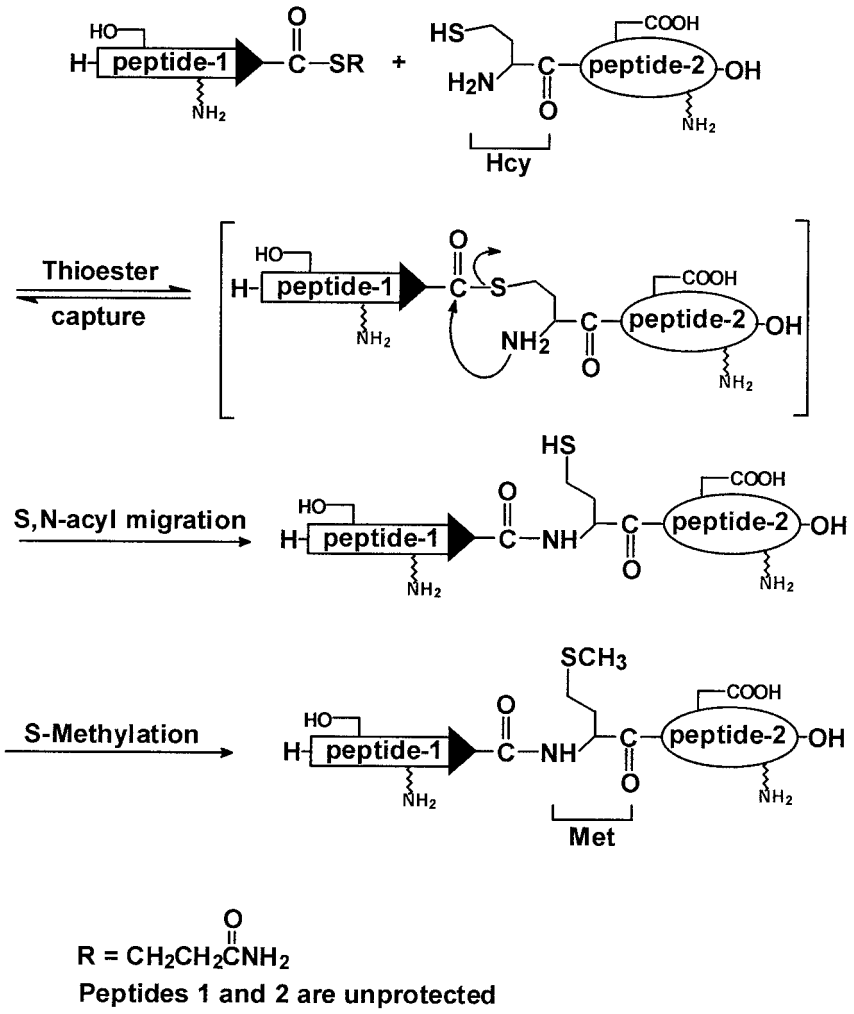

FIGURE 1 Synthetic scheme of orthogonal coupling with Met at the ligation site.

Among various linkages obtained from these ligation methods, such as amide,[6–8] thioether,[9,10] thioester,[11,12] disulfide,[13] oxime,[14] hydrazone,[15–17] and thiazolidine,[18–20] the amide bond formation produces the most stable linkage, but is the most difficult to obtain with two free, unprotected peptide segments. It requires a chemoselective coupling reaction between an acyl moiety on one peptide and a specific amine on another peptide segment independent of other reactive amines present in these segments. Recently, this type of exclusive orthogonal ligation without any protection or activation step has been developed[6–8,18–20] based on a combination of the chemoselective capture of the N^{α}- and C^{α}-termini of two peptide segments as a covalent O- or S-acyl intermediate and the entropic-driven intramolecular O- or S- to N-acyl transfer to form an amide bond (Figure 1).

