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Accessing posttranslationally modified proteins through thiol positioning‡

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The field of peptide synthesis achieved considerable advancement in the last decade with the discovery of native chemical ligation (NCL). With the aim of broader application of ligation methods in the synthesis of proteins several strategies have been developed. One of the significant contributions to NCL based strategies is the desulfurization reaction, which removes the thiol handle to generate the unmodified protein. The principle of NCL coupled with desulfurization is effortlessly executed in the synthesis of posttranslationally modified proteins. This short account will cover the recent developments on how new methods of chemical ligation is being evolved and exploited in achieving posttranslationally modified proteins. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: posttranslational modifications; ligation; ubiquitylation; desulfurization.

Introduction

The bridging of unprotected peptide fragments in aqueous solution through amide bond using native chemical ligation (NCL) has revolutionized the field of chemical synthesis of proteins [1]. From the mechanistic aspect, during NCL a peptide fragment having sulfhydryl group positioned at N-terminal captures chemoselectively another peptide thioester resulting in a transient intermediate that undergoes spontaneous amide bond formation (Scheme 1). Using this strategy hundreds of proteins (10–20 kD) have been assembled and were found to be biologically active as those obtained from natural sources. Another main feature of NCL is its ability to provide modified proteins in enough quantity and purity for the structural and biological studies, which by other means are difficult to obtain. One such example is the synthesis of posttranslationally modified proteins as in the case of glycosylation, methylation, acetylation, and lipidation [2–5].

Native Chemical Ligation and its variations

After the seminal publication in 1994 on NCL by Kent and coworkers [1], efforts have been invested to extend the applicability of this useful concept to other ligation junctions beyond AA-Cys [2–5]. A close look on NCL reveals that two main requirements are needed to be fulfilled in order to extend this approach to other ligation junctions; (i) a peptide fragment with thiol functionality at the N-terminus, (ii) a peptide fragment in the form of thioester at its C-terminus (Scheme 1). Interestingly, Cys is the only natural amino acid that fit in to the former requirement, which unfortunately is not ubiquitous residue in proteins. Hence it is essential to have Cys free ligation methods to enable the synthesis of a wide variety of proteins in their natural form. One of the earliest efforts in this direction was based on removable auxiliary-mediated ligation strategy [4]. In this method a thiol auxiliary mimicking the function of N-terminal Cys is anchored to the N-terminus peptide to promote transthioesterification and the subsequent S–N acyl transfer. In this regard, peptides with N-terminal auxiliaries such as 1-phenyl ethane thiol, 2-mercaptobenzyl and photolabile auxiliaries have been studied and successfully utilized in the synthesis of large peptides [6–9] (Scheme 2). However, steric hindrance at the secondary amine because of the attachment of auxiliary renders ligation at these junctions sluggish and in some cases unsuccessful.

Alternatively, chemists also came up with the idea of using sufhydryl functionality as a temporary handle to function similar to Cys on the side chain of N-terminal amino acid which after ligation could be removed to yield the unmodified peptide. Even though the method of hydrogenolytic desulfurization was known in protein/peptide chemistry nearly a decade before the discovery of NCL, it was Dawson and coworkers who foreseen the power of merging NCL and desulfurization reaction in sequential manner to extend the ligation chemistry to noncysteinyl junctions such as Ala-AA [10]. This idea was successfully demonstrated in the synthesis of both cyclic and acyclic form of peptide antibiotic microcin J25, streptococcal protein G B1 domain, and an analogue of barnase. Comparative studies on desulfurization under different reaction conditions revealed that Raney nickel induced desulfurization is the catalyst of choice with respect to Pd/Al_2O_3 in terms of yield, and side reactions. Kent and coworkers utilized this method to synthesize ubiquitin and its diastereomer bearing D-Glu^{35} in an effort to understand the effect of small structural changes on the global conformation of protein [11]. This study further supported that the desulfurization method is racemization free. The vision of extending this method to other amino acids by Dawson and coworker received attention from different research groups. Thus, Crich and coworker introduced ligation at phenyl alanine, [12] the Sietz group came up with ligation at valine (β-carbon), [13] simultaneously with the Danishefsky group [14]

