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Native chemical ligation at a base-labile 4-mercaptobutyrate N^{α}-auxiliary

Ziv Harpaz, Simon Loibl, Oliver Seitz *

Institut für Chemie, Humboldt Universität zu Berlin, Brook-Taylor-Straße 2, 12489 Berlin, Germany

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ABSTRACT

Native chemical ligation (NCL) proceeds via a S–N acyl shift and, therefore, requires N-terminal cysteine. N^{α}-auxiliaries have long been used to enable NCL beyond cysteine. However, the reversibility of the S–N acyl shift under the acidic conditions used to remove the commonly applied *N*-benzyl auxiliaries limits the scope of this reaction. Herein, we introduce a new class of N^{α}-auxiliary which is designed for removal under mild basic conditions. The 3-*N*-linked 4-mercaptobutyrate auxiliary is readily synthesized in a single step and enables introduction on solid phase by means of reductive amination. The usefulness of the new auxiliary was demonstrated in the synthesis of the anti-microbial C-terminal domain of Dermicidine-1L.

Native chemical ligation[1] has dramatically advanced the field of peptide and protein synthesis by enabling chemoselective coupling of unprotected peptides under mild conditions. The reaction involves a peptide α-thioester and a peptide carrying an N-terminal cysteine (Scheme 1A). Reversible thiol exchange followed by an irreversible S→N acyl shift yields the native peptide bond. The method has gained broad use and is to date perhaps the method of choice for chemoselective synthesis of peptides and proteins.[2]

Unfortunately, the requirement for a cysteine at the N-terminus of the C-terminal segment is a limitation. Cysteine is a rare amino acid and may be located at positions unsuitable for NCL chemistry. Efforts to overcome the Cys restriction have led to the development of two main methods,[3–6] which rely on desulfurization of thiolated amino acid building blocks (Scheme 1B)[7–20] or the use of N^{α}-auxiliaries (Scheme 1C).[21–24] While several impressive feats were accomplished through the ligation–desulfurization approach[8,25–28] the method requires access to a specific thiol-modified amino acid for every ligation junction. The limited commercial availability of thiolated amino acid building blocks calls for dedicated synthesis efforts which are a burden to non-specialist's laboratories.

The N^{α}-auxiliary method involves the introduction of a thiol-bearing scaffold onto the N-terminus of a peptide. Typically, an *N*-benzyl scaffold (see **9a** or **9b**) is designed to enable the removal of the auxiliary once ligation is complete. Though the approach has widened the scope of ligation junctions accessible by NCL, there are problems which limited a broader acceptance.

For example, the amide bond established in the ligation step may break under the acidic conditions used for the removal of the existing *N*-benzyl type auxiliaries.[29] This side reaction is caused by an acid promoted N→S acyl shift which forms the thioester intermediate and, subsequently, hydrolysis products. The sterically demanding *N*-benzyl type auxiliaries typically show low ligation rates at non-glycine ligation junctions. Furthermore, the synthesis of some acid labile auxiliaries is rather tedious and their introduction frequently requires solution steps to prepare preformed amino acid-auxiliary conjugates or special building blocks such as α-bromo-derivatives of amino acids.[24] Photolysis is an alternative method used for the removal of *o*-nitrobenzyl-type auxiliaries, yet the bulky structures cause, again, rather slow ligation kinetics.[30–34] Herein, we present a new class of N^{α}-auxiliary. The design is based on a 3-*N*-linked 4-mercaptobutyrate scaffold (see **9c**), which is rapidly accessible by a single step synthesis, facilitates auxiliary introduction via reductive amination directly on the solid-supported peptide and enables auxiliary cleavage under mildly basic conditions.

In the new auxiliary (**9c**), the thiol handle is positioned two carbon atoms away from the N-terminal peptide amino group enabling a five-membered ring transition state as in NCL. The cleavage characteristic of the auxiliary arises from the presence of an ethyl ester group (Scheme 2). Under mildly basic conditions,

* Corresponding author.

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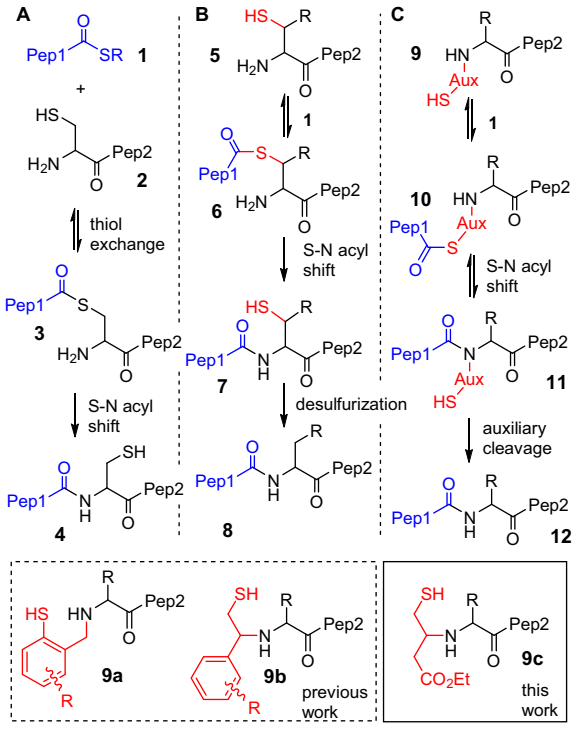
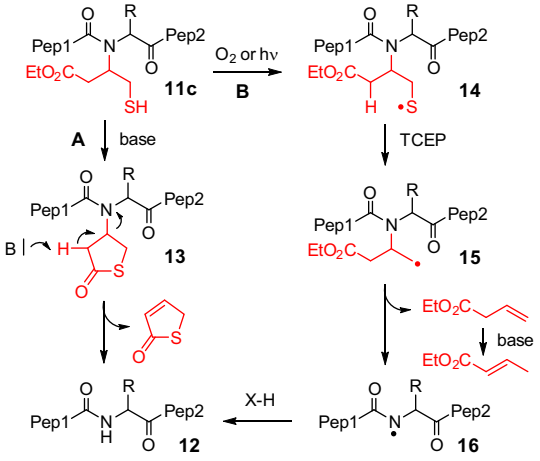