The mechanisms of making or breaking an amide bond in serine and thiol proteases are well recognized. These proteases utilize dyad or triad catalytic residues for the intermolecular N- to O- or S-acyl transfers and hydrolysis. Indeed, the O- or S-acyl intermediates have been exploited successfully for enzymatic synthesis with engineered new enzymes containing high ligase activity.[21–24] Recently, intramolecular acyl transfer reactions have also been found in protein splicing and autoproteolysis. In protein splicing, a series of acyl transfers leads to a final covalent O- or S-acyl intermediate resulting in a spontaneous uncatalyzed O- or S- to N-acyl transfer reaction that forms the corresponding amide bond.[25,26] Thus, there is a convergence in the principle of amide bond formation in which the acyl migration is the centerpiece to overcome chemoselectivity in both the chemical or biological process. This principle of entropic activation forming peptide bonds through proximity effects has been utilized

previously in peptide synthesis as shown by Brenner et al.[27] in their ''Amino Acyl Insertion'' and Kemp et al.[28] in their ''Prior Thiol Capture'' strategy.

In orthogonal ligation, an O- or S- ester is first formed between two free peptide segments (Figure 1, step 1), which is followed by a spontaneous acyl migration to form an amide bond in the desired peptide (Figure 1, step 2). To form the O- or S-acyl intermediate necessary for acyl migration in such a triangulation strategy, the COOH terminus of a free peptide segment generally requires activation to be captured by another free peptide segment. With a free peptide, the conventional activation of the α-carboxylic acid by a chemical activating reagent is often unsatisfactory because of the consequence of random acylation leading to polymerization. However, the problem of activation can be solved through the use of thioester. In nature, thioesters are involved in nonribosomal peptide synthesis through a multienzyme thiotemplate system that forms antimicrobial peptides and in many posttranslational protein modifications such as receptor palmitoylation and myristoylation. In peptide synthesis, Wieland et al.,[29] Schwyzer,[30] and subsequently Blake and Yamashiro[31] have shown that thioesters are relatively unreactive to aminolysis but readily reactive with a thiol through transesterification to form a new thioester at neutral pH. Thus, the thioester linkage forming an S-acyl covalent intermediate between two free peptide segments can be obtained through transthioesterification of a free peptide containing an N-terminal cysteine with another peptide containing a C-terminal thioester. Because the thioester is at close proximity to the α-amine of the cysteinyl peptide, a spontaneous S- to N-acyl migration occurs to form a peptide bond through a five-member ring intermediate. This method has been applied successfully as cysteine ligation for unprotected peptides.[6,8] Other methods to arrive at the O- or S-acyl intermediate have also been developed based on either thiol or aldehyde chemistry to produce disulfide bond[13] and thiazolidine[18] by utilizing the electrophilic or nucleophilic side chain functional groups at the α-amine terminus, thus allowing O- or S- to N-acyl migration to form the amide bond.

Homocysteine (Hcy) is a homolog of cysteine and its thiomethyl ether is the natural amino acid, methionine. Thus, the latent unmethylated thiol moiety of the homocysteine can then be exploited for the biomimetic ligation strategy as in α-cysteinyl peptides to yield homocysteinyl peptides. Subsequent selective S-methylation transforms Hcy to Met (Figure 1). In this paper, we describe the development of the methionine ligation through homocysteinyl peptides with another peptide bearing a thioester via an intramolecular transthioesterification and subsequent S-methylation to give Met at the ligation site. Furthermore, we have also extended this method to the cyclization of unprotected homocysteinyl peptide thioesters under the influence of ring-chain tautomerization equilibrium[32] for the synthesis of cyclic peptides.

EXPERIMENTAL PROCEDURES

Solid-Phase Peptide Synthesis

All thioester and homocysteine peptides were prepared by solid phase synthesis using conventional *tert*-butoxycarbonyl (Boc) chemistry and dicyclohexylcarboimide-N-hydroxybenzotriazole coupling protocol. The synthesis was carried out on the 430A peptide synthesizer (Applied Biosystems, Inc., Foster City, CA) starting with Boc-AA-SCH_2CH_2CO-resin. A procedure described by Hojo and Aimoto[33] was modified for preparation of thioester resins with the first amino acid thioester incorporated on the *p*-methyl-benzhydryl-amine (MBHA) resin in its thiopropionic amide form. After assembly of the sequence, peptides were cleaved from the resin by hydrogen fluoride (HF) anisole and then extracted with 20% acetic acid. All crude peptides were purified by preparative C_{18} reverse phase high performance liquid chromatography (RP-HPLC).

