Engineering nucleophilic catalysis coupled to tandem electrostatic assistance enables native chemical ligation at nanomolar concentrations

Julie Di Adamo[a], Vincent Diemer[a], Oleg Melnyk[a]

[a] Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 - UMR 9017 - CIIL - Center for Infection and Immunity of Lille, Lille, France

https://doi.org/10.17952/38EPS.2026.P039

Introduction

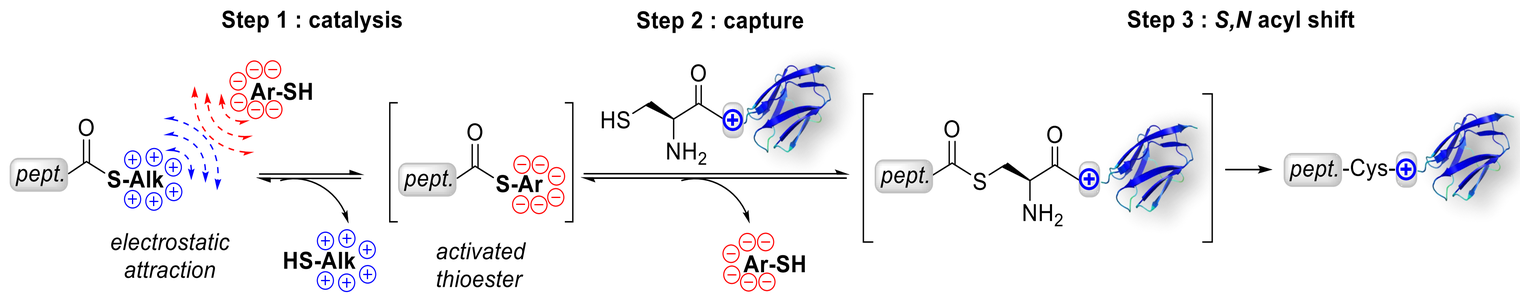
Native chemical ligation (NCL)[1] couples a C-terminal peptide thioester with an N-terminal cysteinyl peptide to form a native peptide bond. This strategy has greatly expanded access to proteins through **semi- or total synthesis**, enabling numerous applications. When **peptide alkyl thioesters**[2] are used, the reaction begins with **nucleophilic catalysis**: a thiol-thioester exchange with an aryl thiol catalyst converts the alkyl thioester into a more reactive aryl thioester. In a subsequent **capture step**, this intermediate reacts with the cysteinyl peptide to generate the native peptide bond.

Despite requiring a **large excess of catalyst**, the reaction exhibits only **moderate rate** constants at millimolar peptide concentrations (~50 M^{-1} s^{-1})[3], promoting side reactions and limiting efficiency at lower concentrations. Considerable efforts have therefore been devoted to **accelerate this reaction** through a variety of strategies.[4] Among them, preliminary work from our laboratory demonstrated that **incorporating charged motifs** into peptide sequences to promote **electrostatic interactions** can significantly **enhance NCL kinetics**.[5]

Objective and Method

Building on this approach, we aimed to retain the kinetic benefits of **electrostatic assistance** while **minimizing or avoiding** charged residues in the final product.

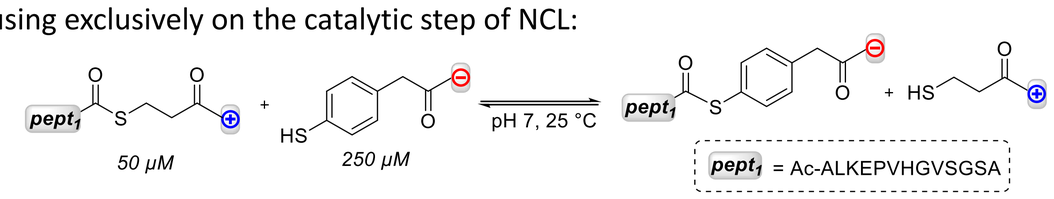
To achieve this, we developed a **tandem electrostatic assistance strategy** that accelerates both key steps of the NCL reaction, **catalysis** and **capture**, as depicted in the scheme on the right.



