Native Chemical Ligation

DOI: 10.1002/anie.200704886

Extending the Scope of Native Chemical Peptide Coupling

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auxiliaries · desulfurization · native chemical ligation · peptides · thioesters

In the early 1990s, a total synthesis of native, functional proteins was considered an almost unachievable goal. However, thanks to Kent and co-workers' development of native chemical ligation, chemical protein synthesis has shifted into the realms of the achievable.[1] The basis of this successful method is the chemoselective reaction of a peptide thioester with a cysteinyl peptide described by Wieland et al.[2] This reaction takes place in aqueous buffer systems, and produces a "natural" peptide bond. The peptide segments can be coupled with one another in unprotected form. It is also possible to synthesize glycosylated or phosphorylated peptides. By combination with molecular biology methods, site-specifically modified proteins can be synthesized by expressed protein ligation, which provides molar masses of up to 52 kDa (β-subunit of F_1-ATPhase).[3]

The course of native chemical ligation is illustrated in principle in Scheme 1. Initially the thiol side chains of cysteine residues participate in reversible exchange reactions in which the thiol RSH of the peptide thioester is also replaced by the cysteinyl peptide **2**. The newly formed thioester intermediate **3** reacts in an S→N acyl transfer to the coupled product **4** via a five-membered transition state. The thiol exchange governs the rate in this reaction sequence. Thiol additives, such as benzylmercaptan, thiophenol, or 2-(4-mercaptophenol)acetic acid (MPAA),[4] are added to accelerate the reaction. These additives lead to the formation of reactive thioesters in an initial equilibrium. In one example, the total synthesis of a covalently coupled HIV-1 protease dimer with the impressive number of 203 amino acids was achieved.[5]

The applicability of the native chemical ligation is restricted in two respects. The synthesis of base-labile peptide thioesters **1** is not always as simple as is customary for peptide acids and peptide amides. Furthermore, cysteine is a relatively rare amino acid (1.4% content), so that in the case of a certain target protein, an artificial cysteine residue must frequently be inserted to provide a suitable coupling site. The present Highlight is concerned with current advances in overcoming these two obstacles.

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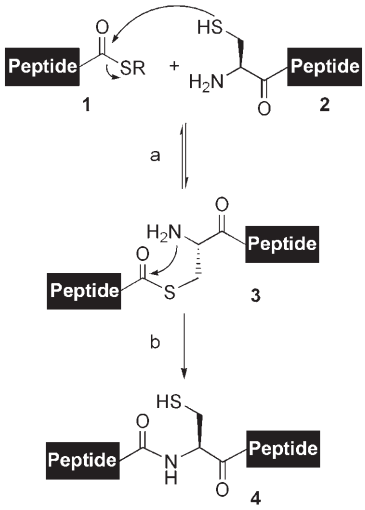
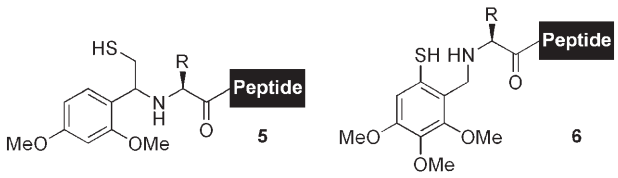


Scheme 1. The principle of native chemical ligation. The cysteinyl peptide initially replaces the thiol component of the thioester in a reversible thiol exchange. Next, a native peptide bond is formed in an S→N acyl transfer step.

