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Chemical synthesis of *N*-peptidyl 2-pyrrolidinemethanethiol for peptide ligation

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ABSTRACT

Peptides carrying a C-terminal 2-pyrrolidinemethanethiol (PMT) unit were synthesized using 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide synthesis (SPPS), and were shown to ligate efficiently with cysteinyl-peptides. This novel PMT-mediated ligation tolerated many different C-terminal residues and was successfully applied to a one-pot N-to-C sequential ligation reaction and the semi-synthesis of lysine 16 acetylated histone H4, demonstrating the utility of the method in peptide and protein synthesis.

Peptide C^{α}-thioesters are the key building blocks for many convergent peptide synthesis strategies, including the well-known native chemical ligation[1] (NCL), traceless Staudinger ligation,[2] and Ag^{+}-mediated thioester ligation.[3] Due to the importance of such molecules, the chemical synthesis of thioesters has become a significant research topic in peptide chemistry.[4] Typically, peptide thioesters are prepared using *tert*-butyloxycarbonyl-(Boc) based solid-phase peptide synthesis (SPPS) whereby the peptide chain is assembled directly on a thiol linker.[5] Despite the effectiveness of this strategy, peptide thioesters bearing acid-sensitive modifications such as glycosylations and phosphorylations are difficult to access using Boc-chemistry due to the repeated use of TFA during deprotection, and the use of HF during the final cleavage step. In addition, the hazardous HF represents a deterring element to many research laboratories. Alternatively, many strategies based on 9-fluorenylmethoxycarbonyl (Fmoc) chemistry have been developed for the synthesis of peptide thioesters.[4,6–11] The direct synthesis of thioesters using Fmoc chemistry was enabled by revising the Fmoc deprotection strategy through the replacement of piperidine with less nucleophilic bases.[6] Nevertheless, aminolysis of the thioester linkage, especially for the first few cycles and racemization of the C-terminal amino acid residue remain problematic. Considerable efforts have been applied for the development of methods for the synthesis of latent thioester precursors, which are compatible with standard Fmoc chemistry. These methods include the activation of protected peptides in solution or on resins,[7] the use of thiol labile safety-catch sulfonamides,[8a–c] acylurea,[8d] pyroglutamyl imides,[8e] peptide azide,[8f] and peptidyl-*N*-acetylguanidine,[8g] and the generation of thioesters through O→S9 or N→S10 acyl transfer.

In our efforts to develop novel and convenient Fmoc-based methods for the preparation of peptide thioesters, we are particularly focused on the N→S acyl transfer reaction.[10] The process is mechanistically reminiscent of intein-mediated protein splicing[12] and is essentially the reverse of peptide ligation. Under normal conditions, the ligation reaction is favored over the thioester formation process. Several methods have been developed to drive the process in the direction of thioester formation. One of the strategies to promote N→S acyl transfer is to make this happen at a tertiary amide rather than a secondary amide under mild acidic conditions. In such a system, the reverse re-ligation process is slowed down as a protonated secondary amine is involved, which enables the transiently presented thioester intermediate to be captured by either excess thiol additives to generate a thioester, or by an in situ cysteine peptide to form the ligation product directly. Based on this rationale, we proposed that a peptide carrying a C-terminal 2-pyrrolidinemethanethiol (PMT) unit could serve as a thioester equivalent and ligate in situ with a cysteine peptide via N→S acyl transfer. The PMT linker can be easily prepared on large-scale quantity from commercially available H-(L)-prolinol. The synthesis of PMT peptides is fully compatible with standard Fmoc solid-phase peptide synthesis (SPPS) on a 2-chlorotrityl chloride (2-ClTrt) resin.

