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Fast protein modification in the nanomolar concentration range using an oxalyl amide as latent thioester

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Abstract: We show that latent oxalyl thioester surrogates are a powerful means to modify peptides and proteins in highly dilute conditions in purified aqueous media or in mixtures as complex as cell lysates. Designed to be shelf-stable reagents, they can be activated on-demand for enabling ligation reactions down to peptide concentrations as low as a few hundreds nM at rates approaching $30 \text{ M}^{-1} \text{ s}^{-1}$.

Thioester based-chemistry has been the source of many innovative breakthroughs in domains such as chemical biology¹ or materials science.^{2,3} In particular, the reaction of a thioester group located at the C-terminus of peptides or proteins with a cysteinyl (Cys) peptide is the cornerstone of the Native Chemical Ligation (NCL) reaction⁴ and related methods (Figure 1a).⁵ The hallmark of this process unquestionably resides in its exquisite chemoselectivity and its capacity to take place in water, under very mild pH and temperature conditions. During the last three decades, this reaction has profoundly remodeled protein chemistry by enabling the routine production of homogeneous batches of native or modified (semi-)synthetic proteins^{6,7,8} Considering the ease with which the Cys residue can be introduced at the N-terminus of peptides and proteins through chemical approaches or recombinant techniques, NCL is also an appealing reaction for accessing protein conjugates.⁹ For the latter application, achieving rapid reactions with N-terminal Cys is highly desirable to accommodate protein limited solubility and minimize side-reactions. Several kinetic studies have shown that the archetypal NCL reaction performed with alkyl or aryl thioesters generally exhibits slow to moderate rates under standard experimental conditions (~ 0.3 to $4 \text{ M}^{-1} \text{ s}^{-1}$).^{10,11} While powerful in the mM concentration range, the NCL reaction shows its limits at μM concentrations due to competitive thioester hydrolysis.¹² Thus, the use of NCL-based approaches becomes challenging for any application proceeding at high dilution such as the modification of proteins, although selenoester chemistry offers some opportunities but to the expense of increased complexity.¹³

In search for fast reacting thioester derivatives, our attention was attracted by oxalyl thioesters. These species were first reported as highly reactive and transient mammalian metabolites, which could be trapped in cell lysate with cysteine following an NCL-like mechanism (Figure 1b).¹⁴ Their reactivity in water remains however largely unexplored, probably due to the difficulties encountered in installing them on biomolecules and to their ease of hydrolysis with a half-lives of a few minutes in water at neutral pH.¹⁵

functionalization (see Supplementary Information), we have opted for a more flexible approach by preparing a conveniently protected lysine derivative **1**, i. e. Fmoc-Lys(^{oxo}SEA)-OH, which has the advantage of being easily introduced at any position into peptide sequences. Lysine derivative **1** was obtained at the gram scale after 6 steps from commercially available starting materials and with 17% overall yield. Its structure was validated by X-ray diffraction crystallography (Figure 2b). Amino acid **1** was easily incorporated into peptides of type **2** using standard Fmoc-SPPS procedures and proved perfectly stable during peptide cleavage and purification steps.

