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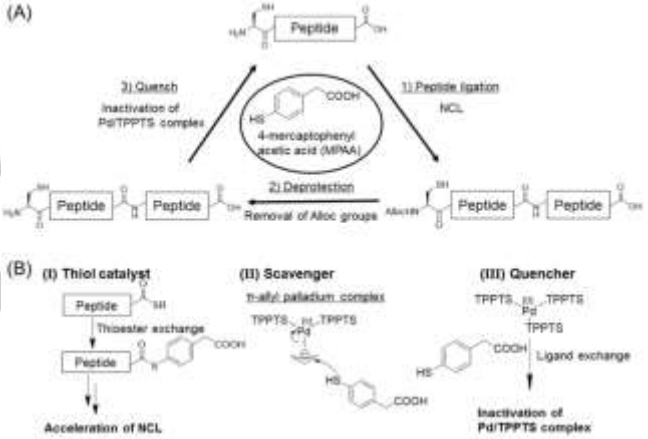
Triple Function of 4-Mercaptophenylacetic Acid Promotes One-Pot Multiple Peptide Ligation

Naoki Kamo,[a] Gosuke Hayashi[a] and Akimitsu Okamoto[a,b]*

Abstract: One-pot multiple peptide ligation is a key technology to improve the efficiency of chemical protein synthesis. One-pot repetitive peptide ligation requires a cycle of three steps: peptide ligation, removal of a protecting group, and inactivation of the deprotection reagent. However, previous strategies are not sufficient because of harsh deprotection conditions, slow deprotection rates, and difficulty in quenching the deprotection reagent. To address these issues, we developed a rapid, efficient deprotection and subsequent quenching strategy using an allyloxycarbonyl group to protect the N-terminal cysteine. 4-Mercaptophenylacetic acid (MPAA), which is a thiol additive of native chemical ligation, functioned not only as a scavenger for a π-allyl palladium complex, but also as a quencher of palladium(0) complex. Utilizing the multifunctionality of MPAA, we achieved the first one-pot five-segment ligation to afford histone H2AX (142 amino acids) in 59% isolated yield.

Chemical protein synthesis has become one of the most powerful methods to obtain a variety of tailor-made proteins. This chemical method has enabled us to incorporate various modifications into proteins at specific sites.[1] To achieve the chemical synthesis of large proteins, native chemical ligation (NCL)[2] is the most widely employed method to condense two side-chain unprotected peptide segments between an N-terminal cysteine (Cys) and a C-terminal thioester. To ligate multiple peptide segments, the N-terminal Cys of middle peptide segments should be protected to avoid polymerization and/or cyclization. For the one-pot multiple peptide ligation to improve synthesis efficiency, there are three key steps: peptide ligation, removal of protecting groups, and quenching of deprotection reagents. However, it has been difficult to repeat these three steps efficiently. For example, thiazolidine (Thz), removed with methoxyamine, is a widely used protecting group of N-terminal Cys for the ligation of several peptide segments.[3] However, the removal of a Thz group requires a long reaction (>3h). Moreover, during the subsequent NCL reaction, methoxyamine would react with thioesters to generate a (*N*-methoxy)carboxamide.[4] Therefore, rapid deprotection under mild conditions and complete quenching of deprotection reagents without affecting the subsequent NCL reaction are required for efficient one-pot multiple peptide ligation.

Recently, Brik and coworkers reported the removal of protecting groups for N-terminal Cys, such as Thz or acetamidomethyl groups, with palladium complexes under NCL conditions.[5] However, a substantial amount of palladium (15–40 equiv.) is required to complete the deprotection, which would not be adequate for the repetitive cycles of ligation in a one-pot approach. Therefore, we decided to employ allyloxycarbonyl (Alloc) protecting groups,[6] which are removed with palladium (0) complexes based on the Tsuji–Trost reaction. It was envisaged that the proposed catalytic mechanism would reduce the amount of the deprotection reagents. We expected that 4-mercaptophenylacetic acid (MPAA), a thiol additive to accelerate NCL reaction,[7] would function as the nucleophile for the π-allyl palladium complex to facilitate the Alloc deprotection.[8] Moreover, it is known that sulfur-containing compounds decrease the reactivity of metal complexes because of the poisoning effects. [9] We expected that MPAA would be utilized for quenching of the palladium complexes after deprotection. Using these three functions of MPAA, we anticipated that efficient one-pot multiple peptide ligation would be accomplished through the repetitive cycles of three steps: NCL, removal of the Alloc groups, and quenching of the palladium complexes (Scheme 1A).



