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Native Chemical Ligation at Glutamine

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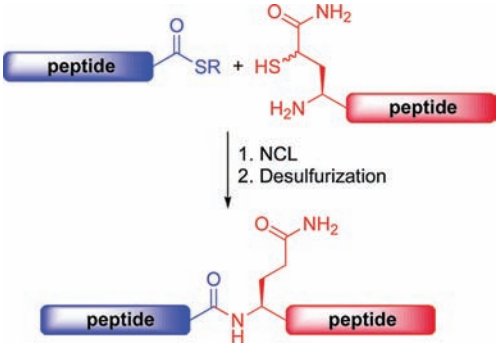
ABSTRACT



The desulfurization reaction introduced by Yan and Dawson as a postnative chemical ligation step greatly expanded the scope of ligation chemistry beyond Xaa-Cys (Xaa is any amino acid) by making ligation at Xaa-Phe, Xaa-Val, Xaa-Lys, Xaa-Leu, Xaa-Thr, and Xaa-Pro junctions accessible in the synthesis of functional proteins. A new ligation site based on Xaa-Gln utilizing γ-mercaptoglutamine is reported, and several examples on the efficiency of ligation coupled with desulfurization are provided.

The combination of native chemical ligation (NCL)[1] and global desulfurization[2] has significantly increased our ability to chemically synthesize proteins for biochemical and structural analyses.[3] To achieve this, an amino acid at the desired ligation junction is modified at the β- or γ-carbon with a thiol group to enable transthioesterification with a peptide thioester and subsequent S−N acyl transfer via a favorable five- or six-membered ring to form the backbone amide bond between the two peptides. The use of Xaa-Ala (Xaa is any amino acid) ligation sites by replacing Ala with Cys to enable NCL followed by a selective reduction of the thiol group on Cys[2] has inspired the development of several other ligation junctions that include Xaa-Phe,[4] Xaa-Val,[5] Xaa-Leu,[6] Xaa-Thr,[7] Xaa-Pro,[8] and Xaa-Lys.[9] Several desulfurization conditions such as nickel boride,[2] Pd/Al_2O_3,[10] and metal-free conditions[11] were found suitable to achieve efficient reduction. More recently, performing ligation and desulfurization in situ was also reported.[12] Moreover, carrying out desulfurization in the presence of Cys residues is possible thanks to the use of orthogonal protecting groups on the Cys[10,13] or by using selective deselenization in the presence of unprotected Cys.[14] Exploiting NCL principles, S−N

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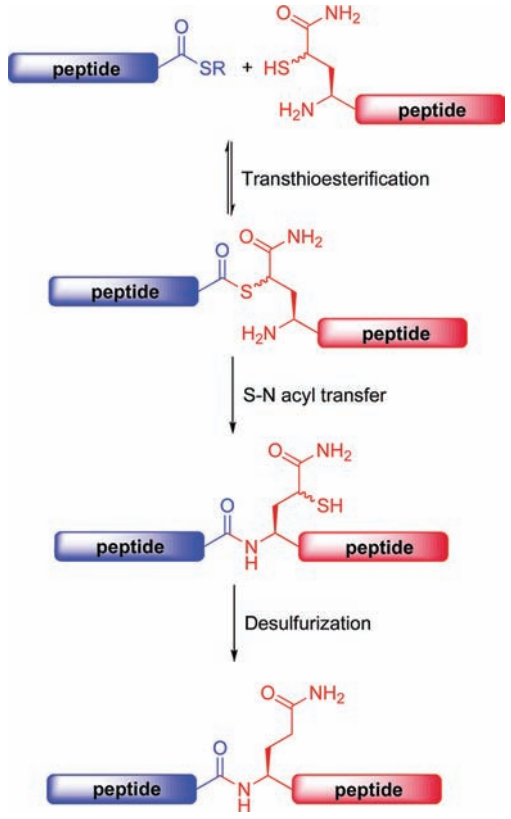
acyl transfer assisted by proximity effect,[15] a method called sugar-assisted ligation for glycopeptide, was also developed.[16] In this strategy, the 2-acetamido group of the glycan unit is modified with a thiol handle to promote amide bond formation via a 14−15-membered ring intermediate for S−N acyl transfer. Our group has also developed a highly efficient method for peptide and protein ubiquitination[9a] by modifying the δ-carbon of a lysine side chain with a thiol group. This promotes a transthioesterification step with a ubiquitin thioester followed by isopeptide bond formation, which after a desulfurization step furnishes the native structure.[17]

