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An Fmoc Compatible, O to S Shift-Mediated Procedure for the Preparation of C-Terminal Thioester Peptides

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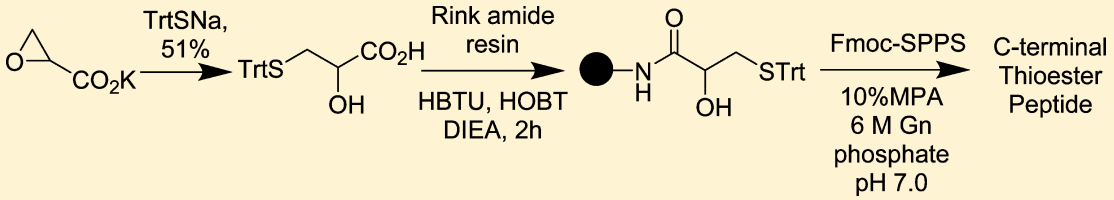
Supporting Information



ABSTRACT: We report a practical 2-hydroxy-3-mercapto-propionic acid (Hmp)/2-methylpiperidine (2-MP) based Fmoc chemistry procedure to prepare the C-terminal Hmp peptides, which serve as the precursors of C-terminal thioester peptides. The subsequent O to S acyl shift and thiol-exchange mediated thioester conversion of the crude precursor peptides can be accomplished smoothly under mild conditions to provide the desired thioester peptides with good yield and high quality. This is a highly adaptable approach, and we envision its broad application in the preparation of C-terminal thioester peptides.

INTRODUCTION

Building upon the well-established techniques of solid-phase peptide synthesis,[1,2] the seminal development of native chemical ligation (NCL) by Kent and co-workers has enabled the efficient assembly of longer peptides (>50 residues) and even of small to medium size proteins.[3] The C-terminal thioester, a key component of NCL, has been traditionally prepared through Boc/Bzl chemistry. However, the required use of hydrogen fluoride as well as the incompatibility with certain synthetic modifications has limited the use of the Boc/Bzl approach and has prompted the search for Fmoc/*t*-Bu based methods. Preparation of peptide C-terminal thioesters by conventional Fmoc/*t*-Bu chemistry is compromised by the instability of the thioester during the Fmoc deprotection step (20% piperidine in dimethylformamide, DMF). To overcome this challenge, numerous alternative approaches have been explored over the past decade.[4−54] Most recently, the unique intramolecular N to S[33−47] and O to S[48−54] acyl shift mediated methods have gained favor, and associated new procedures continue to be reported. In each case, a precursor peptide is synthesized and then is converted to the desired thioester either in situ or prior to the ligation. The Hmp (2-hydroxy-3-mercapto-propionic acid) mediated intramolecular O to S acyl shift, in particular, attracted our attention by virtue of its fast conversion rate, clean product distribution, and mild reaction conditions.[49,50] These characteristics are essential for producing homogeneous, high-quality peptides for pharmaceutical applications. It is known that certain existing post solid-phase treatments have been shown to cause C-terminal epimerization[8,29,55] or side chain modifications.[29,56−58] In addition, the relatively harsh reaction conditions (too acidic or basic, extended reaction time, or high temperatures (>37 °C)) utilized in some procedures contribute to peptide degradation.[59] However, despite the advantages of the Hmp process, the current methods[49−52,60−62] are suboptimal because of their low yields and synthetic complexity, which have limited their practical application. To facilitate broader utilization of the Hmp procedure, we report a practical, Fmoc compatible method to prepare C-terminal Hmp peptides. In addition, we present the results of a systematic examination of the influence of the residue P(−1) (the residue coupled to Hmp) on the thioester yield. Finally, we also disclose a validated, convenient one-pot protocol to generate the final peptide thioesters for use in ligation.

RESULTS AND DISCUSSION

Synthetic Methods of Hmp Peptides. Botti et al.'s elegant approach of directly oxidizing the resin-bound Cys[63] to the corresponding Hmp is compromised by the inefficiency of on-resin diazotization/hydrolysis (Supporting Information Scheme S1).[49] While George et al. have overcome this limitation by preforming the 2-(*t*-butyl-dimethyl-silanyloxy)-3-*t*-butyldisulfanyl-propionic acid building block, the utility of this multistep procedure is limited by its complexity (Supporting Information Scheme S2).[52] To further optimize the synthetic procedure for conciseness and practicality, we have developed an alternative in the form of a trityl protected Hmp linker **3**[64,65] which can be readily obtained via a single-step, regiospecific epoxide ring-opening of the commercially available potassium oxirane-2-carboxylate **1** with sodium triphenylmethanethiolate in DMF (Scheme 1). After a simple workup involving reverse extraction, acidification, and re-extraction, **3** was obtained in 51% yield and was ready for use without additional purification.

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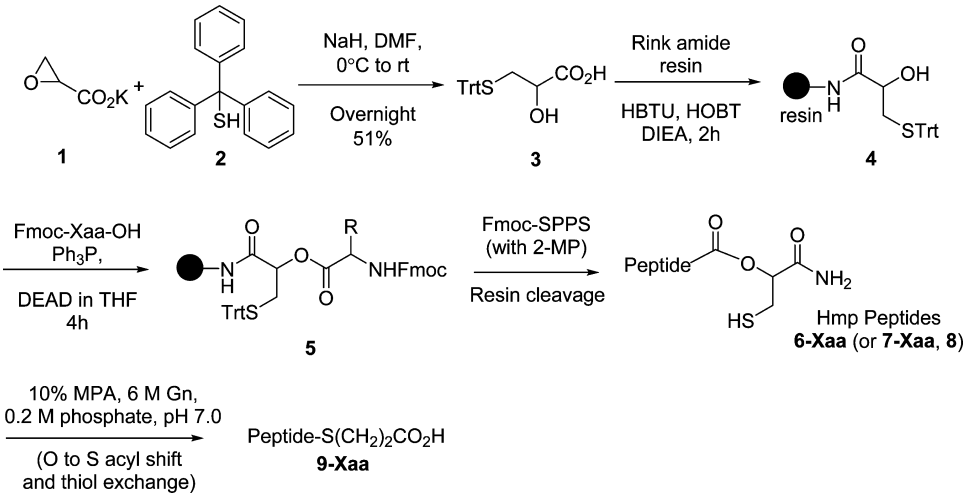
Scheme 1. Hmp Based Procedure for the Preparation of C-Terminal Thioester Peptide



3 can be efficiently coupled without protection of the α-hydroxyl group to commercial Rink amide resin under standard *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU)/hydroxybenzotriazole (HOBT)/Diisopropylethylamine (DIEA)[66] conditions to provide resin **4**. A two-step solid-phase procedure was initially attempted to prepare **4** by coupling **1** to the resin followed by opening of the resin-bound epoxide with sodium triphenylmethanethiolate. Although this procedure yielded the desired product when preparing the random model peptide **6-Gly** (YQHVFLGHmp-NH_2, Table 1), a side product with a 16 Da lower molecular weight was consistently identified during several test syntheses (Supporting Information Figure S1). This side product was presumably the result of a competing epoxide ring-opening by the resin-bound amine group during the loading step.