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Biography

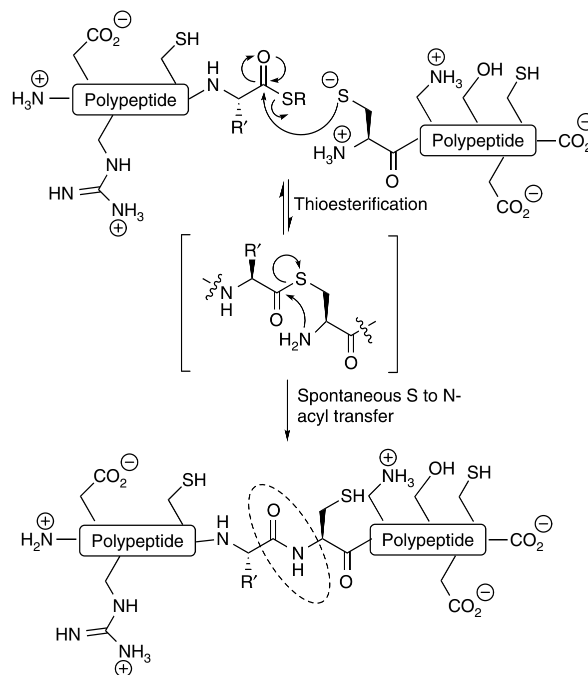
Ajish Kumar K. S. did his MSc (Organic Chemistry) in 2002 in the School of Chemical sciences, Mahatma Gandhi University, Kottayam, India. He completed his PhD in 2008 in organic chemistry (carbohydrate chemistry) under Professor Dilip D. Dhavale on the synthesis and study of monocyclic and bicyclic iminosugars from the University of Pune, India. Since 2008, he is working as a postdoctoral researcher in the laboratory of Dr Ashraf Brik at the Department of Chemistry, Ben-Gurion University, Israel. His current research includes peptide ubiquitylation and synthesis of unnatural amino acids useful for peptide and protein modifications.

Ashraf Brik received his BS from Ben-Gurion University (BGU) in Israel, his MS from the Technion-Isreal Intitute of Technology, and his PhD in Chemistry from the Technion under Professor Ehud Keinan jointly with Professor Philip Dawson from The Scripps Research Institute (TSRI). He received a postdoctoral fellowship from the Israel Science Foundation and spent 3 years as a postdoctoral fellow with Professor Chi-Huey Wong at TSRI. Dr Brik was promoted as a senior research associate and worked for additional 2 years with Prof. Wong. In 2007, Dr. Brik returned to his alma matter as an assistant professor in the Chemistry Department at BGU. His current research involves the design and synthesis of proteins containing peptidomimetic motifs and developing novel chemistries to access proteins with post translational modifications for biological studies. Dr Brik is the recipient of the Ma' of Fellowship and the Marie Curie International Re-Integration award (EU 6th program).