Scheme 1. Summary of the present research. (A) General scheme of multiple peptide ligation in a one-pot manner. (B) Triple functions of MPAA in this study.

Herein, we report the first one-pot multiple peptide ligation utilizing the triple functions of MPAA as (I) a thiol additive for NCL, (II) a scavenger for the π-allyl complex, and (III) a quencher of palladium complexes (Scheme 1B). Finally, we accomplished the chemical synthesis of histone H2AX, a variant of histone H2A, in high yield through one-pot five-segment ligation.

We began to investigate the function of MPAA as a scavenger for the π-allyl palladium complex and a quencher for palladium complexes. We prepared *N*-allyloxycarbonyl-*S*-tritylcysteine (Alloc-Cys(Trt)-OH), which would be applied to Fmoc solid-phase peptide synthesis (SPPS) (Figure S1). This amino acid was condensed with the peptide extended on the resin to afford peptide **1** (Figure S2). Then, we tried to remove the Alloc group of peptide **1** with palladium complexes employing 3,3',3''-phosphanetriyltris(benzenesulfonic acid) trisodium salt (TPPTS) as a ligand for palladium (Figure S3). In the absence of MPAA, the reaction did not reach completion, which indicated that the generated π-allyl palladium complex was trapped without the attack by external nucleophiles (Figure S3B). By contrast, the deprotection reaction with 2 equiv. of Pd/TPPTS complex reached completion within 10 min in the presence of MPAA, and allyl-MPAA was detected in the HPLC chart (Figure S3C). This suggested that MPAA functioned as a scavenger for the π-allyl palladium complex, and facilitated the removal of the Alloc group.

We attempted to achieve complete inactivation of the Pd/TPPTS complex after removal of the Alloc group. We hypothesized that ligand exchange with MPAA on palladium would inactivate the Pd/TPPTS complex. The ligand exchange of the Pd/TPPTS complex with MPAA was confirmed by ^{31}P NMR analysis (Figure 1). In the absence of MPAA, the dissociation of

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TPPTS from palladium was not observed (Figure 1C). However, in the presence of MPAA (10 equiv. versus the Pd/TPPTS complex), a signal derived from free TPPTS was observed after 10 min reaction (Figure 1D), suggesting that MPAA coordinated to palladium and the dissociation of TPPTSs occurred.