The restricted access to peptide thioesters is one of the main obstacles of native chemical ligation. Taking into account the base lability of the thioester structure, peptide thioesters were mostly prepared by *tert*-butoxycarbonyl (Boc) solid-phase synthesis.[6a] However, the necessary use of strong acids for the cleavage of the peptide from the polymeric support is not compatible with acid-sensitive side chain modifications, such as glycosylation or phosphorylation. Therefore, methods that allow the use of the milder 9-fluorenylmethoxycarbonyl(Fmoc) solid-phase synthesis are being intensively investigated. Thus, alternative conditions for Fmoc cleavage under which the thioester function is retained have been sought,[6b,c] and methods have been developed in which the thioester is constructed at a late stage of the synthesis.[6d–r] Recently, a method with a self-purification effect was introduced, which enabled the synthesis of peptide thioesters in high purity without a preparative purification step.[7]

The most important limitation of native chemical ligation is the fact that a cysteine residue must participate in the reaction as nucleophilic reaction partner. To remove this restriction, auxiliary groups have been developed that imitate the cysteine structure in that they provide a cleavable thiol

unit. Normally, the auxiliary group is attached to the terminal α-amino group of the C-terminal fragment. The auxiliaries that are currently the most efficient contain a thiol group in an *N*-benzyl modification (Scheme 2). The electron-rich sub-

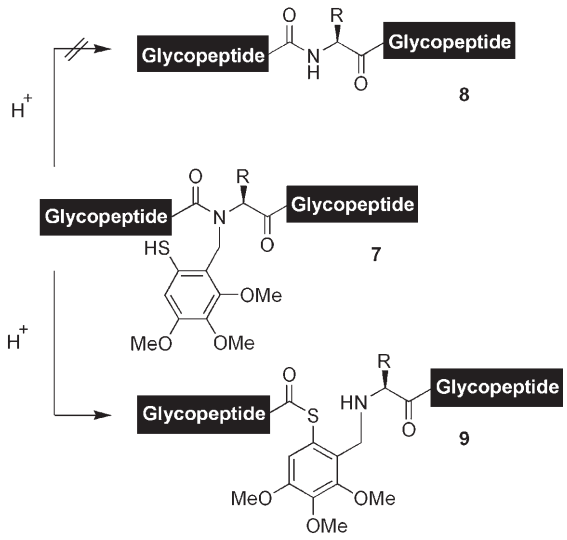


Scheme 2. Auxiliaries for cysteine-free chemical ligation. The mercapto group initially accepts the peptide fragment that is transferred further onto the amino nitrogen atom in the acyl migration step.

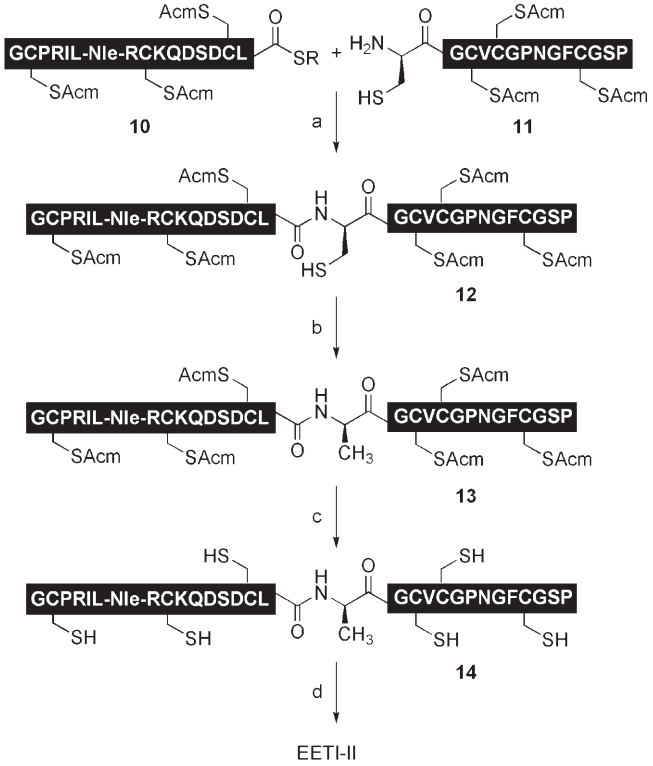
stituents allow the release of the amide formed by acidolysis.[8a,b] The auxiliary-mediated formation of a glycine-glycine peptide bond usually takes place without problems. However, as soon as the steric demand on one of the two reaction partners increases, the achievable reaction rates fall. It is therefore advisable to select the coupling site with participation of at least one glycine residue.