To test our proposal, we first synthesized the PMT linker and loaded it onto 2-ClTrt resin (Scheme 1). To synthesize the linker, H-(L)-prolinol (**1**) was first protected with Boc anhydride. After activating the hydroxyl group of the alcohol **2** with methanesulfonyl chloride (MsCl), the obtained mesyl ester **3** was substituted with potassium thioacetate through an S_N2 reaction. The acetyl

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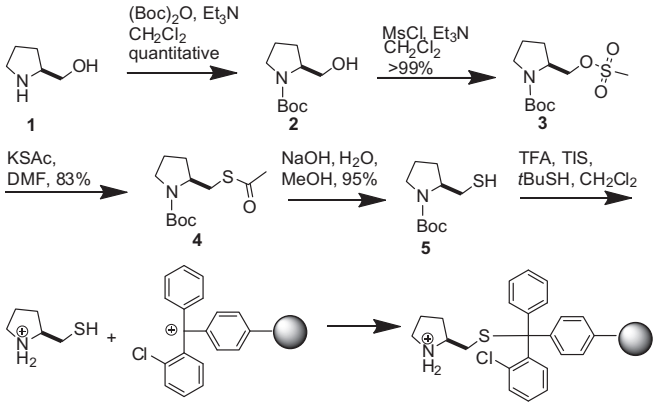

Scheme 1. Chemical synthesis of the PMT linker and loading of the linker onto 2-ClTrt resin.

group of compound **4** was removed by saponification to give N-protected PMT **5**. The overall yield of compound **5** was 78%, thereby allowing the possibility of preparing the linker in large quantity. For the loading of the linker to the 2-ClTrt resin, compound **5** was deprotected, dissolved in dry CH_2Cl_2 and added to the resin which had been pretreated with 30% TFA in CH_2Cl_2. The loading was complete within 1 h (see Supplementary data for the details of the loading process). Fmoc-Gly-OH was then coupled onto the resin. After removing the Fmoc group, a quantitative ninhydrin test[13] was performed with the resin to determine its substitution, which was 0.52 mmol/g. The substitution was comparable to many of the commercial resins and was suitable for SPPS. A model peptide, H-**LSTEG**-PMT (**6**) was synthesized with the PMT-2-ClTrt resin following standard Fmoc-chemistry. After cleavage, the analytical HPLC of the crude peptide **6** showed a relatively clean product. The minor side product with a mass of 56 Da higher was due to the S-alkylation of **6** by a *t*-butyl cation during cleavage. The analytical HPLC and ESI-MS of purified **6** are shown in Figure 1.

To test whether PMT peptides can be used for chemical ligation, a study using **6** and H-**CAKAFA**-NH_2 (**7**) was performed. Peptides **6** (1.6 mg, 16.6 mM) and **7** (3.3 mg, 34 mM) were dissolved in 160 μL of ligation buffer containing 6 M guanidine hydrochloride (Gdn·HCl), 0.2 M NaOAc, 25 mM tris(2-carboxyethyl)phosphine (TCEP), and 2% v/v methyl mercaptoacetate[14] (MMA), pH 5.0. The above ligations at various temperatures were compared (Supplementary data, Figs. S3 and S4). The reaction proceeded slowly at room temperature and the yield was 45% after 24 h. At 37 and 42 °C, the ligation yield reached 83% and 90%, respectively, after 24 h. This indicated that significant activation energy might be required for N→S acyl transfer to occur. It is well-known that microwave irradiation can promote dramatically the rate of peptide synthesis and peptide ligation. Under low-power microwave irradiation, the ligation rate between **6** and **7** was dramatically accelerated. After 7 h, the yield had reached 93%. For the ligation between **6** and **7**, two side products with a mass of 634.45 and 676.4, respectively, were observed on many occasions (peak d and e in Fig. 2). They were likely derived from the cysteinyl-peptide **7** as they were also present when other PMT peptides with different C-terminal residues were ligated with **7**. The nature of these side products is unknown.

To optimize the pH values for PMT-mediated ligation, the ligation between **6** and **7** at different pH (4, 5, 6, and 7) was compared (Supplementary data, Figs. S5 and S6). The ligation was performed in the presence of MMA under microwave irradiation. After 7 h, the ligation yields at pH 4, 5, 6, and 7 were 81%, 93%, 87%, and 67%, respectively. These results indicated that our ligation system preferred mild acidic conditions (pH 5–6). However, the reaction at pH 7 seemed to give a lower amount of the two side products mentioned above.

