2. Abstract

Native Chemical Ligation (NCL) opens the way to convenient preparation of long peptides and proteins that are not affordable by the standard solid-phase peptide synthesis (SPPS). However, the chemical synthesis of peptide thioesters needed for NCL still faces several challenges due to their low stability and slow ligation kinetics. To address these challenges, I developed a one-pot method for the production of peptide thioesters *via* the *N*,*S*-acyl shift of *N*-[2-thioethyl]glycine (*N*-SEt-Gly) followed by native chemical ligation [i]. We expected that cysteamine would undergo *N*,*S*-acyl shift [ii] and incorporated it into the peptidyl resin through nucleophilic substitution with chloroacetic acid. Figure 1a features the examples of developed thioester precursors in time and Figure 1b the scheme of the whole one-pot thioesterification and ligation process. After *N*,*S*-shift, *N*-SEt-Gly can be directly involved in ligation with *N*-terminal cysteinyl peptide or transformed into a corresponding active thioester. We found that the ligation proceeded in both cases and it was fastener when the external thiol was added. The NCL conditions were acidic (pH 3) or neutral (pH 7.4) with respectively: sodium 2-mercaptoethanesulfonate (MESNa) and dithiotreitol (DTT) or 4-mercaptophenylacetic acid (MPAA) and tris(2-carboethoxy)phosphine (TCEP).

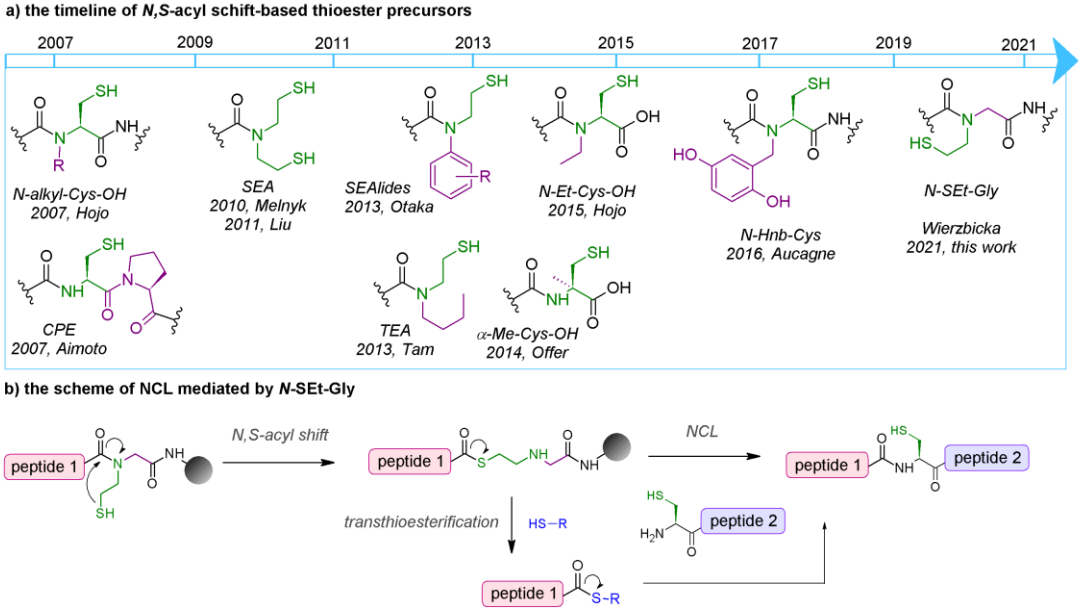

Figure 1 a) the timeline of developed thioester precursors used in native chemical ligation based on N,S-acyl shift; b) the scheme of peptide native ligation with N-[2-thioethyl]glycine, HS-R – external thiol.

The feasibility of this methodology is validated by the syntheses of model linear and cyclic peptides, especially demanding cyclic peptides, including cyclotetrapeptides, the bicyclic sunflower trypsin inhibitor SFTI-1, and rhesus Θ-defensin RTD-1. Synthesis of the whole peptide precursor can be fully automated and proceeds without epimerization or dimerization. For the linear ligation in solution, a library of model Ac-LYRA-Xaa-*N*-[2-thioethyl]glycine sequence was subjected to NCL reaction with H-CKA-OH to test its versatility towards all the natural amino acids in the X position. Next, a library of Zaa-LYRAG-*N*-[2-thioethyl]glycine was tested for self-ligation-desulfurization with unnatural thiolated amino acids like penicillamine or homocysteine as well as selenocysteine with the corresponding deselenization. The reaction kinetics was also improved by microwave heating. The presented method can be also applied in orthogonal cleavage of peptide combinatorial libraries.

[i] Wierzbicka M., Waliczek M., Dziadecka A., Stefanowicz P. One-pot cyclization and cleavage of peptides with N-terminal Cysteine via *N,S*-acyl shift of N-2-[thioethyl]glycine residue. *J. Org. Chem*., **2021**, 86 (17), 12292-12299

[ii] Waliczek M., Wierzbicka M., Arkuszewski M., Kijewska M., Jaremko Ł., Rajagopal P., Szczepski K., Sroczyńska A., Jaremko M., Stefanowicz P., Attempting to synthesize lasso peptides using high pressure. *PLoS One*, **2020**, 15, e0234901/1-e0234901/21