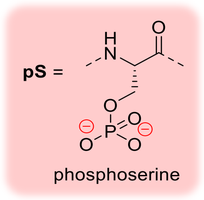
Tandem electrostatic assistance of aryl thiol-catalysed NCL between an alkyl thioester and a cysteinyl protein

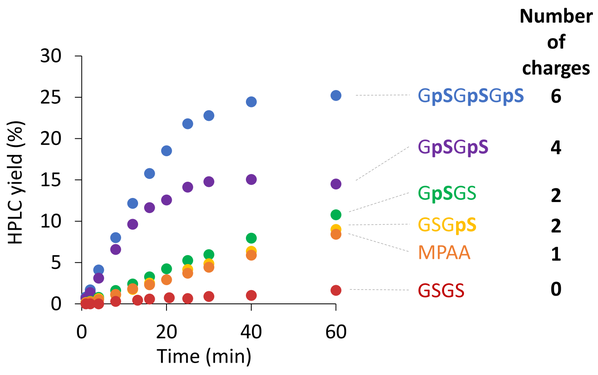
1. Nucleophilic catalysis

First, we sought to identify the **optimal charged moieties** for electrostatic assistance by focusing exclusively on the catalytic step of NCL:



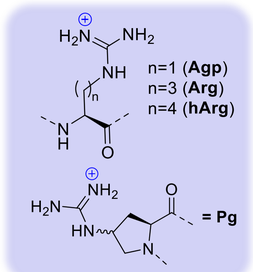
- How does the **catalyst's charge** affect the thiol-thioester exchange rate with Arg_6 thioester?

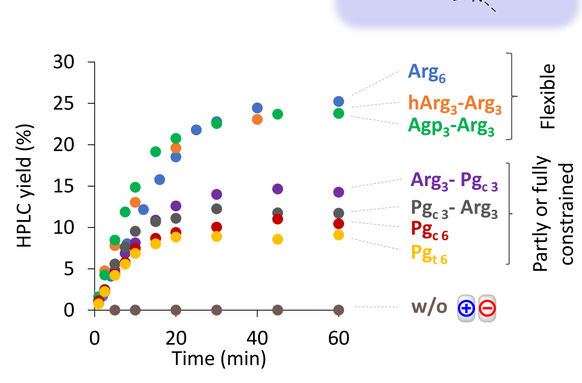




The reaction rate **increases** with the catalyst's charge

- How does **thioester charge pre-organization** affect the thiol-thioester exchange rate with **3pS** catalyst?



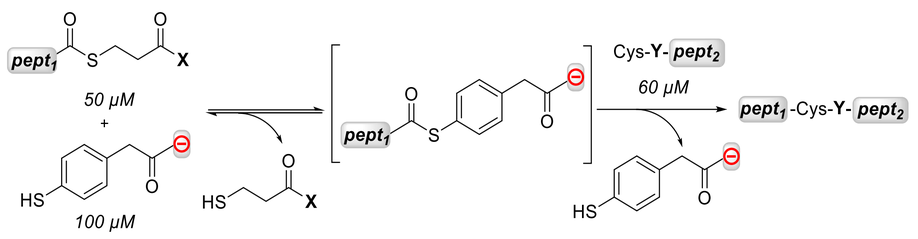


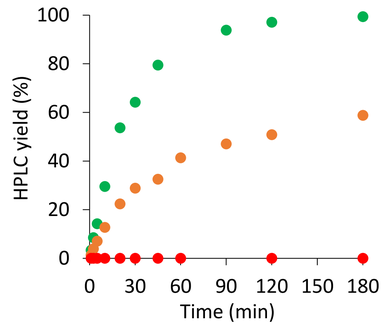
Introducing conformational constraints **decreases** the reaction rate