To evaluate the effect of the thiol additives, three different thiols were tested for the ligation between **6** and **7** at pH 5.0 and microwave irradiation (Supplementary data, Figs. S7 and S8). It was found that the ligation rate was similar when MMA or 4-mercaptophenyl acetic acid (MPAA) was used. Both reactions were nearly complete within 7 h. However, the ligation with MPAA showed less clean HPLC profiles and more side products, possibly originating from **7**, were observed. When sodium 2-mercaptoethylsulfonate (MESNa) was used, the yield was only 66% after 7 h. These results showed that MMA was a better thiol additive for our ligation system.

In summary, the optimal conditions for PMT-mediated ligations are microwave irradiation, MMA as the thiol additive and pH 5–6. The ligation of **6** and **7** under these optimized conditions is shown in Figure 2.

To test whether the newly developed PMT ligation works for other non-glycine C-terminal residues, peptides H-**LSTEX**-PMT (X = A, L, F, S, and V) were synthesized in the same way as peptide **6**. These peptides were ligated with peptide **7** with MMA as the thiol additive at pH 5.0 under microwave irradiation (Table 1). Except for β-branched Val, the PMT peptides with the other four C-terminal residues ligated efficiently with peptide **7**. These ligations were complete within 12 h with yields between 80% and 90%. The ligation between the Val peptide and **7** was slower, and only reached 29% yield after 12 h.

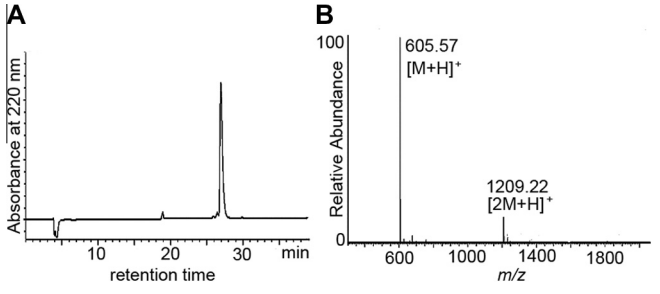

Figure 1. (A) C18 analytical HPLC of purified **6**. Gradient: 40% of buffer B (90% aqueous acetonitrile containing 0.045% TFA) in buffer A (H_2O containing 0.045% TFA) for 40 min. (B) ESI-MS of **6**. Monoisotopic mass, calcd: 604.29; observed: $[M+H]^+$ = 605.57.

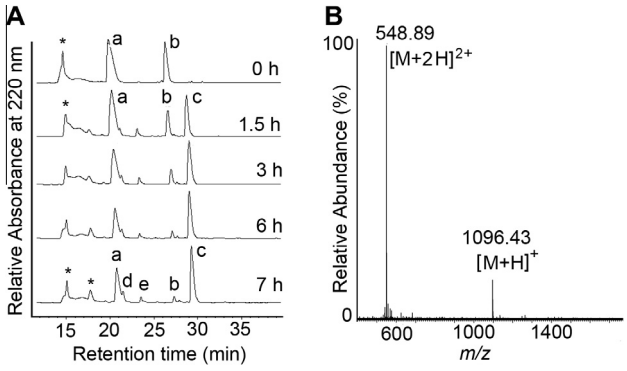

Figure 2. (A) The C18 analytical HPLC of the ligation between **6** and **7** under the optimized conditions. Peak a: peptide **7**; peak b: peptide **6**; peak c: ligation product, H-**LSTEGCAKAFA**-NH_2; peaks d and e indicating molecular masses of 634.45 and 676.4 as detected by ESI-MS, respectively, are unidentified; asterisk peaks: buffer content and non-peptide materials. Gradient: 40% of buffer B in buffer A for 40 min. (B) ESI-MS of the ligation product. Monoisotopic mass, calcd: 1095.54; observed: $[M+H]^+$ = 1096.43.

