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## COMMUNICATION

## Acceleration of thiol additive-free native chemical ligation by intramolecular S→S-acyl transfer

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J. Schmalisch<sup>a</sup> and O. Seitz<sup>a</sup>

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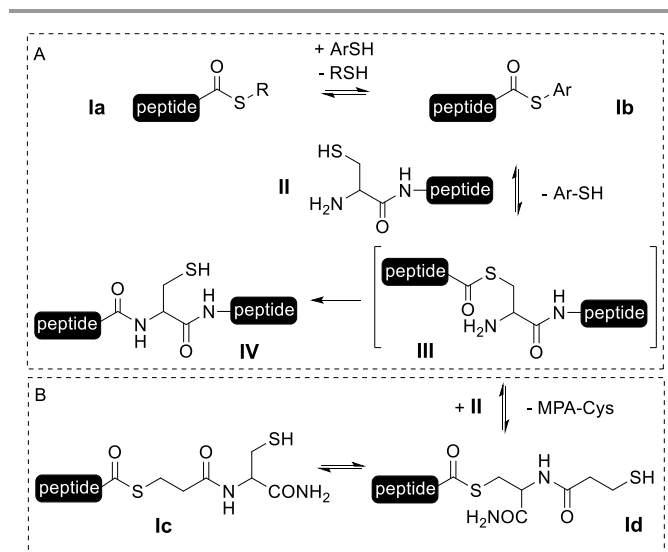
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**Peptide-mercaptpropionylcysteine (MPA-Cys) thioesters show a surprisingly high reactivity in native chemical ligation (NCL) and allow thiol-additive free reactions. This facilitates sequential NCL reactions and ligation-desulfurization reactions in one-pot formats. The synthetic utility is demonstrated by the synthesis of a SH3 domain.**

Next to solid phase peptide synthesis the native chemical ligation (NCL) is amongst the most powerful tools applied in the chemical total synthesis of proteins.<sup>1</sup> The reaction enables the amide-forming connection of two unprotected peptide segments (Scheme 1). In the event, a peptide thioester **I** reacts with a peptide containing an N-terminal cysteine **II**. First, a thiol exchange reaction leads to the formation of a thioester intermediate **III**. A subsequent intramolecular S→N acyl shift establishes the native peptide bond in **IV**. Cysteine is not among the most abundant amino acids and there are many proteins or protein domains which lack cysteine at a position appropriate for ligation. Accordingly, the breadth of NCL chemistry has been extended beyond cysteine.<sup>2,4</sup> The ligation-desulfurization tactic is amongst the most frequently applied approaches for the synthesis of cysteine-lacking proteins.<sup>5-12</sup> This approach involves thiolated amino acids which are converted after NCL to canonical amino acids in a radical desulfurization reaction.<sup>2</sup>

The NCL requires peptide thioesters, which are commonly prepared as alkyl-type thioesters with thiol components based on benzylmercaptane<sup>1</sup> or mercaptpropionamide (MPA)<sup>13-17</sup>. These thioesters have low reactivity, which simplifies synthesis, purification and storage. However, in ligation experiments the reactivity of alkyl-based thioesters usually is too low to enable NCL under challenging (yet realistic) conditions when ligation involves sterically encumbered amino acids or when ligation ought to succeed with sparingly soluble peptide fragments. To increase the reactivity of alkyl peptide thioesters, thiol additives are commonly included in NCL reactions (Scheme 1A).<sup>18</sup> Aryl-based mercaptanes such as thiophenol<sup>18</sup> or mercaptophenylacetic acid (MPAA)<sup>19</sup> lead to the formation of highly reactive phenyl-type thioesters **Ib** and, thereby, confer dramatic enhancements of the ligation rate. In spite of this,



**Scheme 1.** Principle of native chemical ligation via A) in situ conversion of unreactive alkyl thioesters **Ia** into reactive aryl thioesters **Ib** or B) activation of MPA-Cys thioester **Ic** upon intramolecular S→S acyl transfer and formation of a reactive, side-chain cysteine thioester **Id**. (R = alkyl thiol; MPA = mercaptpropionic acid)