Table 1. Yield and Characterization of the Model Hmp Peptides 6-Xaa (YQHVFLXaaHmp-NH_2)

			ESI-MS $(M + H)^+$			
peptide[a]	amount (mg) obtained from 0.10 mmol synthesis	yield	expected	observed	purity by RP-HPLC	residue (P−1) coupling method[b]
6-Ala	51	52%	980.5	980.3	93%	**2**
6-Asp	<5	<5%	1024.4	1024.3	76%[c]	**1**
6-Glu	30	29%	1038.5	1038.2	95%	**1**
6-Phe	30	28%	1056.5	1056.3	90%	**2**
6-Gly	25	26%	966.4	966.2	95%	**2**
6-His	30	29%	1046.5	1046.3	90%	**2**
6-Ile	35	34%	1022.5	1022.3	96%	**2**
6-Lys	30	29%	1037.5	1037.3	96%	**2**
6-Leu	30	29%	1022.5	1022.3	92%	**2**
6-Met	30	29%	1040.5	1040.3	85%	**2**
6-Asn	<5	<5%	1023.4	1023.2	70%[c]	**1**
6-Gln	30	29%	1036.5	1037.3	92%	**2**
6-Arg	<5	<5%	1065.5	1065.3	55%[c]	**1**
6-Ser	30	30%	996.5	996.2	94%	**2**
6-Thr	30	30%	1010.5	1010.3	90%	**2**
6-Trp	30	27%	1095.5	1095.3	95%	**1**
6-Tyr	30	28%	1072.5	1072.3	90%	**2**
6-Val	70	69%	1008.5	1008.3	96%	**2**

[a]All of **6-Xaa** were repared by using piperidine as the Fmoc removal agent. [b]Method **1**: HBTU/HOBT/DIEA/DMAP(cat.); method **2**: Mitsunobu reaction. [c]Crude peptides.

The subsequent coupling the of Fmoc-protected Gly, Ala, and Tyr(*t*-Bu) residues to the α-hydroxyl group of resin-bound Hmp proceeded efficiently by employing a standard HBTU/HOBT/DIEA/DMAP(cat.) protocol. Assembly of the complete sequence by means of automated peptide synthesis followed by cleavage using standard trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/H_2O treatment successfully afforded model peptides **6-Xaa** (Xaa: Gly, Ala, and Tyr). These conditions, however, gave poor results in the case of the C-terminal β-branched residue Val. In the course of investigating an alternative method for coupling Val, use of the Mitsunobo reaction[67−70] was shown to give dramatically improved yields (Supporting Information Figure S2). The efficiency of the Mitsunobu coupling method was then assessed for all naturally occurring amino acids with the exception of Cys. The efficiency was found to be acceptable for the majority of Fmoc-protected residues, however, Arg(Pbf), Glu(*t*-Bu), Pro, Asp(*t*-Bu), Asn(Trt), and Trp(Boc) gave less than satisfactory results presumably because of their bulky side-chain protecting groups as well as the tendency of Pro to undergo diketopiperazine formation. We suspect that the sterically hindered side-chains may have restricted access of the α-carboxy group to the triphenylphosphine activated α-hydroxyl of the resin-bound Hmp. A variety of conditions were examined for coupling Arg(Pbf), Glu(*t*-Bu), Asp(*t*-Bu), Asn(Trt), and Trp(Boc) to Hmp, including the use of symmetric anhydrides (at room temperature (rt) or 60 °C), the bis-(trichloromethyl) carbonate (BTC) mediated in situ generation of acyl chloride,[71] trichloroacetimidate activation,[72] HBTU/HOBT/DIEA/DMAP(cat.), and the Mitsunobu reaction using PMe_3 in place of the sterically hindered PPh_3.[69] Surprisingly, only the HBTU/HOBT/DIEA/DMAP(cat.) method worked effectively, although the yields for Asn(Trt), Asp(*t*-Bu), and Arg(Pbf) remained modest. Most of the model peptides **6-Xaa** for the investigation of O to S acyl shift process were purified by RP-HPLC reverse phase-high performance liquid chromatography; the crude **6-Asn**, **6-Asp**, and **6-Arg** materials were lyophilized and were used directly because of the low recovery (Table 1). Some of the Hmp peptides **6-Xaa** eluted as two close peaks on the RP-HPLC corresponding to the two diastereoisomers

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Table 2. Yield and Characterization of the Model Hmp Peptides 7-8

peptide number	sequence	yield	expected MW	observed MW[c]	purity by RP-HPLC
7-Ala[a]	ADEFGHIKLMNPQRSTVYAHmp-NH_2	17%	2280.6	2279.8	98%
7-Gly[a]	ADEFGHIKLMNPQRSTVYGHmp-NH_2	9%	2266.6	2266.4	98%
7-Gly[b]	ADEFGHIKLMNPQRSTVYGHmp-NH_2	39%	2266.6	2266.4	98%
7-Gln[a]	ADEFGHIKLMNPQRSTVYQHmp-NH_2	28%	2337.6	2337.4	98%
7-Val[a]	ADEFGHIKLMNPQRSTVYVHmp-NH_2	34%	2308.6	2308.4	98%
7-Tyr[a]	ADEFGHIKLMNPQRSTVYYHmp-NH_2	27%	2372.7	2372.2	98%
8[a]	ADEFGHIKLMN(Ac_3AcNH-β-Glc)PQRSTVYLHmp-NH_2	25%	2652.0	2651.6	95%

[a]Fmoc was removed by piperidine (11 min). [b]Fmoc was removed by double 2-MP treatments (11 min × 2). [c]After manual deconvolution of the ESI-MS signals.

resulting from the racemic **3** used in the peptide synthesis (Supporting Information Figure S6).