By using the 1-(2,4-dimethoxyphenyl)-2-mercaptoethyl auxiliary **5** it was possible to construct the 106 amino acid sequence of cytochrome b526 from two fragments. The coupling proceeded between a peptidyl histidine residue and a glycinyl peptide.[8c] Very recently, Danishefsky et al. reported the use of the trimethoxybenzyl auxiliary **6** in the construction of complex glycopeptides. In this case, the auxiliary-mediated coupling between a glycine thioester and glutaminyl peptide did not take place in an aqueous buffer system, but in dimethylformamide.[8d] While attempting the acidolytic removal of the auxiliary group, it was observed that the protonation of the secondary amide nitrogen atom induced a reverse migration of the peptide acyl residue in glycopeptide **7** to the auxiliary SH group (Scheme 3). For this reason it was only possible to obtain the intermediate **9** and its hydrolysis products. Methylation of the SH function of the auxiliary prevented this intramolecular acyl migration, so that subsequent acid treatment yielded the desired result. The fact that the relatively demanding glycine-glutamine coupling succeeded makes the trimethoxybenzyl auxiliary even more interesting. To counter the problem of multiple methylations, reactive groups must possibly be protected. Nonparticipating cysteine residues can be protected from desulfurization by means of protection with the acetamidomethyl (Acm) group.

The use of β-mercaptoamino acids and their subsequent desulfurization offers an alternative to the use of auxiliaries. In the simplest case, recourse is made to cysteine, which is optimal for coupling and is subsequently converted into alanine.[9a] Alanine occurs frequently in proteins, and thus a suitable XX-Ala coupling site (XX ≠ Pro)[10] should be detectable in almost every protein. To protect nonparticipating cysteine residues from desulfurization, they can be provided with the acetamidomethyl (Acm) protecting group.[9b] Scheme 4 illustrates the procedure on the basis of the synthesis of the trypsine inhibitor protein EETI-II. In the first instance, the native chemical ligation of the leucine



Scheme 3. During the acid treatment of the coupling product **7**, an N→S acyl transfer (→**9**) can impede the cleavage of the ligation auxiliary. Quantitative removal of the auxiliary is possible only after methylation of the thiol function in **7**.



Scheme 4. Coupling–desulfurization strategy. Initially cysteinyl peptide and peptide thioester react within the context of the native chemical ligation (a). The cysteinyl product **12** is then converted into the alanyl product **13** in high yield with a large excess of Raney nickel (b). Thereafter follows the removal of the Acm protecting groups from nonparticipating cysteine residues by reaction with I_2, the formation of three disulfide bridges in the presence of a glutiothione redox buffer system, and thus folding of the native EETI-II (c and d).

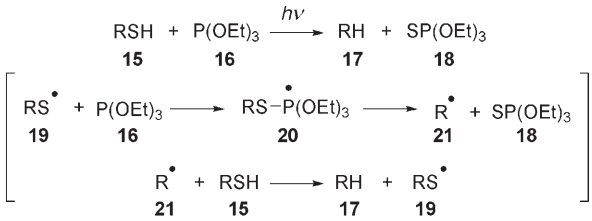
thioester **10** was carried out with the cysteinyl peptide **11**. The sulfur atom was subsequently removed by reaction with a

large excess of Raney nickel, followed by deprotection of the internal cysteine side chains and folding of the native EETI-II in a redox buffer.