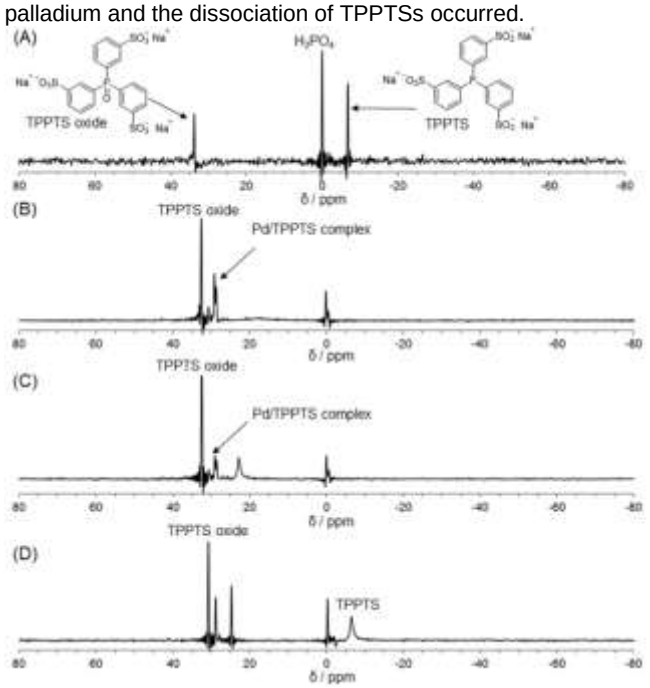


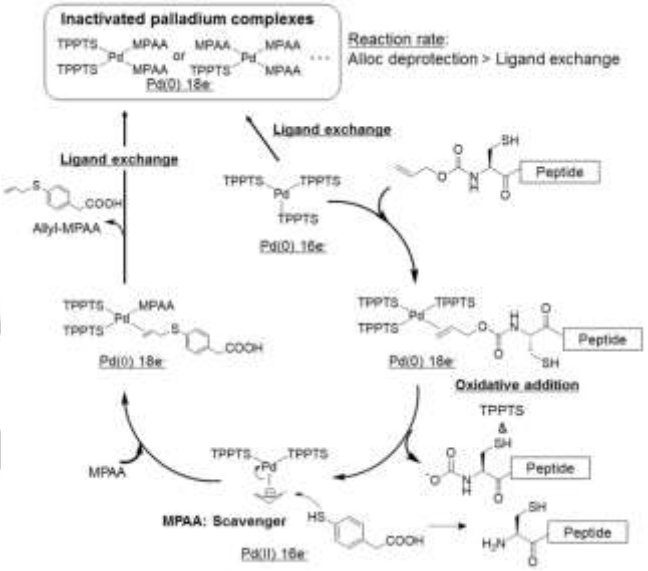
Figure 1. Analysis of ligand exchange of Pd/TPPTS complex with MPAA and TCEP by ^{31}P NMR measurement. NMR spectra of (A) mixture of TPPTS and TPPTS oxide, (B) Pd/TPPTS complex. NMR spectra of Pd/TPPTS complex after 10 min stirring (C) without MPAA, (D) with 10 equiv. of MPAA.

Table 1. Deprotection of the Alloc group of peptide **1** employing the Pd/TPPTS complex after ligand exchange.

		Conversion yield (%)[a]		
Entry		3 min	5 min	10 min
1	Only water	94	82	94
2	100 mM MPAA	69	40	>5
3	NCL buffer	50	23	5

[a] The conversion yields were calculated from peak areas analyzed by HPLC (shown in Figure S4).

We examined the effect of the ligand exchange on the activity of the Pd/TPPTS complex. Pd/TPPTS solutions containing only water (Table 1, entry 1), 100 mM MPAA (entry 2), or NCL buffer (entry 3) were considered. At each time point (3 min, 5 min, or 10 min), peptide **1** bearing Alloc-protected Cys at its N-terminus was added, then the removal of the Alloc group with the Pd/TPPTS complex was analyzed by HPLC (Figure S4). In the presence of only water, the activity of the Pd/TPPTS complex was retained even after 10 min stirring. By contrast, in the presence of 100 mM MPAA or in the NCL buffer, the activity of the Pd/TPPTS complex was gradually reduced as the stirring time increased. Under these conditions, the palladium complex was completely inactivated after 10 min stirring. These results indicated that the Pd/TPPTS complex could be inactivated by ligand exchange with MPAA in the NCL buffer. We also found that 40 mM tris(2-carboxyethyl)phosphine (TCEP) could inactivate the Pd/TPPTS complex (Figure S4). Moreover, the removal of the Alloc group did not proceed with the palladium complex inactivated by ligand exchange, even after 20 h reaction at 37 °C (Figure S5). We concluded that palladium complexes might be saturated after the coordination of MPAA, and the formation of 16- or 14-electron complexes, which are important intermediates for deprotecting the Alloc group, was prevented.[10] Furthermore, the replacement of MPAA with TPPTS changed the electron density of palladium complexes, which could hamper the oxidative addition of Alloc groups. It is notable that during removal of the Alloc group by the Pd/TPPTS complex, inactivation of a palladium complex by ligand exchange proceeded simultaneously and these two reactions reached completion within 10 min (Scheme 2). When we compared MPAA with other thiophenol derivatives as for scavengers and quenchers, MPAA and 4-hydroxythiophenol functioned similarly (Figure S6). We decided to employ MPAA, which is the most water-soluble among the thiophenol derivative, for further experiments.