General Procedure for the Ligation of Homocysteinyl Peptides

The biomimetic coupling was generally performed in 0.2*M* Na_2HPO_4-0.1*M* citric acid buffer containing 3 equivalents of tris(2-carboxethyl)phosphine (TCEP) for 4 h. Typically, 0.13 mg (0.23 μmol) of KG4-*SR* (*SR* = $SCH_2CH_2CONH_2$) **1** and 0.37 mg (0.26 μmol) of HCV12 **5** were dissolved in 200 μL of buffer (pH 7.6) containing 0.20 mg (0.70 μmol) of TCEP for a final concentration of 1 m*M*. Aliquots were withdrawn at hourly intervals for HPLC analysis. The transthioesterification and S,N-acyl migration were usually complete within 4 h. After the completion of ligation, thiopropionic acid (1 μL) was added to convert diacyl peptide to free thiol of the homocysteine product. The products were isolated by preparative HPLC. The coupling yields are summarized in Table I.

Cyclization

N-terminal homocysteinyl peptide thioester **16** (0.2 μmol) was dissolved in phosphate-citric acid buffer (200 μL, pH 7.6) containing TCEP (3 equivalents). After 4 h, cyclic peptide **17** was purified by HPLC. Mass spectroscopy (MS) analysis confirmed the cyclization (calc. 782.9, found 783).

Table I Yields of Methionyl Peptide Synthesized by Biomimetic Ligation of an Acyl Segment with α-Thioester with an Amine Segment with α-Homocysteine

Acyl Segment[a]	Amine Segment	Ligation Product[b]	Yield[c] (%)	Methylated Peptide	Yield (%)
KLYG-*SR* **1**	Hcy-KLQDV **4**	[Hcy^5]-KV10 **7**	97	KV10 **20**	77
	Hcy-ARVELKKLQDV **5**	[Hcy^5]-KV16 **8**	87	KV16 **21**	73
	Hcy-ERVEWLRKKLQDVHNF **6**	[Hcy^5]-KF21 **9**	93	KF21 **22**	96
KYGGFL-*SR* **2**	**4**	[Hcy^7]-KV12 **10**	79	KV12 **23**	94
	5	[Hcy^7]-KV18 **11**	97	KV18 **24**	72
	6	[Hcy^7]-KF23 **12**	80	KF23 **25**	85
SVSEIQLMHNLGK-HLNS-*SR* **3**	**4**	[Hcy^{18}]-SV23 **13**	93	SV23 **26**	68
	5	[Hcy^{18}]-SV29 **14**	97	SV29 **27**	72
	6	[Hcy^{18}]-SF34 **15**	84	SF34 **28**	96

[a] *SR* = $SCH_2CH_2CONH_2$.
[b] KV10 indicates that the peptide contains 10 amino acid residues with Lys at the N-terminus and Val at the C-terminus.
[c] Yield based on acyl segment determined from the peak area of RP-HPLC.

A similar result was obtained for cyclization of HCS18-*SR* (Hcy-SVSEIQLMHNLGGKHLNS- $SCH_2CH_2CONH_2$) **18** to give end-to-end cyclic HCS18 (end-to-end cyclic Hcy-SVSEIQLMHNLGGKHLNS) **19**.