To test the practical utility of this method, we prepared an additional set of longer, random model peptides **7-Xaa** (ADEFGHIKLMNPQRSTVYXaaHmp-NH_2, Xaa: Ala, Gln, Gly, Tyr, or Val) as well as the *N*-linked glycopeptide **8** (ADEFGHIKLMN(Ac_3AcNH-β-Glc)PQRSTVYLHmp-NH_2). Resin cleavage in all cases provided crude products with comparable analytical purities to that obtained from conventional synthesis of similar length of peptides, however, in somewhat lower yield (Table 2 and Supporting Information Figure S7). In one instance, the yield was 9% for peptide **7-Gly** with a Gly at residue P(−1). In another example, the cleavage of a 40-mer C-terminal Hmp peptide with Gly at P(−1) yielded no peptide material. This was presumably a consequence of the instability of the oxo-ester bond between residue P(−1) and Hmp during Fmoc deprotection involving treatment with 20% piperidine in DMF for 11 min for each cycle (total exposure: 7.5 h for a 40-mer peptide). To confirm this hypothesis, the resin-bound peptide **6-Gly** was treated with 20% piperidine in DMF, and the peptide release was monitored through analysis of the DMF supernatant using liquid chromatography-mass spectrometry (LC-MS) (Supporting Information Figure S3). We found that these conditions effectively resulted in aminolysis and premature release of peptide YQHVFLG-$N(CH_2)_5$ from the resin. The aminolysis rate appeared to be residue P(−1) dependent on the basis of the yields of peptides **7-Xaa** with Gly being the most susceptible. To overcome this limitation, we selected the more sterically hindered 2-MP (2-methyl piperidine)[73,74] in the deprotection step as an alternative to piperidine. When testing 2-MP in place of piperidine in the above protocol, we detected no aminolysis even after a 24 h treatment period (an exposure time equivalent to a 65-residue peptide synthesis with double 2-MP treatments per cycle) (Supporting Information Figure S3). The resynthesis of **7-Gly** employing a double 2-MP treatment (11 min × 2) for Fmoc deprotection afforded crude product with identical purity as **7-Gly** prepared using piperidine but in a dramatically improved yield of 39% after RP-HPLC purification (Table 2, Figure 4, and Supporting Information Figure S7).

The Impact of pH and Residue P(−1) on the Yield of Thioester Conversion. Having optimized the synthesis of the Hmp peptides, we next investigated the efficiency of thioester formation through O to S acyl shift and the subsequent thiol-exchange reactions using model peptides **6-Xaa** (Table 3). We initially focused on the pH dependence of this transition and later assessed the impact of the residue P(−1) on the thioester yield at the optimal pH. Finally, we screened several solvent systems to minimize the hydrolysis and to maximize the thioester formation.

Table 3. Sequences of the Model Hmp Peptides 6-Xaa and Their Resulting C-Terminal Thioester Peptides 9-Xaa and 10

peptide number	sequence
6-Xaa	YQHVFLXaaHmp-NH_2 (Xaa: Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Ser, Thr, Trp, Tyr, or Val)
9-Xaa	YQHVFLXaa-S$(CH_2)_2CO_2H$ (Xaa: Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Ser, Thr, Trp, Tyr, or Val)
10	YQHVFLG-S(1,4-C_6H_4)CH_2CO_2H

The conversions were performed in the commonly used 0.20 M sodium phosphate buffer containing 6 M Gn (guanidine). Model peptide **6-Gly** was used to assess the influence of pH on thioester yield with 2% aliphatic MPA (3-mercaptopropionic acid) or 50 mM of the aromatic MPAA (4-mercaptophenylacetic acid) as the free thiol source (Figure 1, Supporting Information Tables S1 and S2). No conversion for either thiol was observed at or below pH 3 at the 6 h time point. Measurable conversion did occur at pH 5, although the rate was relatively low. At pH 6, however, the conversion was completed within 30 min for MPAA and within 90 min for MPA, while at pH 7 or higher, both reactions finished within 5 min. MPA ester **9-Gly** (YQHVFLG-S$(CH_2)_2CO_2H$) was reasonably stable at pH 8 or below; however, the hydrolysis of the corresponding MPAA ester **10** (YQHVFLG-S(1,4-C_6H_4)CH_2CO_2H) became significant at pH 6.5 and higher (Figure 1). Because of the lower stability of the MPAA esters, we focused on the MPA esters when examining the influence of residue P(−1). In addition to C-terminal amide peptide **6-Gly**, the C-terminal acid peptide **11** (YQHVFLGHmp) was also prepared and tested. However, the C-terminal acid **11** was significantly more prone to hydrolysis (~85%) when compared to the amide **6-Gly** (~20%) in the O to S acyl shift step.

In the following experiment, peptides **6-Ala**, **6-Tyr**, and **6-Val** were tested at pH 6.5, 7.0, 7.5, and 8.0 in 2% MPA, 6 M Gn, and 0.2 M sodium phosphate buffer (Supporting Information Tables S3−S5 and Figure S4). At pH 7.0 and above, the thioester formation was judged complete within 30 min for **6-Ala** and **6-Tyr** and within 60 min for **6-Val**. The resulting MPA thioesters **9-Xaa** were stable under these conditions with yields of around 80% for **9-Ala** and **9-Tyr** but disappointingly of less than 30% for **9-Val**. The modest yield of **9-Val** was presumably due to the steric influence of the β-branched side chain which slowed the O to S acyl shift sufficiently to allow the competing hydrolysis of the oxo-ester to prevail to a significant degree. On the basis of this hypothesis, we reasoned that one of the Hmp peptide diastereoisomers may undergo acyl shift more efficiently than the other. The racemic Hmp used in the synthesis yielded two

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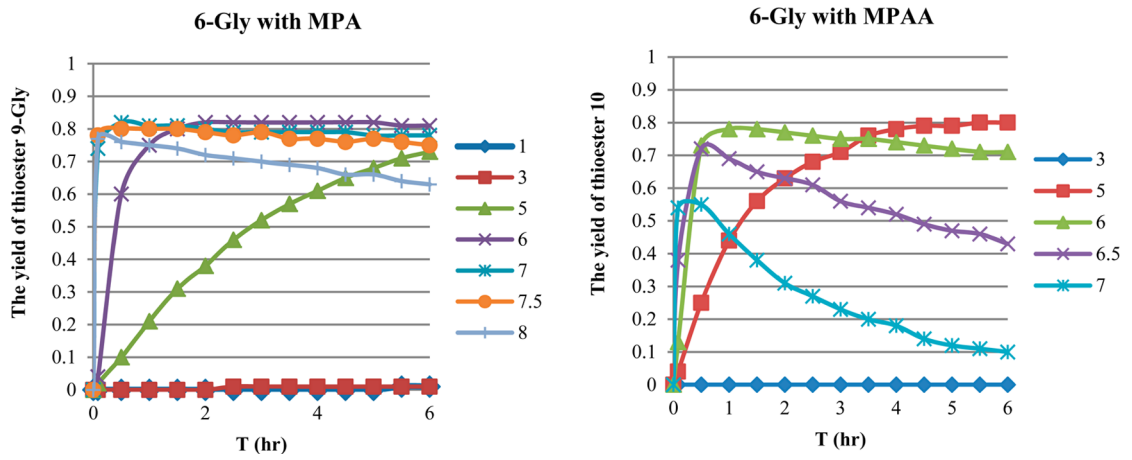

Figure 1. The impact of pH on O to S acyl shift yield (**6-Gly** in 2% MPA or 50 mM MPAA, 6 M Gn, and 0.2 M sodium phosphate buffer).