Scheme 2. Proposed mechanism of removal of Alloc groups and simultaneous inactivation of the Pd/TPPTS complex by ligand exchange with MPAA.

Inactivation of the Pd/TPPTS complex by ligand exchange enabled deprotection of the Alloc group and subsequent NCL reaction in one-pot manner (Figure S7). The Alloc group of peptide **1** was removed with 2 equiv. of Pd/TPPTS complex within 10 min, and the Pd/TPPTS complex was inactivated by ligand exchange with MPAA for a further 10 min. Then, peptide **3**, bearing an Alloc-protected Cys at its N-terminus and a 2-mercaptoethane sulfonate sodium salt (MESNa) thioester at its C-terminus, was added to ligate with **2** for 2 h (Figure S7). The HPLC profile showed a major peak corresponding to the desired ligated product **4**, which was isolated in 90% yield. As a result, a cycle of removal of the Alloc groups, quenching of Pd/TPPTS complex, and subsequent NCL reaction was accomplished utilizing the triple function of MPAA. When the inactivation time was less than 10 min, the self-cyclization and dimerization of peptide **3** were observed as by-products (Figure S8). Therefore, the inactivation of the Pd/TPPTS complex by ligand exchange with MPAA was an important step to suppress the generation of these by-products.

Using these interesting features of MPAA, we tried the first one-pot five-segment ligation to afford a model polypeptide (41 amino acids), which was a modified peptide sequence of C-terminal histone H4. The polypeptide was divided into five peptide segments at the Cys sites (peptides **2**, **3**, **5**, **7**, **9**), and each segment was synthesized through Fmoc SPPS (Figure S9).