S-Methylation

Homocysteinyl peptides (0.1 μmol) were dissolved in 0.2*M* phosphate buffer (100 μL, pH 8.6). To this solution a 50-fold excess of methyl *p*-nitrobenzenesulfonate dissolved in 100 μL acetonitrile was added. After 1 h at ambient temperature, the reaction was quenched with 25% trifluoroacetic acid (TFA)/H_2O (3 μL) and washed with 3 $\times$200 μL ether to remove excess methylating reagent. The aqueous phase was evaporated under vacuum to remove organic solvent and the methylated products were purified by RP-HPLC.

Characterization of Peptide Analogues

Peptides were hydrolyzed with 6 N HCl at 110°C for 24 h and amino acid analysis was performed to confirm the composition of the desired peptides. Analytical HPLC for all peptides was performed on a Vydac column (250 $\times$ 4.6 mm) with a linear gradient of 0–85% buffer B in 30 min at a flow rate of 1 mL/min (buffer A, 0.045% TFA in 5% CH_3CN in H_2O; buffer B, 0.04% TFA in 60% CH_3CN in H_2O). All synthetic peptides were characterized by matrix-assisted laser desorption mass spectroscopy (MALDI-MS).

Verification of Regiospecificity of Transthioesterification

N^{α}-DNP-homocysteic acid and N^{ϵ}-DNP-Lys (DNP: dinitrophenyl) were prepared according to the procedure described by Zhang and Tam.[32] The ligation product was oxidized with formic acid/H_2O_2 to convert homocysteine residue to homocysteic acid and then treated with 2, 4-dinitro-1-fluorobenzene. After hydrolysis of the sample with 6*M* HCl at 110°C for 24 h, the hydrolysate was analyzed with RP-HPLC. Only N^{ϵ}-DNP-Lys but no N^{α}-DNP- homocysteic acid was detected in the hydrolysate obtained from ligation peptides, indicating that the ligation proceeded through the α-amino terminus of homocysteinyl peptide.

RESULTS

Synthesis of Unprotected Peptide Building Blocks

Our proposed scheme of methionine ligation requires the synthesis of two unprotected peptide building blocks: one bearing a thioester at the α-COOH terminus and another bearing an α-Hcy at the amino terminus. The syntheses of peptides containing an α-Hcy at the amino terminus were accomplished by conventional solid-phase peptide synthesis.[34] For the α-Hcy-containing segment, either Boc or 9-fluorenylmethoxycarbonyl chemistry can be used. After cleavage and purification from the resin supports, the free α-homocysteinyl peptides without any protecting groups were used for the subsequent ligations. The syntheses of peptide α-thioesters were also achieved by solid-phase peptide synthesis but only through Boc chemistry. The thioester linkage attached to a resin support is susceptible to strong nucleophiles such as piperidine used repetitively to remove the Fmoc groups. A direct method for preparing the peptide thioester resin support was based on a modified procedure originally reported

by Hojo and Aimoto.[33] The C-terminal Boc-amino acid was converted to its 3-thiopropionic acid ester and then attached to MBHA resin. The peptide sequence was then assembled on the solid support and the peptide thioester cleaved from the resin. Thus, the peptides obtained were 3′-thiopropionic amides **1**–**3** (Table I). All peptides were purified by RP-HPLC and characterized by MALDI-MS. Peptide alkylthioesters were found to be relatively stable and could be purified under acidic conditions.

Conditions for Homocysteine Ligation

Biomimetic ligation involves two reactions, an intermolecular transthioesterification and a rapid intramolecular S,N-acyl migration. Peptide building blocks ranging from 4 to 17 amino acid residues **1**–**6** were used for ligation to yield peptides of 10–34 residues (Table I). The longest sequence of these peptides, SF34 (**28**), is the 34-residue peptide human parathyroid hormone fragment 1-34 (PTH 1-34). Some of these peptides are shorter PTH analogues and contained both Lys and His, which are useful to determine the regioselectivity of the ligation and S-methylation reactions. In all coupling reactions, homocysteine was placed at the N-terminus of the amine peptide segment and a thioester at the C-terminus of the acyl peptide segment. The condition used for transesterification was in general similar to those conditions previously described by our laboratory for α-cysteinyl peptides.[8] They were performed at pH 7.6 in phosphate buffer in a highly reductive environment that contains a 3-fold excess of water-soluble R_3P (TCEP)[35] to prevent disulfide formation and to accelerate the desired reaction. Transthioesterification occurred rapidly and was complete within 4 h when the pH of the reaction solution was constantly adjusted to 7–8. The covalent thioester of two free peptide segments was usually not observed when the reactions were performed under basic condition because the intermediate rapidly rearranges through an S- to N- acyl migration to form the homocysteinyl product.