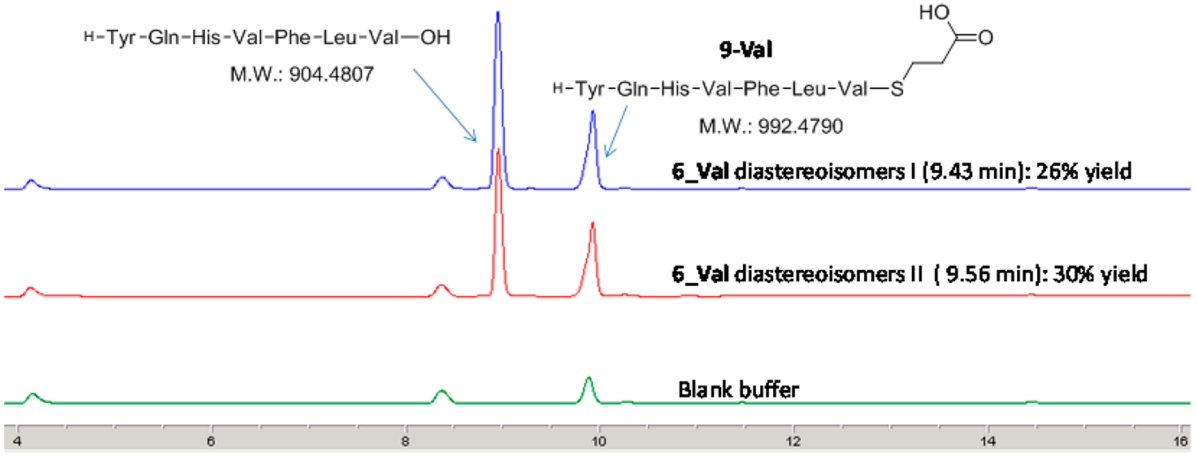

Figure 2. The impact of stereochemistry of Hmp on thioester conversion yield of **6-Val** to **9-Val** (the spectra were collected at 220 nM).

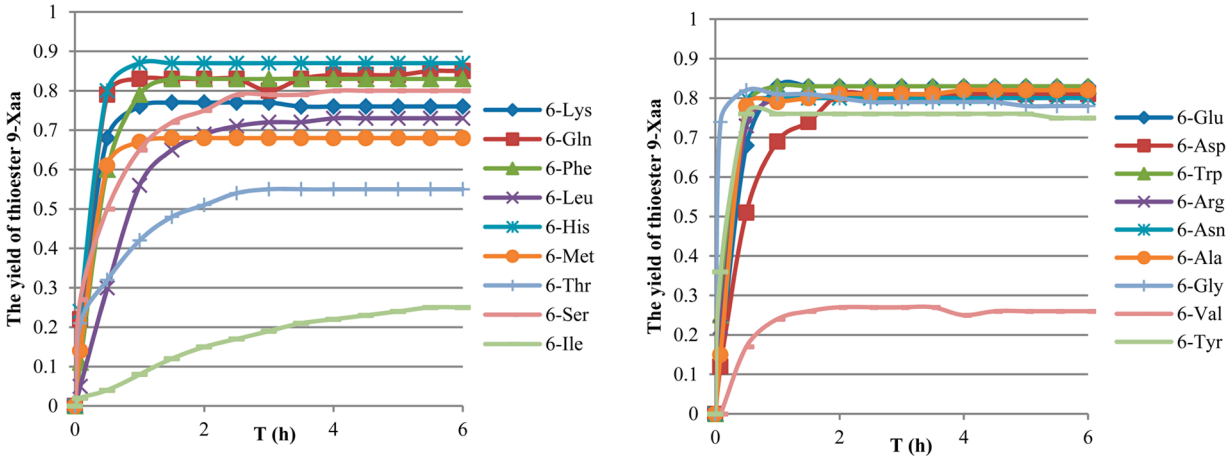

Figure 3. The impact of residue P(−1) on the O to S acyl shift yield (**6-Xaa** in 2% MPA, 6 M Gn, and 0.2 M sodium phosphate buffer, pH 7.0).

diastereoisomers of peptide **6-Val**, which resolved cleanly using RP-HPLC, permitting purification of each to homogeneity (Supporting Information Figure S2). However, the acyl shift yields for the two **6-Val** diastereoisomers were not dramatically different: 26% versus 30% (Figure 2).

All remaining peptides **6-Xaa** were tested at the fixed optimal pH of 7.0 (Figure 3 and Supporting Information Table S6). To summarize, the 2% MPA in 6 M Gn and 0.2 sodium phosphate buffer (pH 7.0) condition afforded 70−80% yield of thioester product for most of the P(−1) residues of model peptide **6-Xaa** while only 25−50% for the β-branced Val, Ile, and Thr.

To improve the O to S acyl shift efficiency, peptide **6-Val** was selected as a model to screen additional conditions. Because the hydrolysis appeared to be the only side reaction, we initially focused on anhydrous conditions. However, it was found that when **6-Val** was treated with thiols MPA, MESNa (sodium 2-mercaptoethanesulfonate), β-mercaptoethanol, MPAA, or thiophenol in anhydrous DMF, the result was either hydrolysis

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(with DIEA or imidazole as base) or no conversion (with pyridine, 2,4,6-lutidine, or no base). We then turned to a mixture of organic and aqueous solvent using various concentrations of NMP (*N*-methylpyrrolidone) and 2% MPA in 6 M Gn and 0.2 M sodium phosphate buffer (pH 7.0). Surprisingly, we found that 100% aqueous conditions gave the best yield (28%) and that no desired thioester was observed when the aqueous component was lower than 50%. With these results, we re-examined the original aqueous condition, and we tested the impact of each buffer component (Supporting Information Figure S5). The results suggested that 10% MPA in 6 M Gn and 0.2 M sodium phosphate buffer (pH 7.0) was an optimal condition, which gave the best yield of 56% for **6-Val** and which also provided excellent solubility for the peptides.