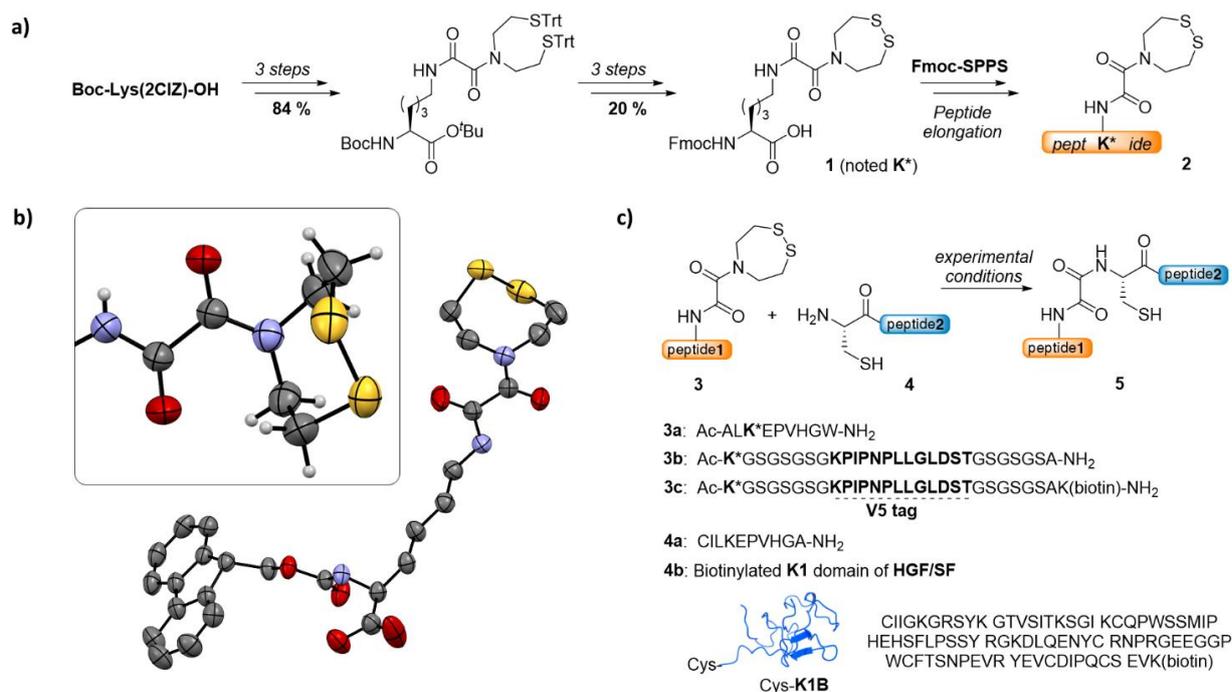


Figure 2. a) Synthesis of the Fmoc-Lys(^{oxo}SEA)-OH residue **1** and its incorporation into peptides of type **2**. b) ORTEP diagram of the X-ray structure of Fmoc-Lys(^{oxo}SEA)-OH **1** with ellipsoids shown at the 50% probability level. c) Model ^{oxo}SEA-mediated ligations examined in this study.

In a first series of experiments, we used model peptides **3a** and **4a** to evaluate the potential of ^{oxo}SEA-containing peptides as acyl-transfer agents in different experimental conditions using

MPAA¹⁸ as the thiol catalyst¹⁹ and TCEP as the reductant (Figure 2c). The presence of a tryptophan residue in peptide **3a** enabled accurate quantification of all the species of interest on HPLC chromatograms by UV-monitoring at 280 nm, including at the lowest peptide concentrations tested. The corresponding data were used for the determination of rate constants by nonlinear regression fitting.

Preliminary experiments established the influence of additives concentration and of the pH on ligation efficiency at 1 mM peptide concentration (see Supplementary Information). The reaction was found to be dependent on the concentration of MPAA but still displayed acceptable conversion ratios in the absence of a thiol catalyst. Importantly, the ^{oxo}SEA group remained stable and silent in the absence of TCEP or at low concentrations of MPAA, giving the possibility to trigger it on-demand. These experiments revealed also that the ^{oxo}SEA group is reacting significantly faster at the mildly acidic pH of 5.5 than at neutral pH. In that, the ^{oxo}SEA group apparently follows the behavior of C-terminal SEA peptides for which mildly acidic conditions are known to promote the *N,S*-acyl shift process.²⁰ Therefore, we initially performed ligations at pH 5.5. The reaction of peptides **3a** and **4a**, respectively introduced at 1 mM and 1.2 mM, yielded the expected ligated product **5a** with an astonishing half-reaction time ($t_{1/2}$) of 200 s for a final HPLC yield >95% (Figure 3a). For comparison, classical SEA ligations performed in the same concentration range require about 5-7 hours to be half-completed, highlighting the extreme difference in reactivity of the SEA and ^{oxo}SEA groups. Gradually, lowering the concentration of peptides **3a** and **4a** to 50 μ M revealed that the reaction was still proceeding very efficiently ($t_{1/2} = 900$ s). Ligation product **5a** was repeatedly obtained with a conversion ratio close to 90%, showing that even used at low molarities, the highly reactive oxalyl thioester surrogates were not significantly hydrolyzed. As a control, NCL reactions performed with classical alkyl and aryl thioesters at the same peptide concentration proceeded more slowly and plateaued at ~65-70% conversion due to hydrolysis of the starting material

(Figure 3b). At 5 μM of peptide **3a** and still at pH 5.5, the hydrolysis of the oxalyl thioester became noticeable, thus reducing the yield of ligated peptide **5a** (~40%). At this point, we reasoned that at such a low concentration the rearrangement of the amide might not be limiting anymore. If this is the case, the optimal pH conditions identified from reactivity studies conducted at mM peptide concentration might no longer apply to dilute conditions. In particular, working at neutral pH would perhaps be more appropriate for promoting the thiol-thioester exchanges and thus the capture of the oxalyl thioester by the Cys peptide.⁵ This proved to be the case since a slight pH adjustment from 5.5 to 7.2 was sufficient to restore satisfying levels of conversion at 5 μM (80%, Figure 3a).