Although the reaction can be conducted at a wide range of pH 5–10, the useful pH range is between 7.0 and 8.0. The optimal reactivity of the coupling reaction observed at this narrow range of pH is in line with the expected basicity of the unprotonated homocysteine amino groups required for a rapid S- to N-acyl transfer reaction. Below pH 5 the reaction is too slow and the yield is low. Analysis of products obtained at pH 7–9 showed three side products in addition to the expected ligated peptides (Figures 2 and 3). At pH 8, thioester hydrolysis and peptide degradation products (A and B in Figure 2) become significant. Degradation occurs due to the lability of the Hcy-X amide bond, which can undergo a five-member ring thiolactone cyclization at pH 8 to give degradation products. All these by-products were indeed observed (Figure 2), identified, and confirmed by MS. For example, in the ligation of KG4-*SR* (KLYG-$SCH_2CH_2CONH_2$) **1** with HCV12 (Hcy-ARVELKKLQDV) **5** at pH 7.6, 5% of the homocysteine fission byproduct was observed (B in Figure 3). Furthermore, this by-product was also observed from the purified ligated product upon prolonged treatment at pH 8.0. The thioester can also link to the free thiol of the homocysteinyl residue, which is regenerated from S,N-acyl migration, to form a diacyl byproduct (C in Figure 2). To minimize these side reactions, a slight excess of amine thiol segment was used. After completion of the reaction, a large excess of thiopropionic acid was added to convert diacyl byproduct to desired peptide. The coupling reaction usually occurred cleanly and efficiently as shown by RP-HPLC (Figure 3) in yields ranging from 79 to 97% (Table I).

For peptides containing both Hcy at the α-amino terminus and thioester at the α-COOH, an intramolecular coupling reaction occurs to yield an end-to-end cyclic peptide. Cyclization on two linear precursors, Hcy-KYGGFL-thioester **16** and Hcy-SVSEIQLMH-NLGKHLNS-thioester **18,** formed cyclic heptapeptide **17** and cyclic 18-residue peptide **19,** respectively. RP-HPLC showed that the intramolecular transesterification occurred rapidly between N-terminal homocysteine and C-terminal thioester at pH 7.6, and was complete within 4 h without any observable dimers or oligomers (Figure 4). Again, the S,N-acyl migration occurred spontaneously and Hcy covalent thioester was not observed in HPLC.

S-Methylation

Selective methylation on Cys was previously demonstrated by Heinrikson in 1971 with methyl *p*-nitrobenzenesulfonate as methylation reagent.[36] This procedure was adopted for S-methylation of homocysteinyl peptides. A large excess of methylating reagent was used and the reaction was acid quenched after 1 h to avoid the danger of undesirable methylation on side chains such as the ε-NH_2 groups of Lys and imidazole rings of His. Mass spectrometric analysis of methylated product **20**–**28** showed an increase of 14 mass units indicating that only one methyl group was added. Higher pH and prolonged reaction time yielded multi-methylated by-products. The integrity of methionine was also confirmed by amino acid analysis of **20**–**28,** which revealed that Hcy was the only methylation