Table 1
The ligation yield (%) between H-**LSTEXaa**-PMT peptides and H-**CAKAFA**-NH_2[a]

Entry	Xaa	1.5 h	3 h	7 h	12 h
1	Ala	21	35	65	81
2	Leu	n.d.	39	68	86
3	Phe	32	60	85	90
4	Ser	24	40	65	82
5	Val	n.d.	12	20	29

[a] Reaction conditions: H-**LSTEXaa**-PMT (15 mM) and H-**CAKAFA**-NH_2 (30 mM) in the buffer containing 6 M Gdn·HCl, 0.2 M NaOAc, 25 mM TCEP and 2% v/v MMA, pH 5 under microwave irradiation. The ligation yield is calculated based on HPLC analysis and the consumption of the PMT peptides. n.d. = not determined.

The conditions for PMT ligation and thioester ligation are relatively orthogonal to each other. Under thioester ligation conditions, PMT peptides prefer the amide form and ligate slowly with cysteine peptides. Therefore, we proposed a one-pot three-segment N-to-C sequential ligation strategy[15] involving thioester and PMT peptides. The first step was the thioester-mediated ligation between the N-terminal thioester and central cysteinyl PMT segment performed at room temperature under mild alkaline conditions. The second ligation was the PMT-mediated ligation between the first-step ligation product and a C-terminal cysteine peptide. To test the proposal, a model sequential ligation was performed. For the first ligation, 4.3 mg (14.2 mM) of H-**FKLAKF**-$SCH_2CH_2CONH_2$ (**8**) and 3.6 mg (14.1 mM) of H-**CLSTEG**-PMT (**9**) were dissolved in 360 μL of buffer containing 6 M Gdn·HCl, 0.2 M phosphate, 25 mM TCEP, and 2% v/v MMA, pH 8.0. After 2 h at room temperature, the ligation was complete (Fig. 3A). Without isolating the ligation product or changing the thiol additive, the second ligation was initiated by adding 7 mg of peptide **7** to the mixture and adjusting the pH to 5.5 with dilute hydrochloric acid. After 5 h under microwave irradiation, the ligation was complete, to give 7 mg of the final ligation product after purification (isolated yield 72%). The second step of ligation was also performed at 42 °C. The reaction was almost complete after 14 h.

To demonstrate that the novel PMT-mediated ligation approach can serve as a useful tool for protein synthesis, we applied the strategy to the semi-synthesis of lysine 16 acetylated histone H4 (H4K16Ac). H4K16Ac is one of the important histone post-translational modifications and has been shown to be involved in the regulation of chromatin compaction and transcription.[16] Previously,

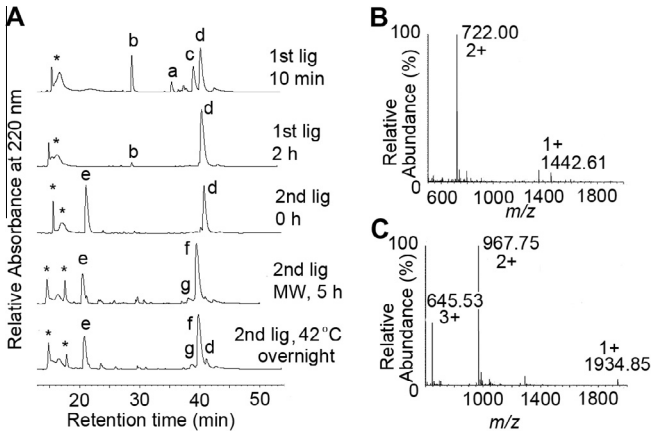

Figure 3. (A) C18 analytical HPLC monitored one-pot N-to-C sequential ligation. Peak a: peptide **8**; peak b: peptide **9**; peak c: H-**FKLAKF**-SCH_2COOMe; peak d: first step ligation product, H-**FKLAKFCLSTEG**-PMT (H-**FG_{12}**-PMT); peak e: peptide **7**; peak f: second step ligation product, H-**FKLAKFCLSTEGCAKAFA**-NH_2 (H-**FA_{18}**-NH_2); peak g: the hydrolysis product, H-**FG_{12}**-OH. B) The ESI-MS of H-**FG_{12}**-PMT. $[M+2H]^{2+}$ obsd: 722.00; monoisotopic mass calcd: 1441.75. (C) The ESI-MS of H-**FA_{18}**-NH_2. $[M+2H]^{2+}$ obsd: 967.75; monoisotopic mass calcd: 1933.00.