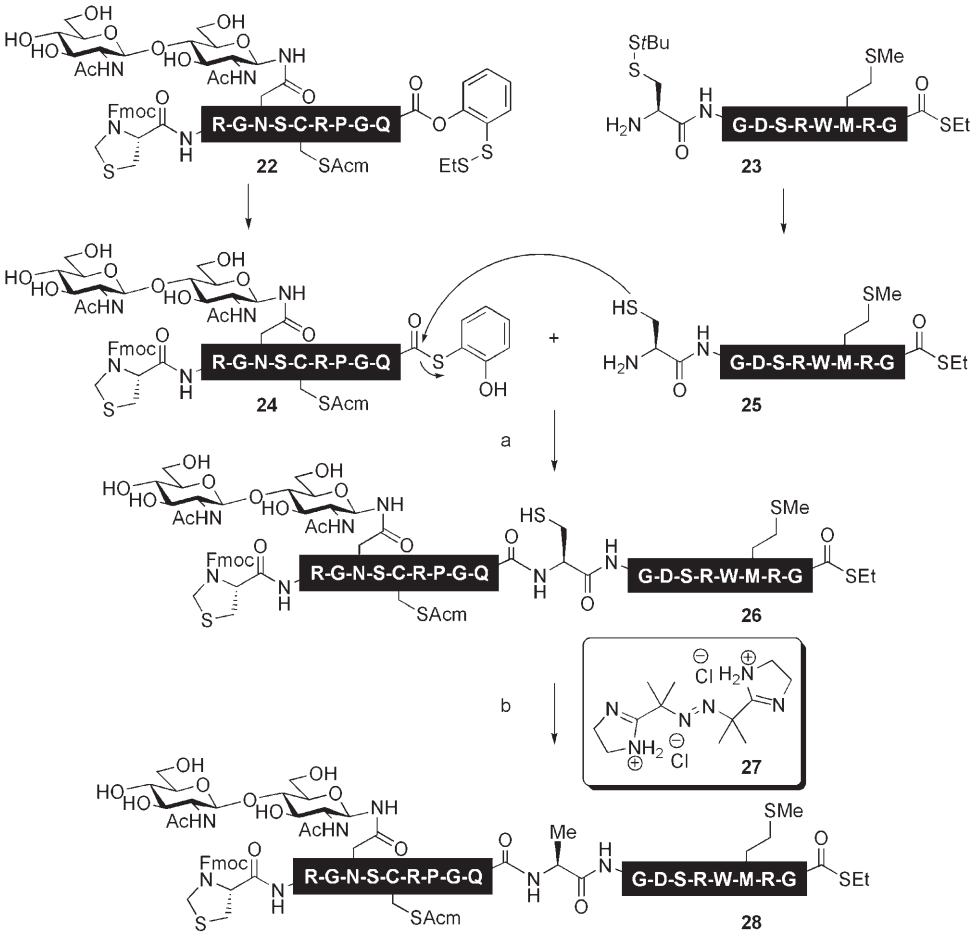
Crich et al. and Botti et al. recently extended the repertoire of this strategy.[11a,b] A β-mercaptophenylalanine building block was used in place of a cysteine residue. After metal-mediated desulfurization, phenylalanine was obtained. This amino acid occurs in proteins with a frequency of 4.1%. Model reactions demonstrated that Met-Phe and Ile-Phe couplings are feasible. In principle the synthesis of the β-mercaptophenylalanine building block is also applicable to histidine, tryptophan, and tyrosine.[11a]

Native chemical ligation to cysteinyl and β-mercaptophenylalanine peptides takes place with adequately high reaction rates, even when the C-terminus of the peptide thioester is made up of sterically demanding amino acids, such as valine or isoleucine. In principle, the method is suitable for multiple dipeptide segments, although it brings with it the possibility of side reactions during the removal of the thiol group. Large excesses of metals or hydrogenation catalysts are typically necessary for desulfurization. However, these also react in an undesirable way with methionine, with the formation of an α-aminobutyric acid residue. Furthermore, the thiazolidine protecting group, which is used for the intermediate masking of N-terminal cysteine groups in consecutive segment couplings, is unstable during desulfurization with a metal. One problem in the synthesis of larger peptides concerns the low recovery rate of the peptide material, whose quantitative extraction is difficult to ensure because of adsorption onto the large metal surfaces. Until recently, the necessary use of large amounts of desulfurization reagent was a critical disadvantage of the strategy. A new method by Danishefky et al. manages without metal reagents, and thus once more sheds new light onto the coupling–desulfurization strategy.[12] The method is based on a reaction for desulfurization of mercaptans with trialkylphosphites, which Hoffmann et al. introduced as early as 1956.[13a]