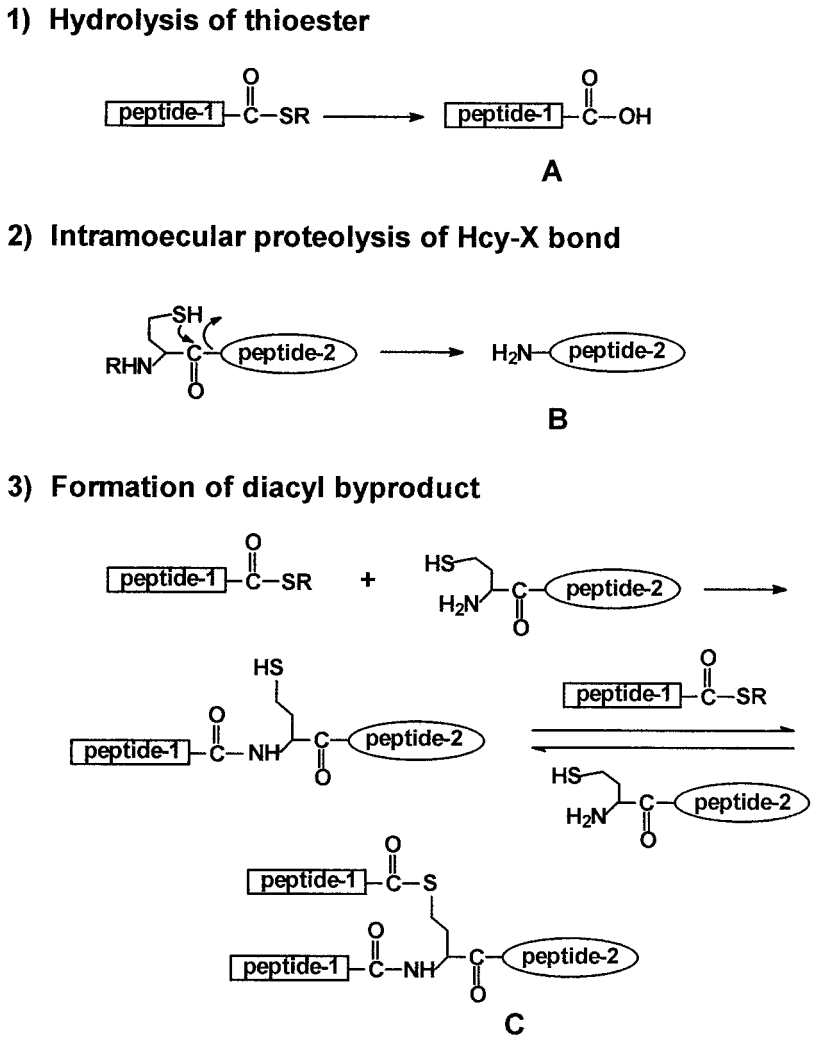

FIGURE 2 Side reactions of methionine ligation of KG4-*SR* ($R = CH_2CH_2CONH_2$) **1** and HCV12 **5**. A: Hydrolyzed product. B: Homocysteinyl fission by-product. C: S,N-diacyl by-product.

residue under the condition described. PTH 1-34 **28** prepared under biomimetic ligation and the S-methylation scheme gave the correct molecular weight and was identical with the known standard obtained from the commercial source prepared by the standard solid phase method (Figure 5).

Regioselectivity

To show that the coupling reactions occurred exclusively through transthioesterification of homocysteine and that no acylation of the side-chain amines of lysine and the imidazole ring of histidine had occurred, we used end-group determination to verify the acylation sites. Homocysteinyl peptide **8** was oxidized with HCO_3H to convert homocysteine into homocysteic acid[37] and then treated with 2,4-dinitrofluorobenzene to convert the amines to the corresponding DNP-capped derivatives.[38] After hydrolysis, RP-HPLC analysis showed only the expected ε-DNP-Lys but no α-DNP-homocysteic acid, indicating the coupling reaction had indeed occurred at the homocysteine N-terminus but not at any other side-chain amines. Peptides were also stable in NH_2OH treatment, indicating that the imidazole was not the coupling site.