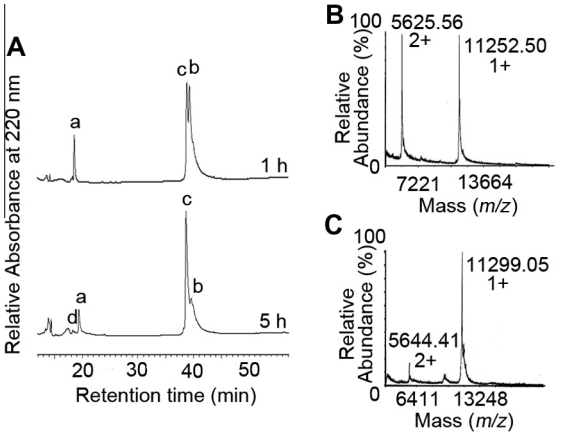

Figure 4. (A) C8 analytical HPLC monitored semi-synthesis of H4K16Ac. Peak a: peptide **10**; peak b: H4 C-terminal domain; peak c: ligation product. B) MALDI-TOF MS of the ligation product. Average isotopic mass calcd: 11253.02, $[M+H]^+$ found: 11252.50. (C) MALDI-TOF MS of the S-alkylated product, H4K16Ac. Average isotopic mass calcd: 11296.09, $[M+H]^+$ found: 11299.05.

we reported the semi-synthesis of H4K16Ac through a traditional NCL/S-alkylation approach.[17] Herein, we synthesized H4K16Ac through PMT-mediated ligation in a similar manner. First, the H4 N-terminal PMT peptide (**10**) containing residues 1–19 and K16Ac was synthesized on a PMT-2-ClTrt resin using Fmoc chemistry. In a typical ligation reaction, 3.4 mg of peptide **10** (4 mM) and 5 mg of C-terminal domain (1.33 mM) were dissolved in 400 μL of ligation buffer containing 6 M Gdn·HCl, 0.2 M NaOAc, 25 mM TCEP, and 2% v/v MMA, pH 5.0. The ligation proceeded efficiently under microwave irradiation. After 1 h, over 26% of the C-terminal domain was consumed based on C8 analytical HPLC. After 5 h, the ligation yield reached 67% (Fig. 4). The ligation mixture was separated by C4 semi-preparative HPLC and 2.4 mg of ligation product was obtained in an isolated yield of 40% (calculated based on the C-terminal domain). To convert the cysteine residue at the ligation site into a lysine analog, the full length H4 was alkylated with 2-bromoethylamine according to the previously reported procedure (Fig. 4).[17] 2 mg of the final H4K16Ac was obtained (83% yield).

In conclusion, we have developed a novel *N*-peptidyl PMT-based method for chemical ligation. This novel method shares many similarities with the peptidyl *N*,*N*-bis(mercaptoethyl)amide (BMEA) method recently reported by Melnyk's group[10i] and our group.[10j] Both linkers can be easily synthesized and the preparations of these peptides are fully compatible with conventional Fmoc-SPPS. Both ligation methods work efficiently under mild acidic and microwave irradiation conditions. Both methods can be applied to one-pot N-to-C sequential three-segment ligations when they are used in combination with normal peptide thioesters.[14,15,18] Despite sharing many common properties, these two methods do behave differently in certain aspects. For example, unlike with BMEA peptides, we were unable to isolate the thioester product when PMT peptides were treated for exchange with an excess thiol compound (data not shown). Nevertheless, the PMT-based method is another example of using the N→S acyl transfer principle for the Fmoc-solid phase synthesis of peptide thioester precursors or equivalents. The method expands the repertoire of chemical ligation methods and provides additional choice for peptide and protein synthesis.

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Supplementary data

Supplementary data (experimental details and data for new compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2013.05.013.

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