Scheme 1. Mechanism of (A) NCL, (B) ligation–desulfurization and (C) auxiliary assisted NCL.

thiolactone **13** may be formed. We assumed that the electron withdrawing thioester group would allow α-deprotonation under mildly basic conditions and a concomitant elimination of the amide group would form the native peptide. The resulting α,β-unsaturated thiolactone product may react further under the reaction conditions. As an alternative to the ionic mechanism and in accordance with the cleavage mechanism proposed for the recently introduced 2-mercapto-2-phenethyl auxiliary,[35] oxygen or light may trigger the formation of thiyl radical **14**. As reported for cysteine, the reaction with triscarboxyethylphosphine (TCEP) would induce desulfurization.[36] In the absence of a powerful hydrogen donor the alkyl radical **15** may undergo a fragmentation reaction, which would deliver the crotonate and the amide radical **16**. The latter should be more reactive than the alkyl radical and may therefore be scavenged by rather unreactive hydrogen donors such as amines.



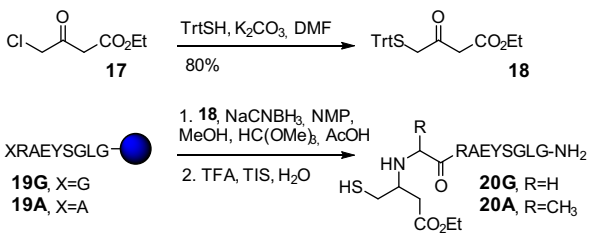
Scheme 2. Hypothetical mechanisms for cleavage of 3-amide-linked 4-mercaptobutyrate auxiliaries under basic conditions via (A) ionic mechanism or (B) radical fragmentation.

To enable the introduction of the auxiliary by reductive amination, ketone **18** was synthesized in a single step starting from commercially available ethyl 4-chloro-3-oxobutanoate **17** (Scheme 3). Subsequently, the auxiliary ketone **18** was attached to two resin-bound model peptides **2** and **3** by using trimethoxyorthoformate under acidic conditions. The conjugate was treated with TFA for liberation and removal of side chain protection and the auxiliary S-trityl group. After HPLC purification, peptides **20G** and **20A** were obtained in 40% and 28% overall yield, respectively (Scheme 3, Fig. S3).

The new auxiliary was evaluated in native chemical ligation reactions (Fig. 1A) involving two peptide mercaptopropionamide thioesters **21G** and **21A**. The first ligations were performed by dissolving the auxiliary peptide **20G** and the thioester **21G** in a ligation buffer (pH 7.5) and incubating at 25 °C. Thiophenol was included for in situ conversion of the mercaptopropionamide esters into the more reactive mercaptophenyl esters. The formation of the Gly-Gly junction upon reaction of **20G** with **21G** proceeded smoothly (Fig. 1B and C) and provided the ligation product **22GG** in 90% yield after 4 h. After HPLC purification auxiliary-modified ligation product **22G** was isolated in 55% yield.

Next we assessed the sterically more demanding Ala-Gly (**21A** + **20G**) and Gly-Ala (**21G** + **20A**) ligations (Fig. 1C). The reactions furnished the ligation products **22AG** and **22GA** albeit at significantly reduced rate. The analysis of the early reaction phase suggests that the Gly-Ala ligation was more challenging for the 4-mercaptobutyrate auxiliary than the Ala-Gly ligation. Nevertheless, after 8 h the Ala-Gly ligation product **22AG** was isolated in 40% yield. The more challenging establishment of the Gly-Ala junction was less successful and **22GA** was obtained in 10% isolated yield after HPLC purification. We also explored the 4-mercaptobutyrate auxiliary in an Ala-Ala ligation (**21A** + **20A**). HPLC/MS analysis suggested that the thiol exchange step succeeded but the initially formed thioester intermediate did not rearrange via the S–N acyl shift (Fig. S8). This is in agreement with the results reported for most other ligation auxiliaries[2a] but the recently introduced 2-mercapto-2-phenethyl scaffold.[35]

We noticed that under the slightly basic conditions (pH 7.5) applied, the maximally achievable yields were limited by a side reaction. Ligation competed with cleavage of the 4-mercaptobutyrate auxiliary (Figs. S5–S8). Of note, under the ligation conditions chosen the auxiliary was exclusively released from the starting auxiliary peptides **20** rather than from the ligation products **22**. This side reaction reduces the concentration of the available auxiliary peptide in the course of the ligation reaction and, therefore, contributes to the rather inefficient ligation at the Gly-Ala junction. On the other hand, the side reaction suggested that a mild auxiliary cleavage is feasible. We screened several conditions to induce auxiliary cleavage from ligation product **22GG**. In the first attempts, we explored various glycine buffers at pH 9.0–10.5. This led to formation of the disulfide without apparent cleavage of the auxiliary (Fig. S9). According to our working hypotheses, a free thiol is



Scheme 3. Synthesis of auxiliary ketone **18** and reductive amination with resin-bound peptides **19G** and **19A** to yield auxiliary peptides **20G** and **20A**. (NMP, *N*-methylpyrrolidone; TIS, triisopropylsilane; Trt, trityl.)