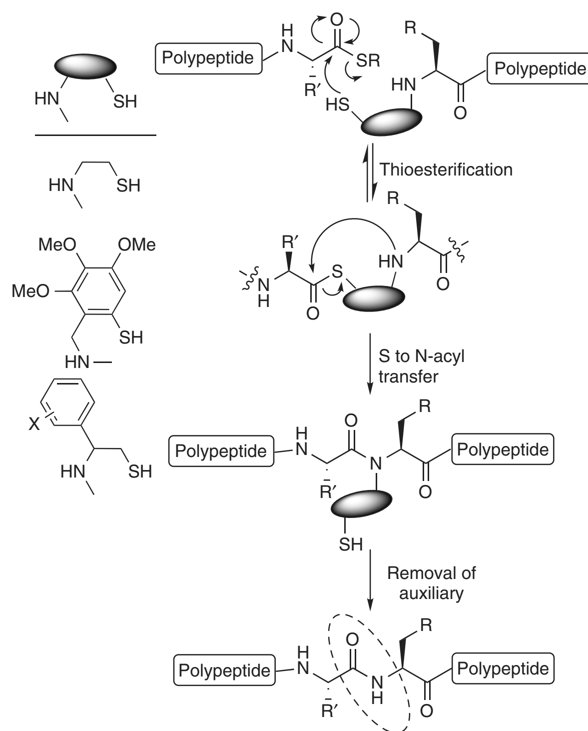
(Scheme 3). Importantly, the development of free radical method for desulfurization led to further improvement of this step in terms of rate and yield [15].

Citing the drawbacks in using N-auxiliary mediated ligation method, Wong and Brik [16–19] reported a conceptionally different extension to NCL by positioning the thiol handle at the glycan part of a glycopeptide to promote peptide bond formation. After sugar assisted ligation (SAL), a desulfurization step would follow to furnish the unmodified N-acetyl glycan (Scheme 4).

The advantage of using SAL lies not only in its ability to assist the native amide bond formation but also to incorporate the glycosyl part site specifically, which has been a prime challenge in the synthesis of homogenous glycoproteins. The fact that a numerous number of proteins are glycosylated during posttranslational modifications adds importance to this approach. The detailed study on this method revealed that reaction proceeds smoothly through a 14/15-membered ring transition state (TS) rather than 12-membered TS. Hence an addition of one amino acid after the glycosylated amino acid is must. The above observation was further clarified by molecular dynamic studies, which revealed a clear correlation between the distance of the functional groups



Scheme 1. Proposed mechanism for NCL.



Scheme 2. N-terminal auxiliary-mediated NCL.

(i.e. thioester and amine) and the rate of the reaction [20]. Unlike in NCL, where a thermodynamically more favored six-membered ring TS is involved, in SAL it is the less favored 14/15 membered ring TS that is being used to give the amide bond. It is also believed that in SAL the restricted conformation plays a major role in the S-N acyl transfer by bringing the N-amino group of glycosyl peptide and the carbonyl group of thioester in close proximity to facilitate the

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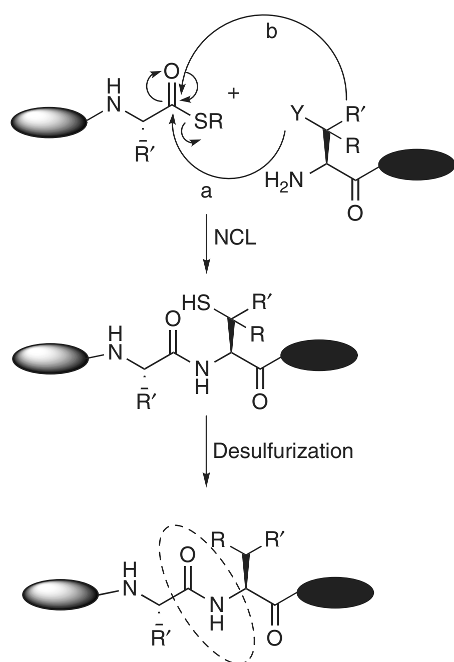