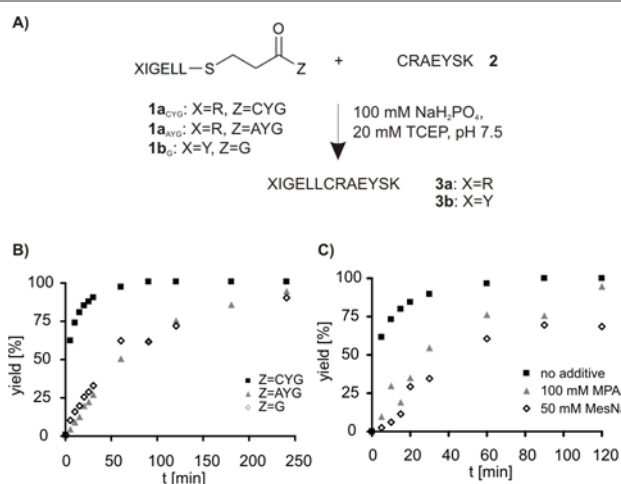
the use of thiol additives also entails a number of drawbacks. Some thiols such as thiophenol are cell toxic. Thiols can provoke undesired side reactions with alkene and alkyne structures.<sup>20</sup> Furthermore, the thyl radicals formed from arylmercaptanes have high stability and act as radical scavengers.<sup>21</sup> This complicates the application of the ligation-desulfurization tactics. The conditions applied in NCL and in desulfurization reactions would, in principle, be compatible with a one-pot reaction format, but the presence of arylmercaptanes is detrimental for the radical desulfurization chemistry. Because of that, tedious HPLC purification and lyophilization is commonly needed prior to desulfurization. To facilitate their removal aryl mercaptanes have been equipped with additional functional groups and captured with aldehyde-functionalized beads.<sup>22</sup> Recently,

trifluoroethanethiol (TFET) was introduced as an alkyl thiol additive which enables one pot ligation-desulfurization reactions.<sup>23</sup> TFET is volatile (bp. 34°C) and therefore difficult to use in NCL/desulfurization reactions which require higher temperatures. Of note, the use of thiol additives must be avoided in kinetically controlled NCL reactions which involve two or more peptide thioester segments of different reactivity.

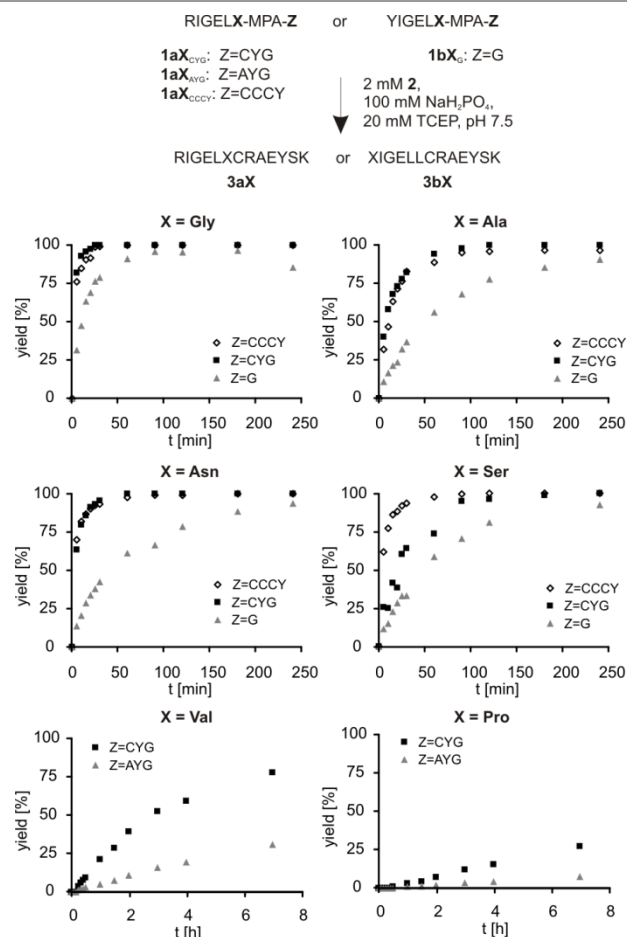
The drawbacks caused by the use of thiol additives prompted us to seek for NCL reactions that can be performed in the absence of thiol additives. We showed that ascorbate can replace thiol additives in NCL reactions.<sup>21</sup> However, ascorbate is a known radical scavenger, which argues against its use in one pot ligation-desulfurization reactions. We were interested in finding a methodology that increases the thioester reactivity *in situ* and at the same time allows one-pot ligation-desulfurization without using thiol additives. We assumed an intramolecular S→S acyl transfer reaction which would convert a relatively stable alkyl thioester into a reactive thioester that drives the NCL reaction (Scheme 1B). In previous studies on nucleic-acid-templated acyl transfer reactions we noticed the high reactivity of side-chain linked cysteinyl thioesters.<sup>24-28</sup> Accordingly, we combined the robust mercaptopropionic acid (MPA) linkage with a C-terminal cysteine residue (**1c**). We envisioned that the synthesis of the peptide-MPA-Cys thioester **1c** should proceed as easy as the synthesis of the commonly used peptide-MPA thioesters. The intramolecular S→S acyl transfer should occur at NCL conditions and the reactive cysteine thioester **1d** formed should provide the high reactivity required for NCL under challenging conditions.