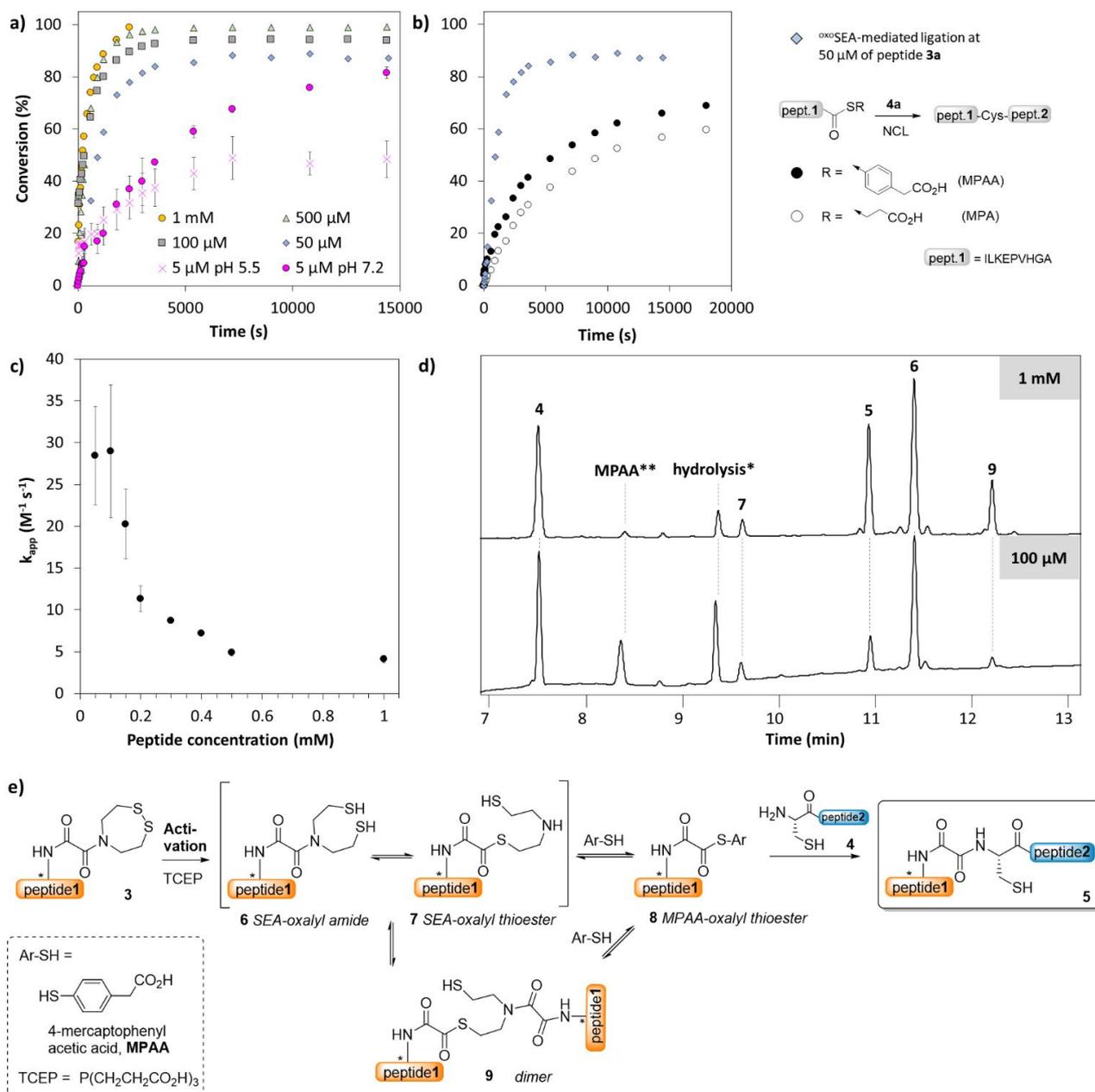


Figure 3. a) Conversion to ligated peptide **5a** from peptides **3a** and **4a**. Unless otherwise specified, standard experimental conditions applied are as follows: 1 equiv. **3a**, 1.2 equiv. **4a**, 200 mM 4-mercaptophenylacetic acid (MPAA), 100 mM TCEP, pH 5.5, 37 °C in 6 M guanidinium chloride in 100 mM phosphate buffer. The series at 5 μ M peptide concentration (squares) are presented as the mean \pm standard error of three independent experiments. For other concentrations, three replicates were made but only one is presented for the sake of clarity. To ensure maximal accuracy of the data, the absence of significant post-quench drifts was checked (See Supporting Information) b) Comparison of oxo SEA-mediated ligation at 50 μ M with the NCL reaction of alkyl and aryl thioesters at 50 μ M (1.2 equiv. **4a**,