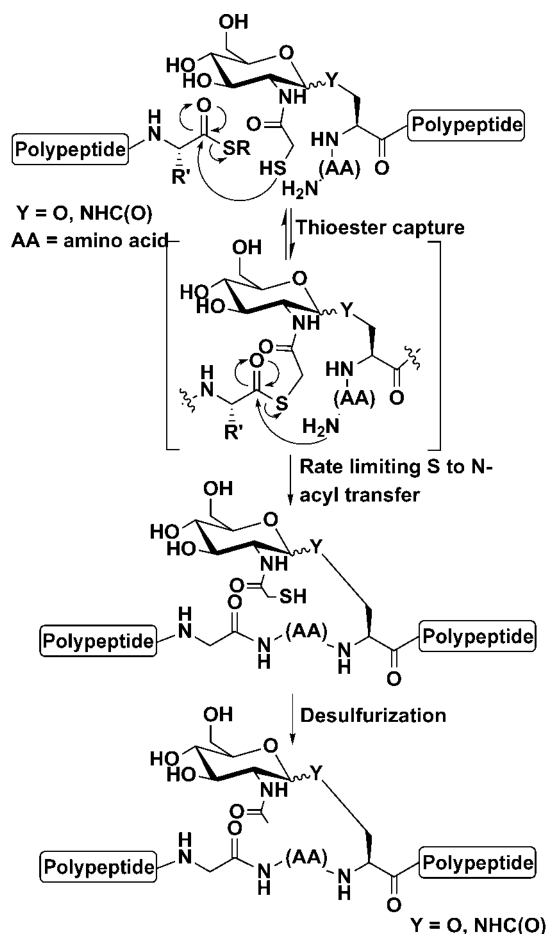
R, R′ = H; Y = SH: ligation at Ala (path a)

R, R′ = CH_3; Y = SH: ligation at Val (Path a))

R = H; R′ = Ph; Y = SH: ligation at Phe (Path a)

R = CH_3; R′ = CH_2SH Y = H: ligation at Valine (Path b)

Scheme 3. Combining NCL and desulfurization.



Scheme 4. Sugar assisted ligation.

intramolecular rearrangement [21]. As stated, faster ligation was observed if the additional amino acid is either aspartic acid/histidine because of the ability of the side chains in these residues to act as base. A study on a model glycopeptide that bears the same sequence but devoid of the thiol handle failed, under similar reaction conditions, to give the ligated product [16]. These results, in addition to the observation of the thioesterification intermediate, gave further support to the proposed reaction pathway. The described strategy holds the flexibility in synthesizing both N- and O-linked glycopeptides/glycoproteins by suitably selecting the sugar substrate with appropriate functionality at the anomeric position and coupling it to side chain functionalities like hydroxyl (Thr, Ser) or carboxylates (Asn) [17,18]. The flexibility of the ligation site revealed the importance of proximity effect as well as the significance of having primary amine in a similar fashion as revealed by studies on amine capture strategy for peptide bond formation through O–N acyl transfer performed by Brenner *et al.*, [22] Wieland *et al.*, [23] and Kemp [24]. One important difference in NCL and SAL is the rate limiting step. While in NCL the thiol capture is rate limiting, in SAL it is the S–N acyl transfer. The efficacy of this method was demonstrated with the successful synthesis of Diptericin, a α-O linked antibacterial glycoprotein [18]. The synthesis takes advantage of the glycosylation site Thr^{54} of diptericin E to promote ligation through Gly–Val junction and was followed with NCL at Ala^{37} which is mutated with Cys37. Subsequently, the thiol functionalities were removed under Dawson's desulfurization conditions to afford the unmodified glycopeptides Deptericn E. Finally, the attachment of thiol handle in more complex sugars has also been examined. Preliminary results indicated that SAL is sensitive to extended glycosylation on the thiol containing sugar. While glucosylation at C-4 and C-6 did not affect ligation, modification at C-3 prevented S–N acyl transfer [25].

The success of SAL in glycopeptide synthesis inspired the development of side chain assisted ligation (SCAL), [26] wherein a removable auxiliary such as cyclohexyl with sufhydryl functionality was attached to the side chain of Asp, Glu, Ser and Thr through ester bond (Scheme 5).