In order to investigate this strategy, we prepared the model peptide-MPA-Z thioesters **1** via solid phase peptide synthesis on the MBHA resin. The amino acids between the MPA linker and the resin contained a cysteine residue in **1a<sub>CYG</sub>** or lacked cysteine in **1a<sub>AYG</sub>** and **1b<sub>G</sub>** (Figure 1A). The peptide thioesters were allowed to react with the cysteine peptide **2** in a thiol additive-free phosphate buffer at pH 7.5 and 2 mM concentration. Tris(carboxyethyl)phosphine (TCEP) was included in the buffer to maintain a reducing environment. The peptide thioesters **1a<sub>CYG</sub>** which contained the

MPA-Cys structure provided ligation product **3a** in quantitative yield within 90 min (Figure 1B). Half maximal formation of product was achieved in less than 5 min. By contrast, the cysteine-lacking peptide thioester **1a<sub>AYG</sub>** required > 240 min to furnish > 93 % ligation product and 60 min for half maximal product formation. Thioester **1b<sub>G</sub>** which contained a glycine residue instead of the tripeptide in **1a<sub>CYG</sub>** or **1a<sub>AYG</sub>** showed a similar behavior. We attribute the rate acceleration provided by the cysteine in the peptide-MPA-Cys thioester **1a<sub>CYG</sub>** to the proposed intramolecular S→S acyl transfer. We examined whether the addition of thiol reagents would enable additional rate increases (Figure 1C). However, neither did the involvement of MesNA nor of MPAA lead to further improvements. Rather, the thiol additives led to decreases of the reaction rate; an effect, which has previously been observed in auxiliary-mediated NCL reactions.<sup>29, 30</sup> We inferred that the side-chain peptide thioester formed upon intramolecular S→S acyl transfer can compete with the reactivity of MPAA-based thioesters. With glycine, alanine, asparagine, serine, leucine, valine and proline we assessed C-terminal amino acids with varied reactivity in peptide thioesters **1** (Figure 1B).<sup>31</sup> In any case, the presence of a cysteine in the MPA leaving group accelerated the NCL with cysteine peptide **2**. For the least hindered amino acid glycine the ligation was completed after 30 min when the MPA-Cys-Tyr-Gly thioester (**1a<sub>G<sub>CYG</sub></sub>**) was



**Figure 1.** A) NCL reaction between peptide-MPA-Z thioesters **1** (2 mM) and cysteine peptide **2** (2 mM). Time course of product formation in ligations of **2** with B) peptide thioesters **1a<sub>CYG</sub>**, **1a<sub>AYG</sub>** and **1b<sub>G</sub>** as a function of the thiol leaving group or C) with **1a<sub>CYG</sub>** in absence or presence of thiol additives.