DISCUSSION

Blockwise orthogonal ligation of unprotected peptides through intramolecular S,N-acyl transfer using

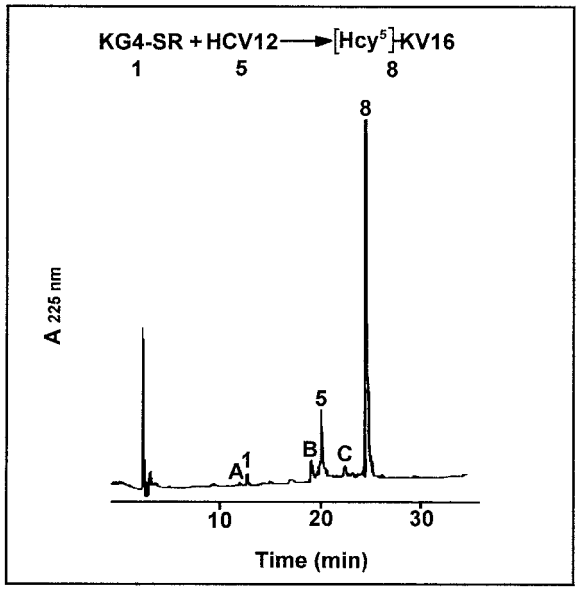

FIGURE 3 RP-HPLC product profile of methionine ligation between starting material KG4-*SR* (R = CH_2CH_2-$CONH_2$) **1** and HCV12 **5** to form [Hcy^5]-KV16 **8** (MS calcd.. 1877.2, found 1877) after 4 h. A: Hydrolyzed thioester KLYG-OH (MS calcd. 479.7, found 480). B: homocysteine fission byproduct (MS, calcd. 1298.6 found 1299). C: S,N-diacyl byproduct (MS, calcd. 2338.9 found 2339).

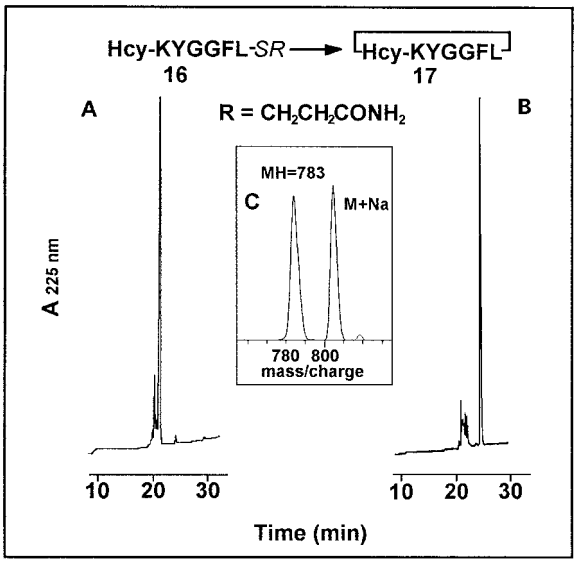

FIGURE 4 HPLC and MS analysis of cyclization from **16** to **17**. A: HPLC profile of crude linear N^{α}-homocysteinyl peptide thioester **16**. B: HPLC profile of crude cyclization product **17**. C: Mass spectrometric analysis of cyclic homocysteinyl peptide **17**.

a biomimetic coupling mechanism has provided a novel solution for overcoming both activation and the requirement of side-chain protection. At present, the repertoire of intramolecular acyl transfers provides ligation sites of cysteine, histidine, glycine. and thiaproline.[6–8,18–20] Our results here show that this chemistry can be successfully extended to Met through ligation at N^{α}-homocysteine, followed by a selective S-methylation. Although we have used the thioalkylation under basic condition, other methods of selective S-methylation under neutral or acidic conditions are also available[39] for optimizing the transformation of Hcy to Met.