The cyclohexyl moiety in SCAL performs the same function as the cyclohexoses in SAL. Thus, the cyclohexyl group, apart from involving in transthioesterification, helps to keep the N-terminal amine and the acyl group in close proximity to promote rapid and chemoselective S–N acyl transfer. After the completion of ligation the auxiliary was removed *in situ* under controlled saponification to afford the fully unprotected peptide. Like in SAL, the ligation using SCAL is tolerant to different amino acids side chain functionalities, and the ligation rate depends mainly on the C-terminal amino acid. Ligation rates were also dependent on the ring size of the reaction intermediate. Thus auxiliary attached to Glu gave the ligation products at a slower rate in comparison to auxiliary attached to Asp. The potential of this ligation method in protein synthesis was verified in the synthesis of HIV-1 Tat protein [27]. After successful polypeptide assembly of HIV-Tat-1 assisted by SCAL, efforts to remove the auxiliary under different saponification conditions were ineffective. We believe this could be solved by using an auxiliary that allow intramolecular cyclization induced detachment. For example, auxiliary based on proline undergoes intramolecular cylization rapidly, even at pH 7, to yield

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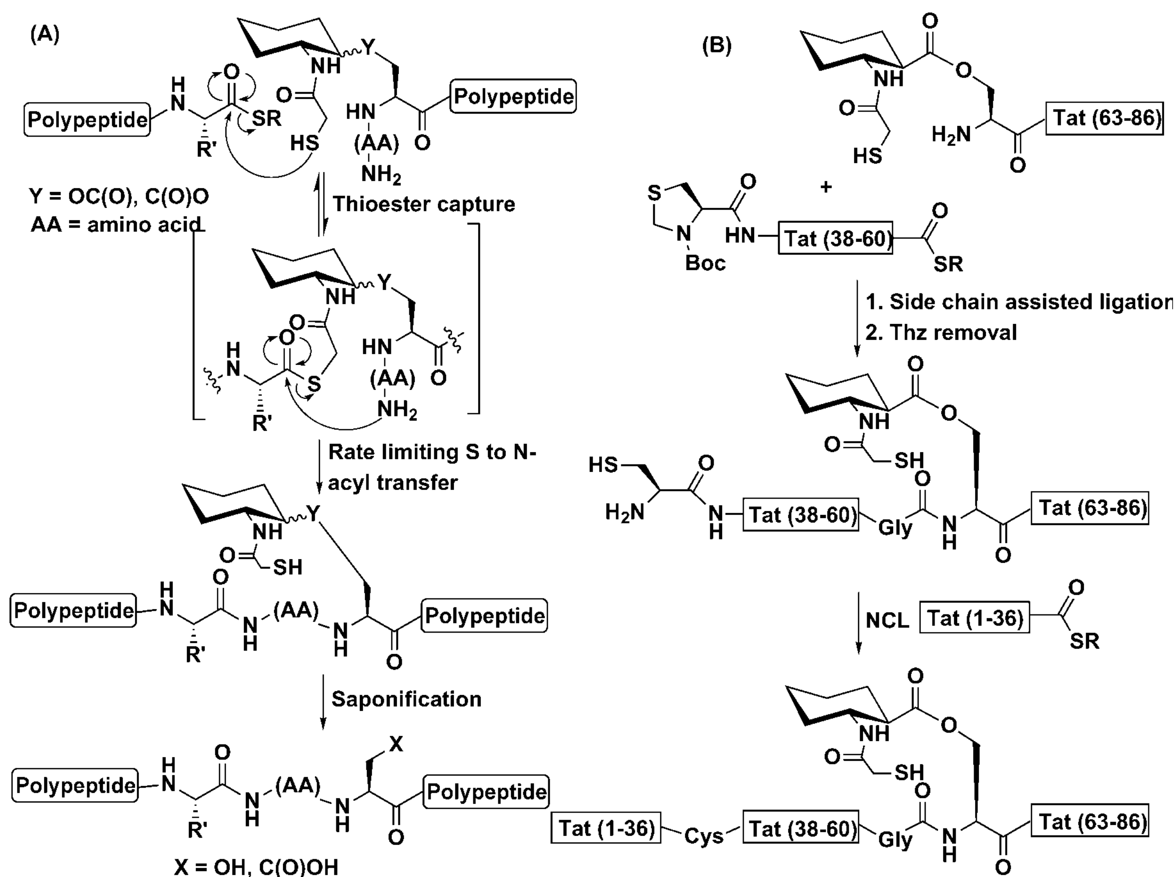