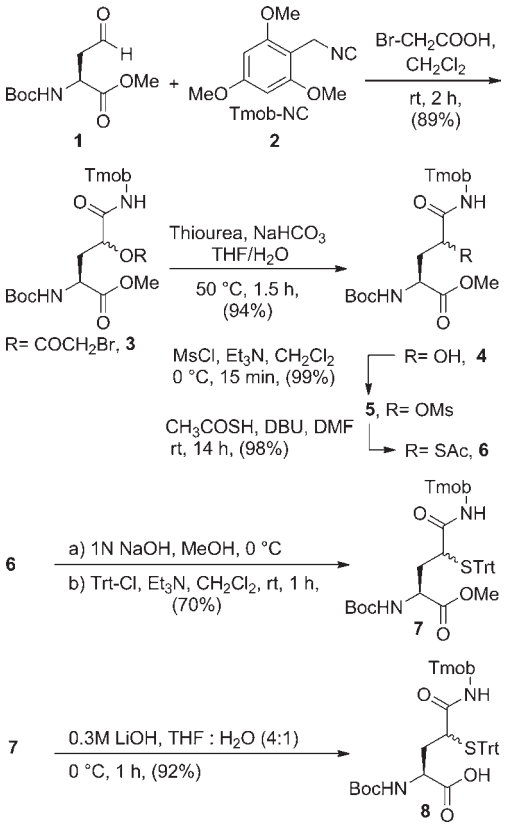
Scheme 1. Ligation at Glutamine Site Employing γ-Mercaptoglutamine-NCL and Desulfurization



The above-described strategies have contributed in several ways to prepare proteins and posttranslationally modified analogues for a variety of studies.[6a,12,18] Motivated by these advances, coupled with the need to have complete flexibility in the retrosynthetic analysis of a protein target by having the ability to perform ligation essentially at any ligation junction, here we report NCL coupled with desulfurization at Xaa-Gln ligation sites (Scheme 1). This ligation is of particular interest due to the high abundance of Gln in protein sequences[19] relative to Cys (2%) and the need to prepare native protein-containing polyQ repeats that are involved in several inherited neurodegenerative diseases for biochemical studies.[20] For example, Huntington's disease (HD) is caused by an expansion of a polyQ repeat in the Huntingtin (Ht) gene.[21] In future endeavors of chemical synthesis of such proteins, the requirement for Gln-Gln ligation site(s) is inevitable.

To enable ligation at Xaa-Gln sites, we sought to develop a straightforward synthesis of thiol-modified Gln residue.

Scheme 2. Synthesis of γ -(*R*,*S*)-Mercapto-L-glutamine



Inspired by the previously reported work on the synthesis of γ-fluorinated Gln,[22] we reasoned that a similar approach could be adopted to prepare the γ-mercaptoglutamine using the Passerini three-component reaction (Scheme 2).

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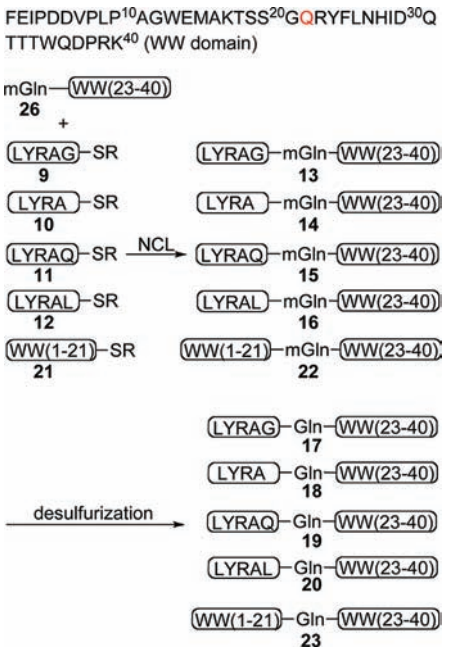
The synthesis of modified glutamine started from commercially available L-aspartic acid, which was converted into aldehyde **1** in three steps according to a previously reported procedure.[23] Subsequently, the Passerini three-component reaction was carried out between precursors **1**, 2,4,6-trimethoxybenzyl isocyanide (Tmob-NC) **2**, and bromoacetic acid. The 4-acyloxy substituted Gln derivative **3** was successfully obtained in 89% yield. Treatment of the Passerini product **3** with thiourea under basic conditions to remove the bromoacetyl group gave Gln derivative **4** with the free hydroxyl. Subsequently, mesylation was carried out to give derivative **5**, followed by nucleophilic attack on the γ-position with thioacetic acid, which resulted in γ-acylthio Gln **6**. Hydrolysis of the acetyl group using sodium hydroxide followed by trityl (Trt) protection yielded the fully protected γ-mercaptoglutamine **7**. Efforts to directly introduce the Trt group by reacting the mesylated derivative with Trt thiol failed to give the desired product. Final basic hydrolysis of the methyl ester gave the γ-(*R*,*S*)-mercapto-L-glutamine (mGln) **8**, ready for solid phase peptide synthesis (SPPS). At this stage, the presence of two diastereomeric mixtures was not disturbing, as in the desulfurization step, this center will be converted to an achiral one, which would furnish enantiomerically pure peptide.