The thioester formation efficiency was then re-examined for all the model peptides **6-Xaa** under this optimized condition. The yield for **9-Val** and **9-Thr** had been significantly improved to 56% and 77%, respectively, while the yields of the additional peptides had been modestly increased to a range of 80−90%, the only exception being **9-Ile** which remained at 30% (Table 4).

Table 4. Yield of Conversion of Model Peptides 6-Xaa to Thioester 9-Xaa under the Optimal Conversion Condition: 10% MPA in 6 M Gn and 0.2 M Sodium Phosphate Buffer (pH 7.0)

peptide	yield	peptide	yield	peptide	yield
9-Ala	85%	**9-Ile**	30%	**9-Arg**	80%
9-Asp	83%	**9-Lys**	82%	**9-Ser**	85%
9-Glu	83%	**9-Leu**	85%	**9-Thr**	77%
9-Phe	80%	**9-Met**	84%	**9-Trp**	80%
9-Gly	90%	**9-Asn**	80%	**9-Tyr**	83%
9-His	90%	**9-Gln**	86%	**9-Val**	56%

One-Pot Hmp/2-MP Based Method for C-Terminal Thioester Peptide. Although the Hmp peptides can be used directly for the native chemical ligation, the competing hydrolysis of the Hmp peptides during the ligation is a significant limitation of the existing methods, which hinders the progress of the reaction and isolation of the final products (see in situ ligation example in Supporting Information Figure S8A).[49−52] In addition, the two diastereoisomer Hmp peptides formed from racemic Hmp **3** elute closely on RP-HPLC (rather than eluting as a single component) which complicates their purification. To address these constraints as well as to simplify the overall process, we conducted the O to S acyl shift and the subsequent thiol-exchange using the crude Hmp peptide immediately following resin cleavage to provide the requisite thioester peptides for the peptide ligation after a single RP-HPLC purification (Scheme 2). This procedure also ensures that the thioester conversion takes place at the optimal condition to maximize the yield (see examples in Supporting Information Figure S8A and S8B). To validate this approach, the crude **7-Gly** (prepared by the 2-MP method with double treatments) obtained directly from resin cleavage was treated with 10% MPA in 6 M guanidine and 0.2 M sodium phosphate (pH 7.0) to facilitate the O to S acyl shift and thiol-exchange and was judged to be completely converted within 20 min by LC-MS. Subsequent RP-HPLC purification provided the desired peptide C-terminal MPA thioester **12** in 38% yield after lyophilization (Figure 4, Table 5, and Supporting Information Figure S9). A 34-mer thioester **13** with Phe at the C-terminus (SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF-S$(CH_2)_2CO_2H$, N-terminal 34 residues of human parathyroid hormone) was succefully prepared by this method with 28% overall yield and high purity as confirmed by LC-MS (Figure 4, Table 5, and Supporting Information Figure S9). Interestingly, although 2-MP was considered less effective for Fmoc removal than piperidine,[73,74] we found that a single 2-MP treatment (11 min) actually gave identical crude purity as the double treatments when preparing **12** (Figure 4, Table 5, and Supporting Information Figure S9). The efficiency of single 2-MP treatment was further confirmed by the successful preparation of a 35-mer peptide thioester **14** with Gly at the C-terminus (SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNFG-S$(CH_2)_2CO_2H$) with a 14% yield and excellent purity as determined by LC-MS (Figure 4, Table 5, and Supporting Information Figure S9).

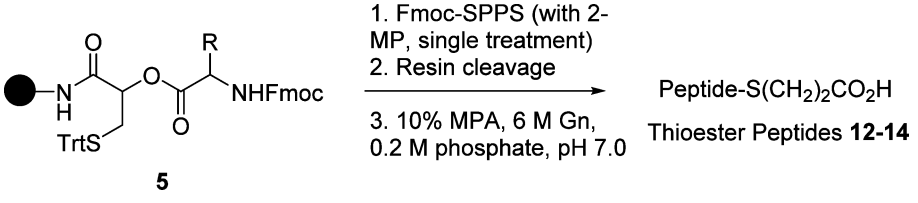
CONCLUSION

In conclusion, we have developed a practical, Hmp/2-MP based Fmoc chemistry procedure enabling efficient preparation of C-terminal Hmp peptides. Subsequent O to S acyl shift and thiol-exchange-based conversion of the crude precursor to the thioester can be quickly accomplished under very mild conditions to afford the desired thioester after a single RP-HPLC purification in good yield. We believe this optimized Hmp/2-MP procedure is an effective and readily adaptable method for the preparation of homogeneous peptide C-terminal thioesters, and we envision its utilization in NCL and other applications[75−81] requiring peptide thioesters.

EXPERIMENTAL SECTION

General Information. All solvents [*N*,*N*′-dimethylforamide (DMF), methanol (MeOH), dichloromethane (DCM), acetonitrile (ACN), diethyl ether (Et_2O), etc.] and reagents [hydroxybenzotriazole (HOBT), *N*,*N*′-diisopropylcarbodiimide (DIC), *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), piperidine, 2-methylpiperidine (2-MP), trifluoroacetic acid (TFA), 3-mercaptopropionic acid (MPA), 4-mercaptophenylacetic acid (MPAA), thiophenol, sodium 2-mercaptoethanesulfonate (MESNa), *β*-mercaptoethanol, potassium oxirane-2-carboxylate (vendor: Acesys Pharmatech), guanidine (Gn), triisopropylsilane (TIS), diethyl azodicarboxylate solution (40% in toluene) (DEAD), etc.] were purchased and used directly. Water (H_2O) was obtained from Milli-Q water purification system (Millipore). Polystyrene Rink amide (RAM)

Scheme 2. One-Pot Hmp Based Procedure for the Preparation of C-Terminal Thioester Peptide