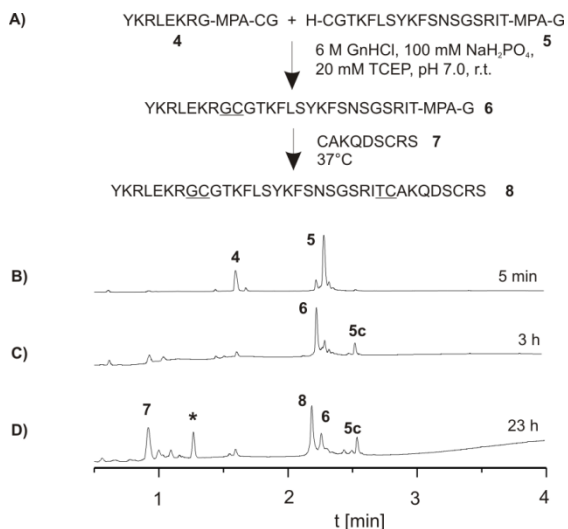


**Figure 2.** Dependence of NCL on the MPA-Z leaving group and the C-terminal amino acid **X**.

used, whereas 78% was obtained at the same time with the MPA-Gly thioester **1bG<sub>G</sub>**. The ligation acceleration induced by cysteine in the thiol component was more obvious when the C-terminal amino acid was sterically hindered. For example, the valine-MPA-Cys-Tyr-Gly thioester **1aV<sub>CYG</sub>** afforded 78 % ligation product in 7 h, whereas the 'classical' valine MPA-Ala-Tyr-Gly thioester **1aV<sub>AYG</sub>** provided only 31 % yield. We also tested whether additional cysteine residues allow for further increases of the NCL efficiency. For the peptide thioesters with glycine, alanine or asparagine as C-terminal amino acid there was little difference in reactivity between the MPA-Cys-Tyr-Gly and MPA-(Cys)<sub>3</sub>-Tyr leaving groups. However, the two additional cysteine residues proved advantageous for the NCL of the peptide thioester at serine (**1aS<sub>CCCY</sub>**). The decrease in reactivity observed for the peptide thioester at leucine was attributed to the limited solubility of **1aL<sub>CCCY</sub>**.

The experiments showed that the reactivity of the peptide-MPA-Z thioesters can readily be adjusted. Encouraged by these findings the idea of using controlled thioester reactivity for ligation reactions with more than two segments was rising.<sup>32, 33</sup> In N-to-C directed sequential ligations,<sup>32, 34-36</sup> a N-terminal peptide segment is equipped with a highly reactive thioester that drives a rapid NCL with a bifunctional middle fragment. The latter contains an N-terminal cysteine residue as well as a C-terminal peptide thioester, which must have low reactivity to minimize oligomerization or cyclization. However, the reactivity must still be sufficient to enable the second NCL with a C-terminal peptide segment which is added after the first NCL without intermediary purification.

We tested the peptide-MPA-Cys thioesters in the synthesis of a 34mer segment from human type II secretory phospholipase A2 (residues 51-85, Figure 3).<sup>37</sup> The protein sequence provides several

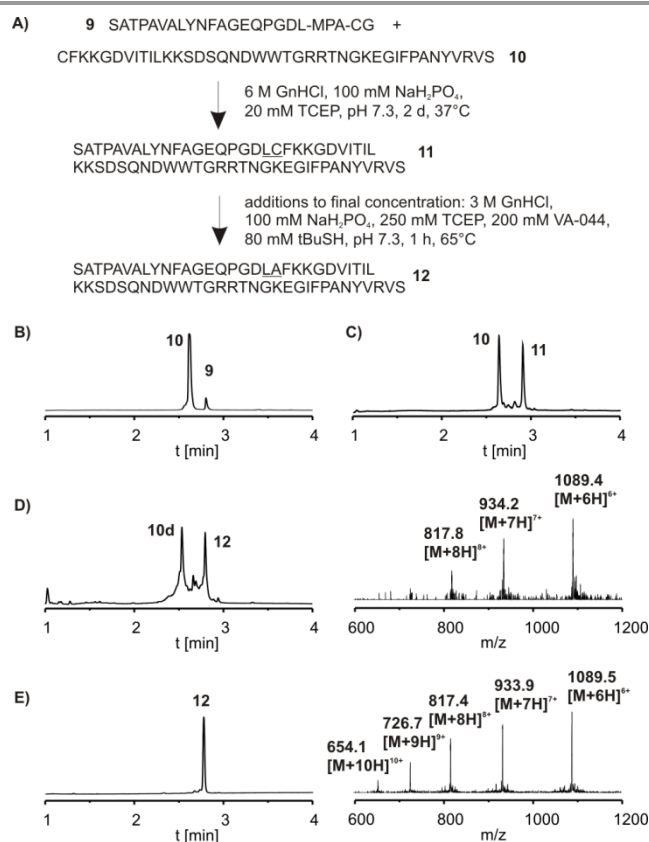


**Figure 3.** A) One-pot sequential NCL reactions under kinetic control. UPLC trace ( $\lambda = 210$  nm) after B) 5 min or C) 3 h of the reaction between **4** (2 mM) and **5** (3 mM) and D) 4 h after addition of **6** (4.5 mM). (\*, unknown, non-peptidic material)