Scheme 3. Model Thioester Peptides Used in Our Ligation–Desulfurization Study with mGln-WW(23–40)



With the thiol modified glutamine derivative **8** in hand, we incorporated it into a model peptide, mGln-WW-(23–40) **26** derived from YAP65 WW domain polypeptide. This peptide was successfully prepared using SPPS and isolated in 20% yield (Scheme 3). To test the ligation using mercaptoglutamine, four different model thioester peptides **9**–**12** were prepared as previously reported (Scheme 3)[6a] and subjected to ligation with peptide **26** (Figure 1 and the Supporting Information). Gratifyingly, HPLC coupled with MS analysis showed that all ligation reactions were successful and resulted in the ligation products **13**–**16**. The isolated yields with thioester peptides **9**–**12** were 70, 62, 56, and 50% respectively. The ligation time for reaction completions, as measured by the disappearance of the mGln-WW(23–40) **26** peak, in buffer containing mercaptophenylacetic acid (MPAA) were as anticipated,[24] with Gly being the fastest (0.5 h), while Leu was the slowest (1.5 h). In the case of the slow ligation, a side product corresponding to dehydration of mGln-WW(23–40) was observed (~30%). A preliminary study indicated that this side product is occurring because of the presence of mGln residue, which might undergo nitrile formation of its side-chain. We are currently further characterizing this side reaction and providing solutions to minimize it.

Next, we examined the desulfurization step to generate the native Gln residue. Interestingly, when using the metal-free desulfurization conditions, no product was observed,

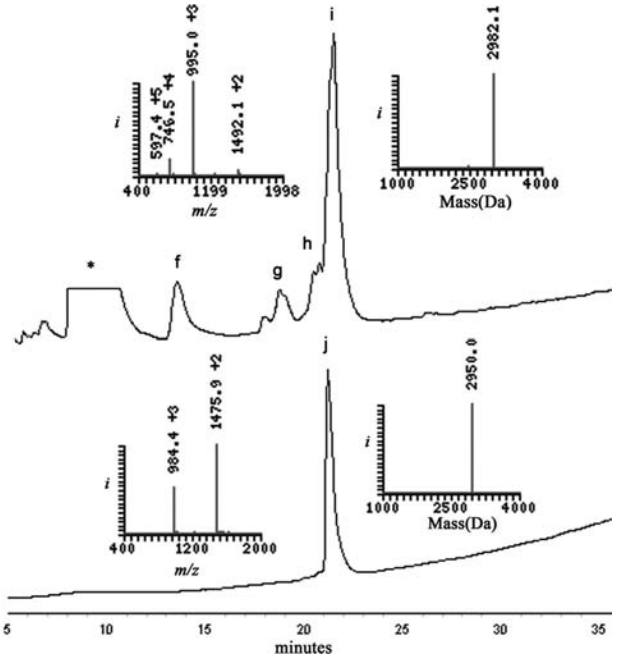


Figure 1. A representative example of ligation (top) and desulfurization (bottom) of mGln-WW(23–40) **26** with LYRA-thioester **10** after 1 h. Peak f corresponds to LYRA-MPAA with the observed mass of 671.2 Da (calcd *m*/*z* 671.6 Da); peak g corresponds to mGln-WW(23–40) **26** with the observed mass of 2478.6 Da (calcd *m*/*z*. 2478.7 Da); peak h corresponds to the dehydration byproduct of mGln-WW(23–40) with the observed mass of 2460.6 Da; peak i corresponds to the desired ligation product **14** with the observed mass of 2982.1 Da (calcd *m*/*z* 2982.3 Da); peak j corresponds to the desired desulfurization product **18** with the observed mass of 2950.0 Da (calcd *m*/*z* 2950.3 Da); peak * is thiol additive.