Scheme 5. (A) Side chain assisted ligation(SCAL); (B) SCAL mediated synthesis of HIV-Tat-1 protein.

an indolizidine type skeleton. Thus, by careful selection of auxiliary that is less prone to cyclization because of the conformational restriction can be handy in ligation (neutral pH) and can be effectively removed under saponification conditions.

Posttranslational Modification: Ubiquitylation

Looking at the backyard of the chemical ligation of proteins, the number of methods based on NCL is still expanding (i.e. increasing potential ligation sites) and hence the constraints toward the synthesis of a variety of proteins are diminishing. Apart from the synthesis of natural proteins, the preparation of posttranslationally modified proteins is also gaining the interests of chemical biologists because of the difficulties associated with the expression of homogeneous posttranslationally modified proteins and with obtaining enough quantity for structural and functional analysis. Among the different posttranslational modifications, the ubiquitylation of protein is receiving tremendous attention because of the numerous cellular processes that it is involved in. The task of preparing homogeneous product can be met chemically by carefully selecting the tools and the site of modifications. It was Muir and coworkers who successfully materialized this idea with the successful semi-synthesis of site-specifically ubiquitylated H2B by covalently linking three polypeptide fragments [28]. Because of the absence of cysteine in both ubiquitin and H2B two traceless ligation strategies were pursued. Thus, H2B fragment with residues 117–125 bearing a photolabile ligation auxiliary through a Gly linker at K120 was synthesized chemically (Scheme 6). This linker is the future Gly76 of C-terminus of ubiquitin, most importantly serve as the most favorable Gly–Gly junction for the sterically hindered auxiliary-mediated ligation [29].

Even though the well planned strategy was converted to targeted ubiquitylated H2B, the N-auxiliary mediated ligation took ~72 h for partial ligation, triggering the quest for the development of new and efficient method for ubiquitylation of proteins. Subsequently, we introduced the idea of ubiquitylation at mercaptolysine containing peptides (Scheme 7) [30].

Our strategy relied on the development of modified lysine with sulfhydryl function at its δ-position of the side chain thus achieving a ligation site similar to that of Cys. The amino acid was then incorporated in the α-synuclein peptide (1–17) and the resultant peptide was ligated to ubiquitin thioester in a highly efficient manner. The effectiveness of the strategy was further studied in sequential ligation of ubiquitylated α-synuclein with another peptide thioester. In order to apply the modified Lys amino acid in various ligation schemes mercaptolysine with variable protecting groups were synthesized applicable for both Boc and Fmoc-SPPS [31].

In a parallel study, Liu and coworkers [32] reported dual NCL at Lys by positioning thiol functionality at γ-position of the side chain of Lys (Scheme 8). Using this strategy the group was successful in demonstrating the usefulness of thiol functionality at the γ-position performing dual function i.e. the backbone ligation as well as in the side chain ligation of ubiquitin or biotin.

Recently, Chan and coworkers [33] introduced a genetically encoded pyrrolysine analogue with a ligation handle directly into the recombinant protein to facilitate the synthesis of ubiquitylated protein.