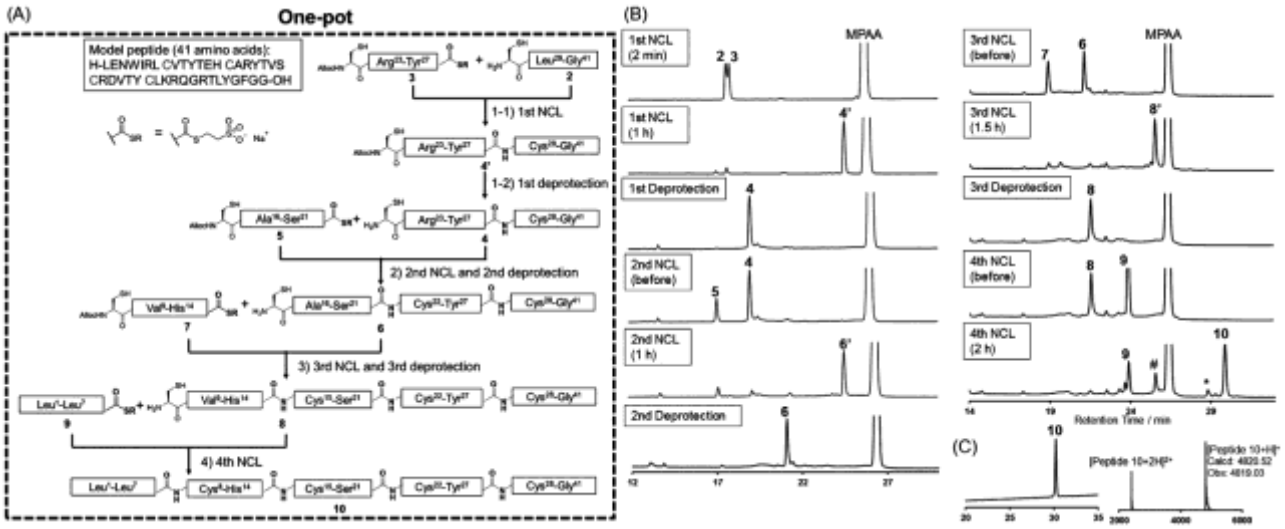


Figure 2. Synthesis of a model peptide using one-pot five-segment ligation. (A) Synthetic route. (B) One-pot ligation monitored by analytical HPLC (gradient: 10–45% for 35 min) monitored at 220 nm. ***4′***, ***6′*** and ***8′*** = Alloc-protected peptides **4**, **6**, and **8**. # = a ligation product between peptides **7** and **9**. * = a ligation product between peptides **5**, **7**, and **9**. (C) HPLC profile (gradient: 10–45% for 35 min) and MALDI-TOF mass spectrum of purified peptide **10**.

One-pot ligation of these five peptide segments was conducted according to the synthetic procedure shown in Figure 3A. All the NCL reactions and the removal of Alloc groups were conducted in NCL buffer (6 M Gn·HCl, 200 mM NaH_2PO_4, 100 mM MPAA, 40 mM TCEP) at 37 °C. Peptides **2** and **3** (1.1 equiv versus peptide **2**) were dissolved in NCL buffer and the NCL reaction reached completion within 1 h to afford peptide **4** (Figure 2B). Pd/TPPTS complex (2 equiv.) was added to the reaction solution, and the mixture was stirred for 20 min. Then, powdered peptide **5** was added to initiate the second NCL reaction. These operations were repeated for the second deprotection and the third NCL and deprotection (Figure 2B). Finally, powdered peptide **9** was added to initiate the fourth NCL reaction. HPLC analysis after 2 h showed a major peak corresponding to the desired ligated peptide **10** (Figure S10). We identified the other HPLC peaks as deriving from the hydrolysis of thioesters or cyclization of added peptide segments. The reaction product was purified by HPLC, the desired peptide **10** was obtained in 66% isolated yield from **2**, suggesting that each reaction step (four ligations and three deprotection steps) proceeded in 94% yield on average. The purity of peptide **10** was confirmed by HPLC and MALDI-TOF MS (Figure 2C).

Based on the successful one-pot five-segment peptide ligation, we next attempted the chemical synthesis of full-length human histone H2AX, a variant of histone H2A. This protein is exchanged with canonical H2A in chromatin fibers mainly in S phase. When a double-strand DNA break occurs during DNA replication, the S139 at the C-terminus of H2AX is phosphorylated by some kinases to form gamma-H2AX (γ-H2AX).[11] This modification contributes to the recruitment of DNA-repairing proteins. Recently, relationships between PTMs on H2AX have been energetically investigated.[12]

H2AX bearing 142 amino acids does not contain Cys residues, which are necessary for peptide ligation by the NCL reaction. Therefore, we introduced four Cys mutations at four Ala sites ($A^{21}C$, $A^{53}C$, $A^{86}C$, $A^{122}C$), and full-length H2AX was divided into five peptide segments (Scheme S1, peptides **11**, **12**, **14**, **16**, and **18**), which were synthesized by Fmoc SPPS (Figure S11).

The one-pot five-segment ligation using the Pd/TPPTS complex was conducted according to the synthetic procedure shown in Scheme S1. Each NCL reaction and the deprotection of the Alloc groups by the Pd/TPPTS complex were monitored by HPLC (Figure S12). Finally, 1.2 equiv of peptide **18** was added to initiate the fourth NCL reaction. After 2 h, full-length H2AX with four Cys mutations (**19**) was observed as the major product (Figure 3A, S13). The reaction product was purified by HPLC, and the desired peptide **19** was obtained in 59% isolated yield from **11**, suggesting that each reaction step proceeded in 93% yield on average. The purity of peptide **19** was confirmed by HPLC and MALDI-TOF MS (Figure 3A). Such a high isolated yield of the synthetic H2AX after one-pot five-segment ligation suggests that our ligation method is more efficient than previous attempts, which achieved one-pot four-segment ligation (trifluoroacetamidomethyl group, 45%; seleno- and thioesters, 29%; *N*-sulfanylethylanilide, 20%).[13-15] A total of seven steps were completed within 15 h, and furthermore, the concentration of the peptide elongated from the first peptide did not change greatly (initial, 2.1 mM; final, 1.7 mM) because only small amounts of additives are used for the entire synthetic procedure.