Both the ligation and S-methylation proceed with high efficiency and regiospecificity. There is no observable random acylation on the side-chain functionalities such as the amine of lysine, guanidine of arginine, or imidazole of histidine. This is clearly illustrated in the synthesis of PTH 1-34 (**28**), which contains three histidine, three lysine, and two arginine. There are, however, four observable side reactions associated with the reactivity of thioester and thiols in the reaction scheme. The most noticeable is the S,N-diacylated product, which can be minimized by using an excess homocysteinyl peptide segment and reverted to the desired homocysteine peptide with the addition of thiol compounds such as 3-thiopropionic acid after

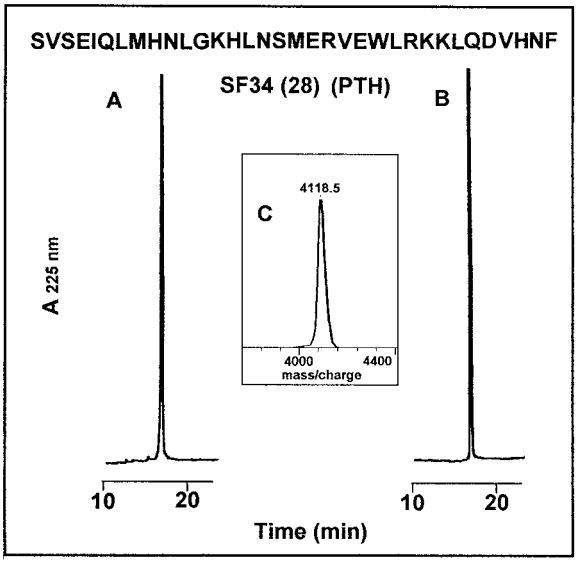

FIGURE 5 HPLC and MS analysis of SF34 **28** synthesized with orthogonal coupling-S-methylation method. A: HPLC profile of SF34 **28**. B: HPLC profile of coinjection of **28** and PTH standard. C: Mass spectrometric analysis of SF34 **28**.

completion of reaction. The second side reaction is the formation of disulfides of either the starting material or the product. Again, this side reaction is reversible by a strong reductive environment during the reaction conditions using R_3P. The third is hydrolysis of thioester to carboxylic acid. This side reaction is often accelerated when histidine is present in the peptide sequence but appears to be $< 5\%$ in the synthesis of PTH 1-34. The fourth is peptide degradation caused by intramolecular proteolysis of Hcy-X amide bond via a five-member thiolactone ring formation. The latter two side reactions become severe at high pH and prolonged reaction conditions, but are insignificant when the reaction was performed at pH < 8.0.

Although the present study focuses on methionine ligation, this two-step strategy can be utilized to afford nonnatural amino acids for a variety of purposes. Chemoselective thioalkylation of cysteinyl sulfhydryl has been used for covalent attachment of fluorophores, chelating agents, radiolables, and coenzymes. Thus, the homocysteine in the ligated peptide resulting from our ligation strategy can be used for similar purpose. In addition, the peptide bearing homocysteine can also be exploited to generate a library of analogues through thioalkylation with a diverse family of electrophiles readily available from commercial sources. Finally, the same thioalkylation can be used for semisynthesis by ligating the homocysteinyl peptide to proteins as antigens.

Recently, we have shown that orthogonal ligation can be applied successfully to intramolecular cyclization to give end-to-end cyclic peptides with high efficiency.[4,32] Our results show that the homocysteinyl peptide thioesters readily undergo cyclization to give end-to-end cyclic peptides. Previous methods for cyclization of peptides in solution rely on a combination of enthalpic activation of α-carboxyl groups and a protecting strategy for side chains to prevent random acylation. In addition, cyclization reactions need to be carried out in high dilution to minimize unwanted competing intermolecular oligomerization. In contrast, our method uses a combination of a weakly activated thioester and a chemoselective transthioesterification, resulting in a proximity-driven acyl transfer that avoids the limitations of conventional methods. Furthermore, because the intramolecular transthioesterification is favored under equilibration conditions of ring-chain tautomerization, moderate to high concentrations of peptides can be used for the cyclization reaction and no polymerization is observed. In summary, the biomimetic ligation of unprotected peptides at methionine site provide a useful entry to the repertoire of ligation methods, particularly when there is no cysteine in the peptide sequence.

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