Walling and co-workers replaced phosphites by phosphines as desulfurization reagent and proposed the mechanism shown in Scheme 5.[13b,c] The thiyl radical **19** formed under the influence of light reacts with the phosphite **16** to provide phosphoranyl species **20**. Subsequent decomposition of **20** produces the alkyl radical **21**. The homolytic abstraction of a hydrogen atom from remaining mercaptans generates the desulfurized product, and at the same time continues the radical chain by formation of a new thiyl species **19**. Valencia and González used triethylborane in acetonitrile as radical initiator to convert cysteine into alanine with the highly nucleophilic triethyl phosphite.[14] The reagents applied by Danishefsky et al. set the stage for the implementation of alanine as a potential ligation site in protein synthesis. Triscarboxyethylphosphine (TCEP), almost ubiquitous in peptide chemistry, acts as reducing agent. Danishefsky et al. used VA-044 (2,2′-azo-



Scheme 5. Proposed mechanism for radical desulfurization.[13b]



Scheme 6. Kinetically controlled ligation and selective, metal-free desulfurization. a) 6 M Guanidine hydrochloride, 0.2 M Na_2PO_4, 0.19 mM TCEP·HCl buffer (pH 6.3), 67%; b) EtSH, *t*BuSH, TCEP, **27**, 37°C, 87%.

bis[2-(2-imidazolin-2-yl)propane] dihydrochloride (**27**)) for the initiation of the radical chain. This reagent and an analogue, AVCA (4,4′-azobis(4-cyanovaleric acid), have recently been introduced as initiators for the radical coupling of thiolglycopeptides with olefin conjugates.[15] TCEP and VA-044 are both water soluble. In the example in Scheme 6, which demonstrates the high selectivity of this desulfurization method, the two peptide fragments **22** and **23** were first subjected to reductive cleavage of the disulfides whereby the phenolic ester **22** reacted to form the thioester intermediate **24**. This was followed by the fusion of the formed fragments **24** and **25**. This coupling of the glutamine thioester and the cysteinyl peptide occurred rapidly and unproblematically so that the ligation product **26** was isolated in a 67% yield after only two hours' reaction time. A reaction of the cysteine residue with the C-terminal ethyl thioester occurred only within the coupling product **26**, forming only a slight extent of a cyclic thiolactone. The product of the cysteinyl ligation **26** was subsequently incubated at 37°C in an aqueous solution with the radical initiator VA-044 and TCEP for a period of just two hours. Further reaction components *t*BuSH and EtSH (both in large excess) were also added as hydride donors to accelerate the reaction. The numerous sulfur-containing groups of the cysteine peptide **26** remain unchanged during the reaction. Thus, in spite of the presence of a thiazolidine, an Acm-protected internal cysteine residue, a methionine residue, and a C-terminal thioester, **26** could be selectively desulfurized exclusively at the unprotected cysteine residue. The numerous sulfur-containing structural units were as equally tolerated as the asparagine-bound disaccharide, whose secondary hydroxy groups would have been at risk of an epimerization under the conditions of a Raney nickel desulfurization. After desulfurization the from now on alanine-containing peptide **28** was isolated in a yield of 87%. In comparison to the conventional methods, the desulfurization strategy of Danishefsky et al. provides a high yield and peptide recovery rate, takes place without side reactions, and is also metal-free. Only in this way will the combined use of native chemical ligation and desulfurization be a robust, competitive method.

Over the last ten years, native chemical ligation has become one of the most efficient tools in protein chemistry. The increasing number of publications with a biological background whose results are based on the coupling of peptide fragments emphasizes the potential of this procedure. The development of auxiliaries and coupling alternatives, such as the ligation–desulfurization strategy, continuously extends the repertoire of these methods. Improved methods, such as the desulfurization method introduced by Danishefsky et al., simplify the synthesis to a degree that opens up access to tailored proteins of choice—even for the less experienced researcher.

Published online: January 31, 2008

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