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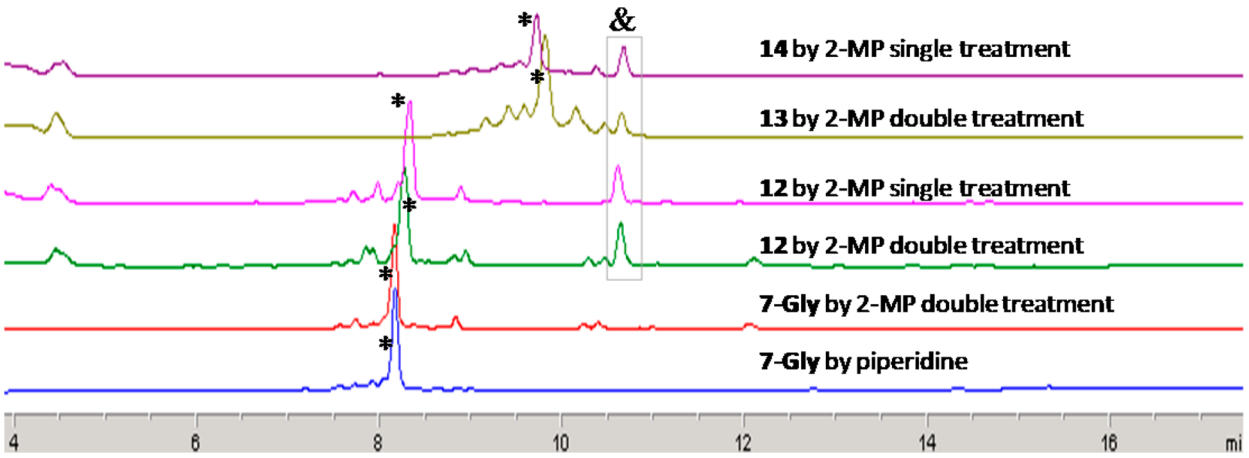

Figure 4. Analytical HPLC traces of crude Hmp peptide **7-Gly** and thioester peptides **12**–**14** (at 220 nm). Peaks marked with * are the desired products; peaks marked with & are MPA related compounds (not peptides as determined by mass spectrometry).

Table 5. Yield and Characterization of the Model MPA Thioester Peptides 12–14

peptide number	sequence	yield	expected MW	observed MW[c]	purity by RP-HPLC
12[a]	ADEFGHIKLMNPQRSTVYG-S$(CH_2)_2CO_2H$	38%	2251.5	2251.0	98%
12[b]	ADEFGHIKLMNPQRSTVYG-S$(CH_2)_2CO_2H$	36%	2251.5	2250.6	98%
13[a]	SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF-S$(CH_2)_2CO_2H$	28%	4205.8	4205.4	98%
14[b]	SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNFG-S$(CH_2)_2CO_2H$	14%	4262.9	4262.0	98%

[a]Fmoc was removed by double 2-MP treatments (11 min × 2). [b]Fmoc was removed by single 2-MP treatment (11 min). [c]After manual deconvolution of the ESI-MS signals.

resin and Chlorotrityl Polystyrene resin were purchased from Rapp Polymere GMBH. Fmoc chemistry based amino acid cartridges were purchased from several vendors and were used directly. LC-MS system: 1100 series liquid chromatography mass spectrometer, model G1956A/B. General analytical RP-HPLC condition: All the samples were analyzed by using a Waters SymmetryShield RP18 Column (cat. no. 186000179, 3.5 μm, 4.6 × 100 mm.): a linear gradient from 6% aqueous ACN (0.1% trifluoroacetic acid) to 60% aqueous ACN (0.1% trifluoroacetic acid) over 14 min at a flow rate of 1.0 mL/minute.

2-Hydroxy-3-(triphenylmethyl)thio-propanoic Acid 3. Triphenylmethanethiol (11.0 g, 40 mmol) was dissolved in anhydrous DMF (150 mL) under the protection of dry N_2 gas, and this solution was cooled to 0 °C by an ice–water bath. NaH (60% in mineral oil, 1.6 g, 40 mmol) was added carefully through several portions, and the resulting solution was stirred at 0 °C for an additional 15 min. Potassium oxirane-2-carboxylate solid (2.60 g, 20 mmol) was added in one portion, and the resulting reaction mixture was then gradually warmed to rt and was stirred overnight before being poured into H_2O (500 mL) and being extracted with Et_2O (100 mL × 3). The Et_2O layer was discarded, and the aqueous phase was acidified by 1 N HCl to pH 3–4 (determined by pH strips) and was extracted by EtOAc (100 mL × 3). The combined EtOAc was washed by brine and was dried over anhydrous Na_2SO_4 for 2 h. EtOAc was then removed by rotary evaporator to provide product **3** as a pale yellow solid (3.73 g) in 51% yield, which was ready for use without further purification. ESI-MS (+VE) m/z: 387.2 $(M + Na)^+$. HR-ESI MS calcd for $C_{22}H_{20}NaO_3S$ $(M + Na)^+$, 387.1025; found, 387.1029. ^{1}H NMR (400 MHz, DMSO-d_6) 7.35–7.15 (15H, m), 5.55 (1 H, bs), 3.85 (1 H, m), 2.32 (2 H, m). ^{13}C NMR (100 MHz, DMSO-d_6) 174.1, 144.9, 144.4, 130.0, 129.8, 129.6, 129.3, 128.6, 128.5, 128.3, 128.0, 127.6, 127.4, 127.2, 69.4, 66.2, 36.4.

Preparation of Resin 4 by Using 3. Rapp Polymere Rink amide resin (300 mg, 0.20 mmol) was treated by 20% piperidine in DMF (6 mL) for 20 min to remove the resin-bound Fmoc group. After DMF washes (6 mL × 6), the resin was treated with a mixture of HBTU (380 mg, 1.0 mmol), HOBt (135 mg, 1.0 mmol), DIEA (0.18 mL, 1.0 mmol), and Hmp **3** (365 mg, 1.0 mmol) in DMF (3 mL) for 2 h at rt. The resulting resin **4** was washed by DMF (6 mL × 6) and Et_2O (3 mL × 3).

General Procedure of Coupling Residue P(−1) by Mitsunobu Reaction. The above resin **4** (160 mg, 0.10 mmol, prepared through Hmp **3**) was washed by anhydrous THF (3 mL × 3) to remove any moisture and was treated with a mixture of Fmoc-Xaa–OH (1.0 mmol), DEAD (40 wt % in toluene) (0.46 mL, 1.0 mmol), and triphenylphosphine (262 mg, 1.0 mmol) in anhydrous THF (5 mL) at rt for 4 h. The resulting resin was washed by DMF (6 mL × 3).

General Procedure of Coupling Residue P(−1) by HBTU/HOBT/DIEA/DMAP(cat.) Condition. The resin **4** (160 mg, 0.10 mmol, prepared through Hmp **3**) was treated with a mixture of Fmoc-Xaa–OH (1.0 mmol), HBTU (380 mg, 1.0 mmol), HOBt (135 mg, 1.0 mmol), DIEA (0.18 mL, 1.0 mmol), and a catalytical amount of DMAP (1.2 mg, 0.01 mmol, 10% of resin substitution) in DMF (3 mL) for 2 h at rt. This coupling step was repeated once. The resulting resin was washed by DMF (6 mL × 3).