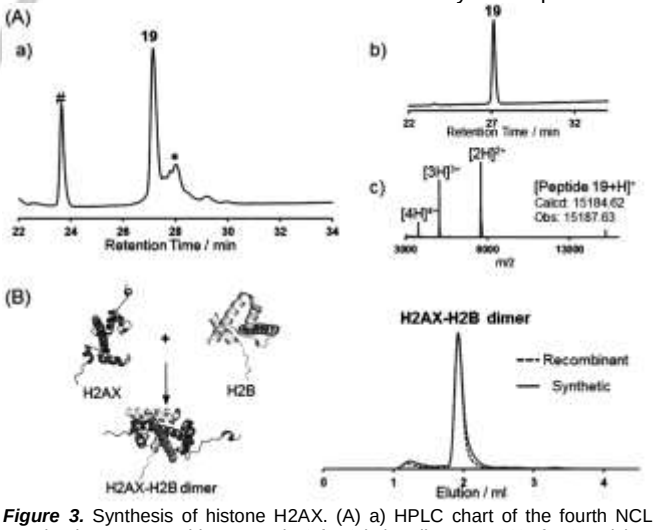


Figure 3. Synthesis of histone H2AX. (A) a) HPLC chart of the fourth NCL reaction between peptides **17** and **18** for 2 h (gradient: 10–66% for 35 min). # = allyl-MPAA. * = mixture of a self-cyclized formation of peptide **14** and a ligation product of peptides **14**, **16**, and **18**. b) HPLC chart, and c) MALDI-TOF mass spectrum of purified peptide **19**. (B) Size-exclusion chromatography analysis of reconstituted H2AX–H2B dimer (Protein Data Bank entry 3AFA of H2A–H2B dimer) containing either recombinant H2AX or synthetic H2AX.

Subsequent desulfurization, which converted mutated Cys residues into the original Ala residues, afforded peptide **20** (full-

length H2AX) in 79% isolated yield (Figure S14). Analysis by atomic absorption spectrophotometer showed that palladium content attached on chemically synthesized H2AX was 0.00096% (Figure S15).

The heterodimer of synthetic H2AX (**20**) and H2B was reconstituted to examine whether synthetic H2AX would be folded properly. Recombinant H2AX or synthetic H2AX were dissolved in denaturing conditions, and recombinant H2B was added in separate experiments. These solutions were dialyzed for one day, and analyzed by size-exclusion chromatography. The chromatography profile showed a major peak corresponding to the H2AX–H2B heterodimer (Figure 3B). Each heterodimer was collected and analyzed by HPLC. The ratios of the peak areas of synthetic H2AX to H2B were almost the same as those of recombinant H2AX to H2B (Figure S16). These results indicated that synthesized H2AX was properly folded to form a H2AX–H2B heterodimer, just as recombinant H2AX was.

In conclusion, we have devised an efficient one-pot multiple peptide ligation employing the triple function of MPAA. We focused on the scavenging and poisoning effect of MPAA on palladium complexes, and utilized these features to accomplish the inactivation of the Pd/TPPTS complex after the removal of Alloc groups. From clear HPLC data and the high isolated yield of H2AX, we envisage that six or more peptide segments could be also assembled in a one-pot manner using our method. Therefore, our strategy is adequate for the chemical synthesis of large multifunctionalized proteins. We expect that this technique will lead to the elucidation of biological functions of proteins and the development of new medicines.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: chemical protein synthesis • peptides • palladium • native chemical ligation

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Layout 2:

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Efficient one-pot multiple peptide ligation was accomplished through repetitive native chemical ligation (NCL) and the removals of allyloxycarbonyl (Alloc) groups with palladium complexes. In this reaction system, we utilized triple functions of 4-mercaprophenylacetic acid (MPAA).

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Triple Function of 4-Mercaptophenylacetic Acid Promotes One-Pot Multiple Peptide Ligation