cysteine ligation sites that enable sequential ligation of peptide fragments. We prepared the N-terminal peptide segment **4**, the middle fragment **5** and the C-terminal segment **7** by solid-phase synthesis. According to the logics of N-to-C directed sequential ligations the thioester at the N-terminal fragment **4** was equipped with a highly reactive leaving group i.e. MPA-Cys. The middle

fragment **5** contained a rather unreactive threonine as C-terminal amino acid and the MPA-Gly leaving group. As expected, the ligation with of the thioester **4** with the middle fragment **5** proceeded rapidly (Figure 3C) and product **7** was obtained in 89% yield after 3 hours. For the second ligation, the C-terminal segment **6** was added at 37°C to promote conversion of the bulky threonine thioester of **7**. The ligation did not proceed further after 23 hours but the yield of the full-length segment was already 65% (Figure 3D). As expected, the cyclization of the middle fragment was observed as a side reaction. However, the amount of cyclized peptide **5c** was less than 10% during the entire duration of the one pot sequential ligation procedure. Products arising from dimerization or oligomerization of **5** were not detected.

We were next interested in exploring the suitability of peptide-MPA-Cys thioesters for one-pot ligation-desulfurization chemistry. We embarked on the synthesis of the SH3-domain YSC84 from yeast. This protein domain is devoid of cysteine. Drawing on Cys→Ala desulfurization the ligation site was chosen between Leu20 and Ala21 (Figure 4A). Again, the peptide thioester (1-20) **9** was built on



**Figure 4.** A) One-pot sequential ligation-desulfurization for the synthesis of the YSC94 SH3 domain. UPLC trace ( $\lambda = 210$  nm) after B) 5 min or C) 2 d reaction of **9** (2 mM) and **10** (3 mM). D), E) UPLC analysis (left) and MS analysis (right) of product **12** D) 1 h after addition of the desulfurization cocktail or E) after HPLC purification.

a mercaptopropionic acid linker equipped with a cysteine residue on a MBHA resin and allowed to react with an excess of Cys-peptide (2.2 eq.) **10** in a thiol additive-free solution of guanidinium hydrochloride buffer. After 48 hours at 37°C the ligation product **11** was formed in 88% yield based on integration of the UPLC trace (Figure 4C). Subsequently, the reaction mixture was diluted with a

degassed solution of the radical initiator VA-044, tert-butyl mercaptane and TCEP to final peptide concentration of 1-2 mM. This mixture was agitated for one hour at 65°C. UPLC-MS analysis showed the completeness of desulfurization (of both ligation product **11** and excess cysteine peptide **10** (Figure 4D)). HPLC purification furnished the pure desulfurized full length SH3 domain **12** (Figure 4E) in 53% isolated yield over the two steps.

## Conclusions

The use of and synthesis on the mercaptopropionic acid (MPA) linker is well known since the early days of peptide thioester chemistry<sup>13</sup> and native chemical ligation chemistry<sup>14, 16</sup>. The MPA linker has frequently been coupled to glycine or norleucine functionalized resins.<sup>15</sup> The data presented by us points to a remarkably high reactivity of peptide-MPA-Cys thioesters in native chemical ligation reactions. We assume that the high reactivity is the result of an intramolecular S→S acyl transfer reaction that leads to a highly reactive side-chain linked cysteine thioester intermediate. Of note, under the conditions assessed by us we saw no indication for accelerated hydrolysis of the peptide-MPA-Cys thioesters. Owing to these properties NCL reactions can be performed by omitting the commonly applied (but barely appreciated) thiol additives. This, in turn, facilitates one-pot sequential NCL reactions and one-pot ligation-desulfurization protocols, which save time and cost of materials because intermediary purification is redundant. Of note, this approach introduced by us does not require new compounds. The required building blocks are commercially available and can be used in solid phase peptide synthesis without further modifications. This should facilitate applications of peptide-MPA-Cys thioesters in NCL chemistry in general.

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## Notes and references

<sup>a</sup> Humboldt-Universität zu Berlin, Institut für Chemie, Brook-Taylor-Str. 2, 12489 Berlin, Germany. Fax: +49 30-2093-7266; Tel: +49 30 2093 7446; E-mail: oliver.seitz@chemie.hu-berlin.de.

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