Preparation of Resin 4 by Two-Step Solid-Phase Procedure. Rapp Polymere Rink amide resin (300 mg, 0.20 mmol) was treated with 20% piperidine/DMF (6 mL) at rt for 20 min to remove the Fmoc protection. After DMF washes (6 mL × 6), the resulting resin was mixed with potassium oxirane-2-carboxylate **1** (126 mg, 1.0 mmol), HBTU (380 mg, 1.0 mmol), HOBt (135 mg, 1.0 mmol), and DIEA (0.18 mL, 1.0 mmol) in DMF (3 mL) at rt for 2 h and then was washed by DMF (6 mL × 3). In a separate flask, a fresh sodium triphenylmethanethiolate solution was prepared by adding triphenylmethanethiol (556 mg, 2.0 mmol) into a NaH suspension (60% in mineral oil, 72 mg, 1.8 mmol) in anhydrous DMF (5.0 mL) at 0 °C and by stirring for additional 15 min. The above resin was briefly washed by anhydrous DMF (6 mL) to remove the moisture before being mixed with the fresh sodium triphenylmethanethiolate solution for 60 min at rt. The resulting resin **4** was then washed by DMF (6 mL × 6) and Et_2O (3 mL × 3). DIEA, DBU, and Et_3N were also tried as the base for the epoxide ring-opening by triphenylmethanethiol; however, this conversion occurred efficiently only when NaH was used.

Peptide 6-Gly (YQHVFLGHmp-NH_2). Starting with the resin **4** (160 mg, 0.10 mmol) prepared through Hmp **3** or the two-step solid-

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phase procedure as described above, the next Gly was coupled by mixing the resin with Fmoc-Gly-OH (300 mg, 1.0 mmol), HBTU (380 mg, 1.0 mmol), HOBt (135 mg, 1.0 mmol), DIEA (0.18 mL, 1.0 mmol), and a catalytical amount of DMAP (1.2 mg, 0.01 mmol, 10% of resin substitution) in DMF (3 mL) for 2 h at rt. This coupling step was repeated once. The rest of the residues were assembled by using peptide synthesizer ABI 433 with the standard Fmoc_HOBT_DCC method (see the protocol details as described below). The resin cleavage was conducted by treating the 0.10 mmol resin with 10 mL TFA solution containing 2.5% TIS, 2.5% H_2O at rt for 2 h with gentle shaking. The resin was filtered off, and the filtrate was treated with 80 mL cold Et_2O. The crude peptide was collected by centrifugation (see Supporting Information Figure S1 for the analytical HPLC traces of the crude products).

Peptide 6-Val (YQHVFLVHmp-NH_2). Starting with the resin **4** (160 mg, 0.10 mmol, prepared through Hmp **3**), the next Fmoc-Val-OH was coupled by either HBTU/HOBT/DIEA/DMAP(cat.) or Mitsunobu reaction as described above. The rest of the residues were assembled by using peptide synthesizer ABI 433 with the standard Fmoc_HOBT_DCC method (see the protocol details as described below). The resin cleavage was conducted by treating the 0.10 mmol resin with 10 mL TFA solution containing 2.5% TIS, 2.5% H_2O at rt for 2 h with gentle shaking. The resin was filtered off, and the filtrate was treated with 80 mL cold Et_2O. The crude peptide was collected by centrifugation and was washed with cold Et_2O (40 mL × 2) (see Supporting Information Figure S2 for the analytical HPLC traces of the crude products).

General Procedure for Synthesizing Model Peptides 6-Xaa, 7-Xaa, and 8. All the syntheses started with resin **4** (160 mg, 0.10 mmol, prepared through Hmp **3**). The residue P(−1) was coupled by using the Mitsunobu reaction. The rest of the residues were assembled on the automatic peptide synthesizer ABI433 with standard Fmoc_HOBT_DCC 0.10 mmol protocol or with the modified 2-MP protocol (see Table 3 for details). The only difference between these two protocols is that the deprotection step was repeated in 2-MP protocol (11 min × 2 vs 11 min).

Resin cleavage was conducted by treating 0.10 mmol resin with 10 mL TFA solution containing 2.5% TIS, 2.5% H_2O at rt for 2 h with gentle shaking. The resin was filtered off, and the filtrate was treated with cold Et_2O (80 mL). The crude peptide was collected by centrifugation, was washed with cold Et_2O (40 mL × 2), and was purified by preparative RP-HPLC using a Waters SymmetryPrep C18 Column (cat. no. WAT066245, 7 μm, 19 × 300 mm) with a linear gradient from 5% aqueous ACN (0.05% TFA) to 50% aqueous ACN (0.05% TFA) over 80 min at a flow rate of 15 mL/minute. The peptides were eluted between 30 and 45 min depending on the sequence. The final RP-HPLC fractions with desired product were pooled together and were freeze-dried to afford the lyophilized powder.

Table 6. Standard ABI433 Fmoc_HOBT_DCC Protocol and the Modified 2-MP Protocol (Double Treatments)

	standard Fmoc_HOBT_DCC (0.10 mmol) protocol	2-MP (0.10 mmol) protocol
coupling reagent	1 M DIC/DMF (1.0 mL)	1 M DIC/DMF (1.0 mL)
coupling reagent	1 M HOBt/DMF (1.0 mL)	1 M HOBt/DMF (1.0 mL)
Fmoc amino acid	1.0 mmol cartridge	1.0 mmol cartridge
solvents	DMF and DCM	DMF and DCM
activation time	16 min	16 min
coupling time	37 min	37 min
deprotection agent and time	20% piperidine in DMF, 11 min	20% 2-MP in DMF, 11 min, and 20% 2-MP in DMF, 11 min

Oxo-Ester Bond (between Residue P(−1) and Hmp) Stability Study with 20% Piperidine and 20% 2-MP in DMF. RAPP Polymere resin bound-peptide **6-Gly** (50 mg) was treated with 20% piperidine or 20% 2-MP in DMF (1 mL). At each selected time point, the supernatant (DMF phase) (50 μL) was removed and treated with TFA (0.5 mL) for 20 min before diluted by ACN/H_2O (1/1, 3 mL). The resulting solution was subjected to LC-MS analysis (inject 50 μL); the HPLC traces shown below are collected at wavelength 220 nM (see Supporting Information Figure S3 for the analytical HPLC traces).