200 mM 4-mercaptophenylacetic acid (MPAA), 100 mM TCEP, pH 7.2, 37 °C in 6 M guanidinium chloride in 100 mM phosphate buffer). c) Apparent second order rate constant of peptide **5a** formation determined by nonlinear regression fitting from the traces in Figure 3a. The data correspond to the mean \pm standard error (95% confidence limit interval) determined from three independent replicates. d) UPLC trace of the reaction of peptides **3a** and **4a** at 1 mM ($t = 30$ s) and 100 μ M ($t = 60$ s). *The hydrolysis byproduct observed here originates from the hydrolysis of unreacted starting material in the HPLC vial and not from the reaction. ** Traces of residual MPAA after extraction e) Proposed mechanism for the ^{oxo}SEA-mediated ligation.

Intriguingly, the determination of apparent second order rate constants for the ^{oxo}SEA-mediated process at various peptide concentrations revealed an uncommon behavior (Figure 3c). While the rate constant obtained at 1 mM was $\sim 4 \text{ M}^{-1} \text{ s}^{-1}$, it counterintuitively increased as peptide concentration decreased, with a remarkable break at 150 μ M. Below this concentration threshold, the apparent second order rate constant stabilized at a high level of $\sim 30 \text{ M}^{-1} \text{ s}^{-1}$. UPLC-MS monitoring of the reaction mixture at various peptide concentrations furnished useful information regarding the nature of some reaction intermediates involved in this process (Figure 3d). The observation of reduced ^{oxo}SEA species **6** and rearranged thioester **7** are expected according to previous studies on the C-terminal SEA group in peptides (Figure 3e).²⁰ The ^{oxo}SEA-derived thioester **7** likely undergoes a thiol-thioester exchange with the aryl thiol catalyst MPAA to form **8**, which could not be observed in our experiments for being probably quickly consumed by the Cys peptide to produce the desired ligated product **5**.

Here, the peculiarity arises from the formation of an unusual dimeric intermediate **9** observed in UPLC. This adduct significantly accumulates within the mixture when the reaction is conducted at 1 mM peptide concentration but falls to basal levels at 100 μ M and below. The accumulation of dimer **9** at high peptide concentration shows that it is slowly reacting. It must first dissociate to evolve into ligated product **5** and its formation likely accounts for the

dependence of the rate constant to peptide concentration. Importantly, ligation proceeds extremely fast at low peptide concentration, that are the conditions for which the chemical system is primarily intended for.

Thus far, the results obtained for the ^{oxo}SEA-mediated ligation on model peptides **3a** and **4a** provided suggestive evidence that the reaction might still operate in the nanomolar concentration range. However, 5 μ M is the lowest working concentration limit allowing reliable analytical detection and reproducible determination of ligation yields by HPLC. To further explore the performance of the reaction in the nanomolar concentration range, we opted for SDS-PAGE separation followed by Western-Blot as the analysis tool and to work at the protein level. For this purpose, we produced the ^{oxo}SEA peptide derived from V5 peptidic tag **3b** and the 10 kDa C-terminally biotinylated kringle 1 (K1) domain of the hepatocyte growth factor (K1/HGF) equipped with an N-terminal Cys **4b**.²¹ V5 tag can be detected with high sensitivity using a specific anti-V5 monoclonal antibody labelled with horseradish peroxidase (HRP), while streptavidin-HRP enables to trace the K1 protein domain. The latter was reacted at concentrations ranging from 2 μ M to 270 nM in the presence of ~6 equivalents of V5 tag ^{oxo}SEA peptide **3b**.