Combination of Arg_6 **thioester** and **3pS catalyst** enable to achieve the fastest rate

2. Tandem NCL

Next, we demonstrated the **feasibility and efficiency** of the tandem NCL strategy using **3pS catalyst** :





Tandem assistance → **X = Y = Arg_6**
No aryl thioester accumulation
Significant rate enhancement

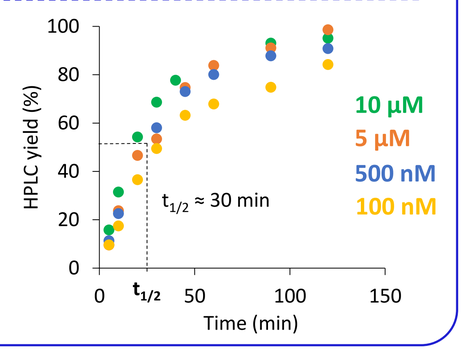
Catalysis assistance → **X = Arg_6 and Y = GSGSGS**
Aryl thioester accumulation

w/o assistance → **X = OH and Y = GSGSGS**
No ligation product

The **potential** of this approach at low concentrations was determined with ligations from **10 µM to 100 nM** between:

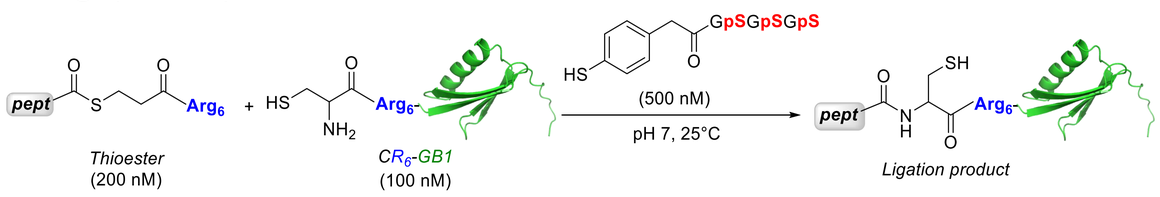
Arg_6 thioester (1 equiv.)
3pS catalyst (5 equiv.)
Arg_6 cysteinyl peptide (1.2 equiv.)

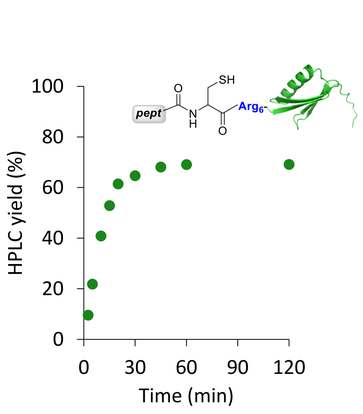
Kinetic profile almost **unchanged** upon 100-fold dilution

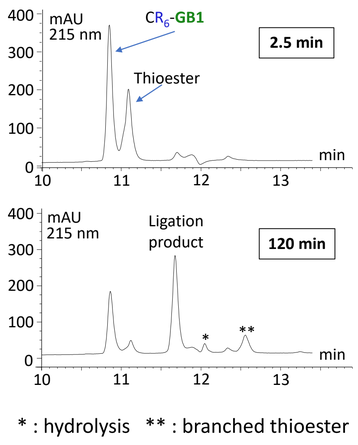


3. Protein modification

After demonstrating its efficiency on model peptides at low concentrations, we applied this tandem strategy to **protein modification** at 100 nM with only submicromolar catalyst loadings (500 nM):