Preparation of Peptide C-Terminal Acid 11 and the Conversion to Thioester. Chlorotrityl polystyrene resin (0.20 mmol) was treated with Hmp **3** (0.40 mmol) and DIEA (1.0 mmol) in DCM (3.0 mL) at rt for 2 h. After DMF washes (6 mL × 3), the resin was treated with 5% piperidine in DMF (3 mL) to substitute all the remaining trityl chloride. The resulting resin was treated with Fmoc-Gly-OH (1.0 mmol), HBTU (1.0 mmol), HOBt (1.0 mmol), DIEA (1.0 mmol), and a catalytical amount of DMAP (0.01 mmol, 10% of resin substitution) in DMF (3 mL) for 2 h at rt. This step was repeated once. After DMF washes (6 mL × 3), the rest of the residues were assembled on the peptide synthesizer ABI433 using standard Fmoc_HOBT_DCC method. The resulting peptide was treated with 2% MPA, 6 M Gn, and 0.2 M sodium phosphate buffer (pH 7.0) for 90 min and was analyzed by LC-MS, which indicated that 15% was converted to the thioester and that 85% was hydrolyzed.

Assessment of Peptide Thioester Yield. Each peptide (1 mg/mL) was dissoved in 6.0 M guanidine, 0.20 M sodium phosphate buffer at the desired pH with 2% MPA or 50 mM MPAA. The sample was then immediately monitored by LC-MS for up to 6 h. The yield of peptide thioester was calculated by dividing the peptide thioester HPLC peak area by the sum of the peptide thioester, the peptide acid (hydrolyzed product), and any conversion intermediate. The further optimization was conducted with 5 mg/mL peptide in each testing condition with LC-MS monitoring the conversion progress (see Supporting Information Tables S1−S6).

Impact of Stereochemistry of Hmp on O to S Acyl Shift Yield by Using Two 6-Val Isomers. These two **6-Val** isomers were separated from the **6-Val** prepared with racemic Hmp (see crude analytical HPLC in Supporting Information Figure S2); each was freeze-dried. The lyophilized powder was then treated with 2% MPA, 6 M Gn, and 0.2 M sodium phosphate at pH 7.0 (∼1.0 mg/mL peptide concentration) at rt for 90 min to facilitate the O to S acyl shift and thiol-exchange. The yield of resulting thioester was calculated by the UV absorption areas at 220 nM with the baseline subtraction.

Examination of the Impact of Each Buffer Component on the Thioester Formation Yield of 6-Val. Five milligrams/milliliter of **6-Val** was dissolved in the following buffers (all at pH 7.0): 2% MPA, 6 M Gn, 0.2 M sodium phosphate; 10% MPA, 6 M Gn, 0.2 M sodium phosphate; 20% MPA, 6 M Gn, 0.2 M sodium phosphate; 10% MPA, 6 M Gn; 10% MPA, 0.2 M sodium phosphate; 10% MPA. The samples were analyzed by LC-MS after 2 h at rt (see Supporting Information Figure S5 for the analytical HPLC traces).

12 (ADEFGHIKLMNPQRSTVYG-$SCH_2CH_2CO_2H$) Prepared by the One-Pot Hmp/2-MP Procedure with Double 2-MP Treatment. Fmoc-Gly-OH was coupled to resin **4** (160 mg, 0.10 mmol, prepared through Hmp **3**) by the Mitsunobu reaction, and the rest of the residues were assembled by ABI433 using the 2-MP protocol (double treatments, 11 min × 2). The resulting resin (0.10 mmol) was treated with TFA (9.5 mL), TIS (0.25 mL), and H_2O (0.25 mL) for 2 h at rt. The resin was then filtered off, and the filtrate was treated with cold Et_2O (90 mL); the resulting crude peptide **7-Gly** was collected by centrifugation, was washed with cold Et_2O (40 mL × 2), and was air-dried (30 min). A freshly prepared 10% MPA in 6 M Gn, 0.2 M sodium phosphate (pH 7.0) 5 mL was added to the dried crude **7-Gly**. This mixture was vortexed until fully dissolved and was kept at rt for 20 min before being subjected to LC-MS analysis which indicated that the conversion to thioester had completed. This mixture was then diluted by 0.1% TFA containing H_2O (20 mL) and was purified by preparative RP-HPLC column with the same conditions as described above. The pooled fractions were freeze-dried to afford 86 mg **12** in

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38% yield and 98% purity (by RP-HPLC), ESI-MS (+VE) *m/z*: observed, 1126.5 $(M + 2H)^{2+}$ (calculated: 1126.0).

12 (ADEFGHIKLMNPQRSTVYG-SCH$_2$CH$_2$CO$_2$H) Prepared by the One-Pot Hmp/2-MP Procedure with Single 2-MP Treatment. **12** was prepared as previously described when preparing thioester **12** (using double 2-MP treatment) except single 2-MP treatment was used; the yield was 36%, and the purity was 98% by RP-HPLC (40 mg of product was obtained from 0.05 mmol resin **4**), ESI-MS (+VE) *m/z*: observed, 1126.3 $(M + 2H)^{2+}$ (calculated: 1126.0).

13 (SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF-S-(CH$_2$)$_2$CO$_2$H) Prepared by the One-Pot Hmp/2-MP Procedure with Double 2-MP Treatment. **13** was prepared as previously described for the synthesis of thioester **12** (double 2-MP treatment) in 28% yield and 98% purity (by RP-HPLC) (118 mg of product was obtained from 0.10 mmol resin **4**), ESI-MS (+VE) *m/z*: observed, 1402.8 $(M + 3H)^{3+}$ (calculated: 1402.9).

14 (SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNFG-S-(CH$_2$)$_2$CO$_2$H) Prepared by the One-Pot Hmp/2-MP Procedure with Single 2-MP Treatment. **14** was prepared as previously described for the synthesis of thioester **12** (double 2-MP treatment) except the single 2-MP treatment was used; the overall yield was 14% with 98% purity (by RP-HPLC) (20 mg of product was obtained from 0.033 mmol resin **4**), ESI-MS (+VE) *m/z*: observed, 1421.9 $(M + 3H)^{3+}$ (calculated: 1422.0).

■ ASSOCIATED CONTENT

Supporting Information

^{1}H and ^{13}CNMR spectra of **3**, the HPLC traces and mass spectrometry data, the conversion kinetic study data of model Hmp peptides **6-Xaa**, and the example of native chemical ligation by directly or stepwisely using C-terminal Hmp peptide **8**. This material is available free of charge via the Internet at http://pubs.acs.org

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Notes

The authors declare no competing financial interest.

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