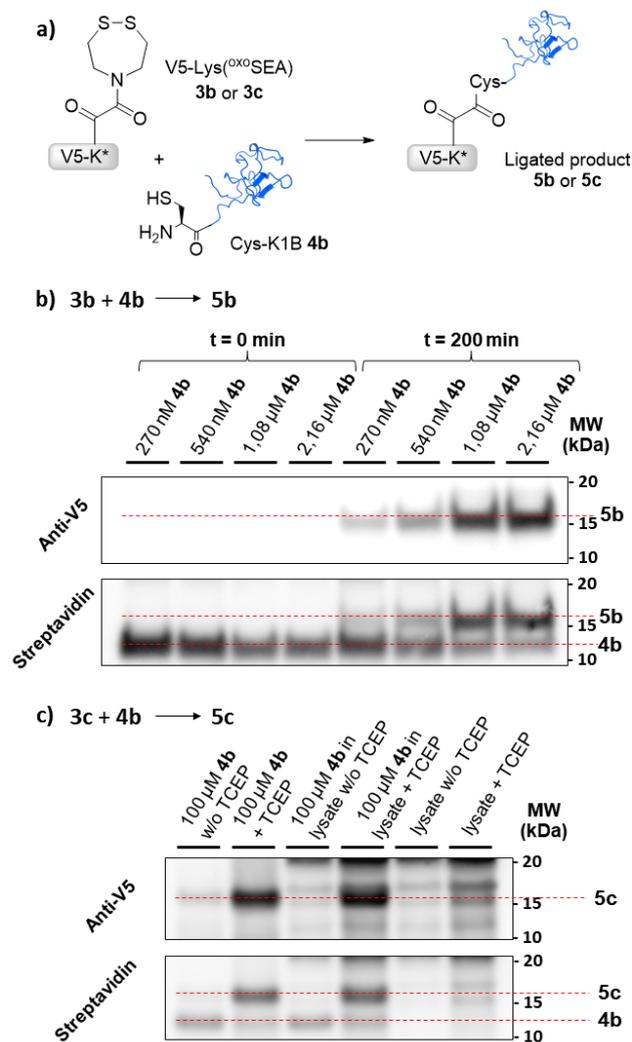


Figure 4. a) oxoSEA -mediated ligation between V5 containing peptide **3b** or **3c** and biotinylated K1 domain of HGF/SF **4b**. b) Western-blot analysis of the reaction in the nanomolar concentration range confirms the formation of protein **5b**. Conditions: **4b** 270 nM to 2.16 μM , **3b** 5.8 equiv., 0.1 M sodium phosphate buffer, 5 mM MPAA, 0.5 mM TCEP, pH 7.2, 37 °C. Note that reaction conditions were optimized to minimize the large excesses of additives. c) Western-blot analysis of the ligation reaction in a crude THP-1 whole cell protein extract ($1.6 \mu\text{g } \mu\text{L}^{-1}$) supplemented with 100 nM (i. e. $93 \text{ ng } \mu\text{L}^{-1}$) **4b** (see Supplementary Information)

The analysis shown in Figure 4 allows to appreciate the successful formation of the ligation product **5b** after 200 minutes, even when K1 polypeptide **4b** was introduced at a concentration of 270 nM. Importantly, the control experiments performed in the absence of TCEP or in the

presence of a biotinylated K1 domain lacking the N-terminal cysteine residue failed to produce any ligation product, respectively demonstrating the latency of the system and its chemoselectivity (see Supplementary Information). After having characterized the ^{oxo}SEA-mediated ligation in a purified media, we were interested to know if the same reaction could operate in mixtures as complex as a cell lysate (see Supplementary Information). To this end, the protein extract of a THP-1 whole cell lysate was supplemented in Cys-K1B **4b** at 100 nM final concentration and reacted in the presence of 15 equivalents of V5-Lys(^{oxo}SEA) peptide **3c**. Strikingly, a band revealed by both anti-V5 antibody and streptavidin-HRP conjugate at ~15 kDa suggested the formation of the ligation product **5c**. Its formation was however accompanied by an important background noise and the appearance of other bands. Controls at different reaction times, in the presence or in the absence of a strong reducing agent enabled to conclude that a nonspecific interaction of peptide **3c** introduced in excess with unidentified elements of the protein extract accounted for this observation. The successful formation of ligated product **5c** in such a complex mixture again highlighted the high selectivity and efficiency of the ^{oxo}SEA-mediated ligation for protein modification.

In conclusion, we herein report the exceptional reactivity of a bioinspired and activatable oxalyl thioester surrogate for peptide and protein modification at high dilution. This highly chemoselective reaction proceeds remarkably fast in aqueous media, under mild conditions and allows to perform ligations in purified and highly complex media such as cell lysates. Beyond protein modification, this work extends the scope of oxalyl thioesters as valuable reagents by addressing the issue of their poor stability in water through the synthesis of bench-stable and activatable surrogates. In doing so, these compounds or any derivative thereof might be used for future works as a complementary approach for practical and widely applicable crosslinking strategies or polycondensation of water-soluble (bio)molecules.

Acknowledgements

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Keywords: non-native ligation • thioester • protein modification • *N,S*-acyl shift systems • latency

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