pept = Ac-ALKEK(dnp)VHGVSGSA

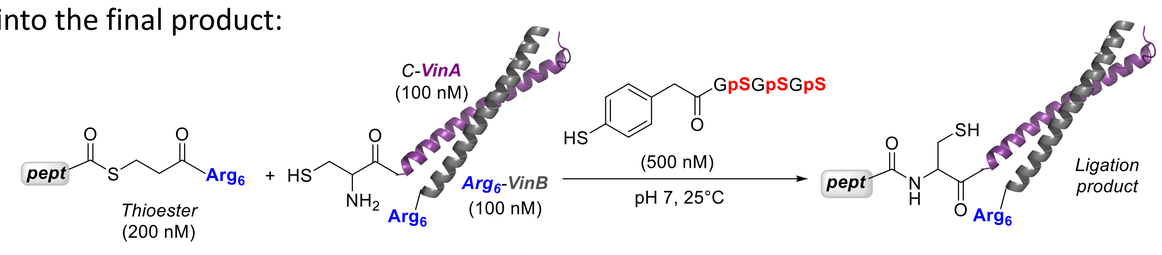
GB1 = GSTYKLILNGKTLKGETTTEAVDA ATAEKVFKQYANDNGVDGEWTY DDATKTFTVTEGSGSHHHHHH

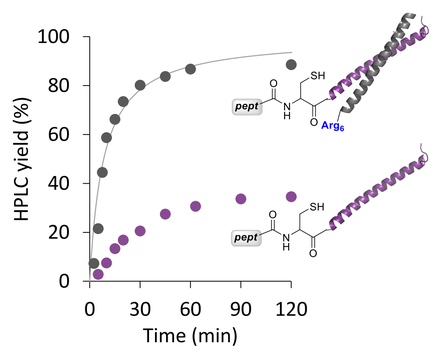
CR_6-GB1 was produced by recombinant expression

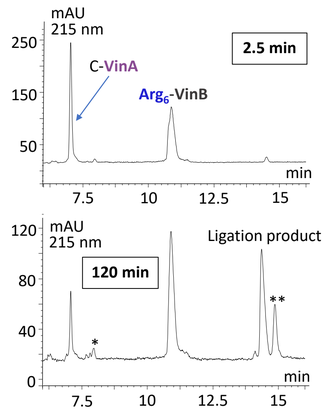
* : hydrolysis ** : branched thioester

4. Protein modification in trans

Unlike the previous approach, introducing the charged module ***in trans*** brings the charged group **close to**, but not directly next to, the cysteine, avoiding its incorporation into the final product:







Theoretical kinetic profile of a second-order reaction (k = 2 10^4 M^{-1} s^{-1})

pept = Ac-ALKEK(dnp)VHGVSGSA

VinA = LEIEAAFLERENTALETRVAELRQRV QRLRNRVSQYRTRYGPLGGGK$_{biot}$

VinB = LEIRAAFLRQRNTALRTEVAELEQE VQRLENEVSQYETRYGPLGGGK

* : hydrolysis ** : branched thioester

Conclusion

Overall, **tandem electrostatic assistance** of the NCL reaction offers an **efficient** and **catalyst-sparing** approach for protein modification under dilute conditions, with reaction rates reaching ~ 10^4 M^{-1} s^{-1}.

Building on these promising results, the ***in trans*** approach could ultimately enable **fast** and **traceless** protein modification.

References:

[1] P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. H. Kent, *Science* **1994**, 266, 776–779

[2] J. Di Adamo, N. Ollivier, S. Cantel, V. Diemer, O. Melnyk, *J. Org. Chem.* **2024**, *89* (18), 13719–13724

[3] J. Di Adamo, V. Agouridas, V. Diemer, O. Melnyk, *Org. Lett*. **2025**, 27, 13991–13996

[4] V. Agouridas, O. El Mahdi, V. Diemer, M. Cargoët, J.-C. M. Monbaliu, O. Melnyk, *Chem. Rev.* **2019**, *119*, 7328–7443

[5] N. Ollivier, M. Sénéchal, R. Desmet, B. Snella, V. Agouridas, O. Melnyk, *Nat. Commun.* **2022**, *13*, 6667

Acknowledgements to:

Agence de la Recerche Nationale (ANR-23-CE07-0016, TURBO project)
Jean ZAY supercomputer (IDRIS,CNRS) AD010816084R1
European Peptide Society (EPS) for providing a registration fee waiver