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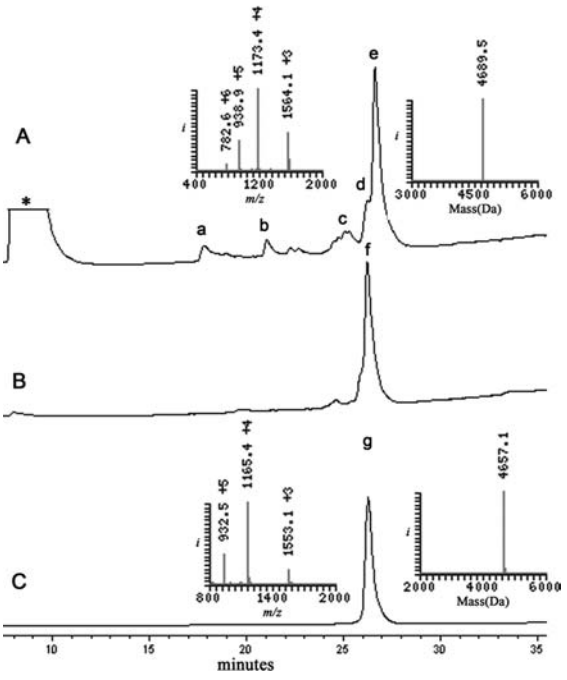

Figure 2. (A) Ligation of WW(1−21)-thioester **21** and mGln-WW(23−40) **26** after 80 min. (B) Desulfurization of mGln-WW(1−40) **22**. (C) WW(1−40) prepared by SPPS with L-Gln at position 23. Peak a corresponds to mGln-WW(23−40) **26** with the observed mass of 2478.6 Da (calcd *m*/*z* 2478.7 Da); peak b corresponds to WW(1−21) thioester **21** with the observed mass of 2317.4 Da (calcd *m*/*z* 2317.4 Da); peak c corresponds to WW(1−21)-MPAA with the observed mass of 2379.0 Da (calcd *m*/*z* 2379.4 Da); peak d corresponds to the dehydration byproduct of mGln-WW(23−40) with the observed mass of 2460.6 Da; peak e corresponds to the desired ligation product WW-(1−40) **22** with the observed mass of 4689.5 Da (calcd *m*/*z* 4690.1 Da); peak f corresponds to the desired desulfurization product, WW domain **23** with the observed mass of 4657.1 Da (calcd *m*/*z* 4658.1 Da); peak g corresponds to WW domain prepared by SPPS with L-Gln at position 23 with the observed mass of 4657.1 Da (calcd *m*/*z* 4658.1 Da); peak * is thiol additive.

and the purified ligation product was abolished to give products with unidentified masses. This could arise from undesired reactivity of the formed radical on the γ-position during the desulfurization step. Consequently, we turned our attention to the nickel boride desulfurization conditions,[2] which do not involve radical intermediates. To our delight, under these conditions the reaction was completed within 1 h and afforded the desired products **17**−**20** in high purity and ∼60% yield (Figure 1 and the Supporting Information).

With the lessons gained from our study on the model peptides, we then turned our efforts to the synthesis of WW domain (Scheme 3). The N-terminal fragment, i.e., WW-(1−21) thioester **21**, was prepared using *N*-methylcysteine-mediated thioester formation[25] in 30% yield (Supporting Information). With the two fragments in hand, we then employed our optimized conditions for Gln-NCL to construct the full length WW domain (1−40) **22**, which upon a desulfurization step furnished the native WW domain **23** with an overall yield of 25%.

To verify that the chiral integrity of the α-carbon in γ-(*R*,*S*)-mercaptoglutamine was not affected during any step of the synthesis, we carried out the following study. We synthesized the WW domain (1−40) using SPPS, where either L-Gln or D-Gln was introduced at position 23 to yield the peptides **24** and **25**, respectively. The two isomers were separated by HPLC, and to our delight, the desulfurization product **23** overlapped with the L-Gln containing WW domain (1−40) prepared by SPPS (Figure 2B,C and the Supporting Information). Moreover, the circular dichroism (CD) measurement of the ligation−desulfurization product **23** and the prepared WW domain (1−40) **24** using SPPS exhibited similar spectra (Supporting Information). These spectra were also very similar to the previously reported one.[26] These results further support the integrity of the Gln-mediated ligation.

In summary, we have presented a straightforward and high-yielding synthesis of γ-mercaptoglutamine. Using this γ-mercaptoglutamine, we were able to perform several Xaa-Gln ligation sites and the synthesis of WW domain. In these cases, the desulfurization using nickel boride was efficient, contrary to metal-free desulfurization that led mainly to unidentified byproduct. We believe that ligation at Gln junctions will be useful in the synthesis of several other proteins, in particular Gln rich proteins such as proteins containing polyQ repeat. We are currently exploiting the applicability of this method.

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Supporting Information Available. Experimental procedures and spectroscopic data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.