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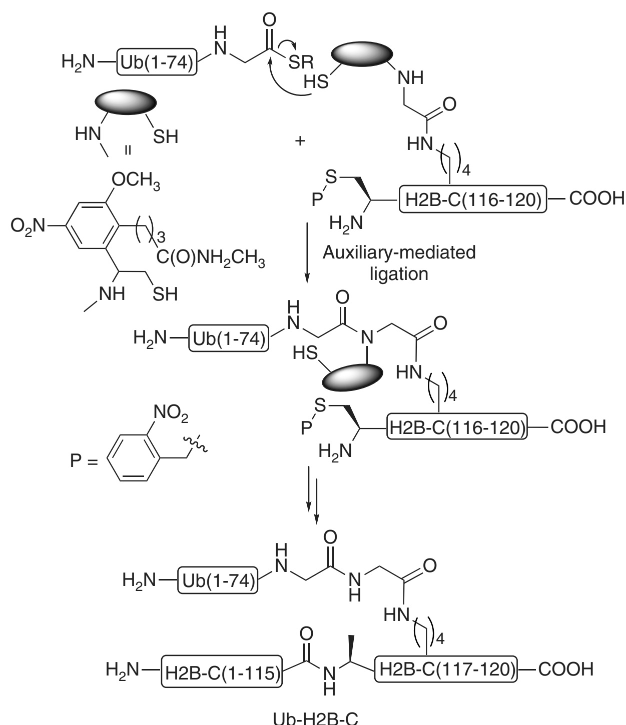
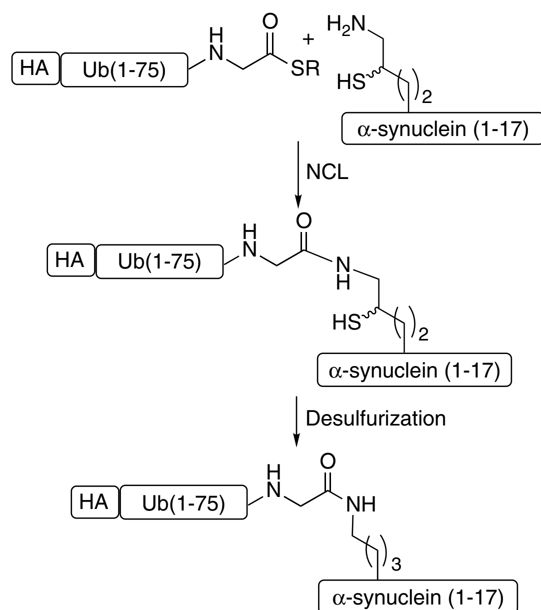


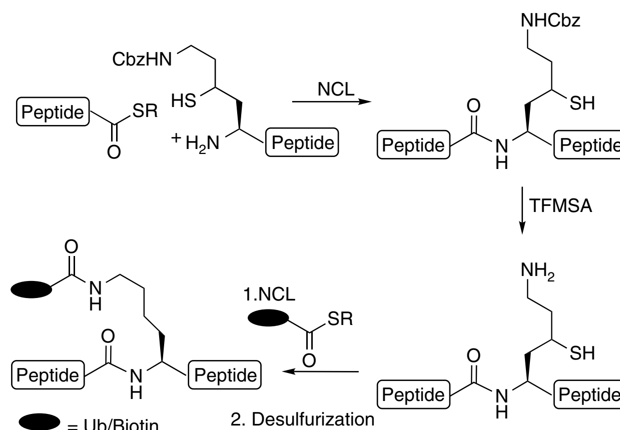
Scheme 6. Site specific ubiquitylation of H2B-C.



Scheme 7. Highly efficient and chemoselective peptide ubiquitylation through δ-mercaptolysine [30].

Conclusions

The chemical synthesis of posttranslationally modified proteins is entering a new phase with the discovery of new ligation methods. For example, the incoming research articles related to peptide ubiquitylation in such a short span of time clearly vindicates the pace and urgency with which the research is being performed in this field. The success of the above ligation strategies revolved around positioning of sufhydryl functionality



Scheme 8. Dual function of lysine.

at a suitable position of the amino acid side chain coupled with selective desulfurization methods. With the discovery of these new chemical tools, understanding the role of posttranslational modifications in cellular process will derive a major boost.

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