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Engineered oxalyl thioester-containing protein domains as a building block for synthetic biopolymers

Francesco Terzani,[a] Simindokht Rostami,[b] Chen Wang,[a] Benoît Snella,[a] Birgit Wiltschi,[b] Oleg Melnyk,[a] Vangelis Agouridas[a]

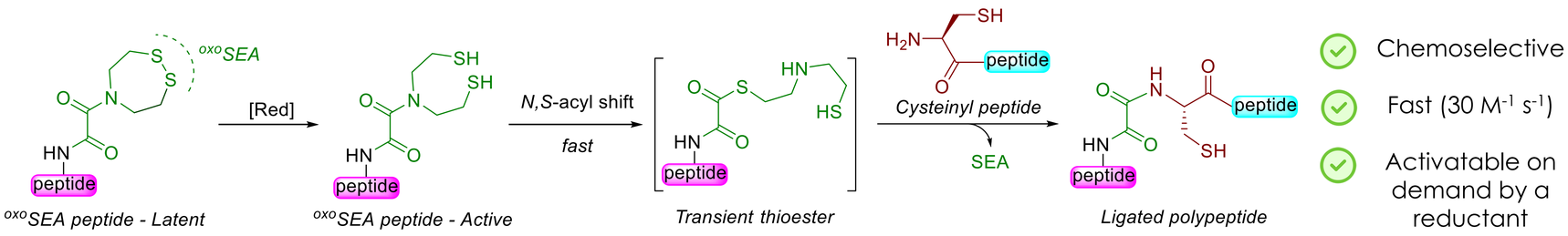
[a] *Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 - UMR 9017 - CIIL - Center for Infection and Immunity of Lille, F-59000 Lille, France*

[b] *Institute of Bioprocess Science and Engineering, University of Natural Resources and Life Sciences, Vienna*

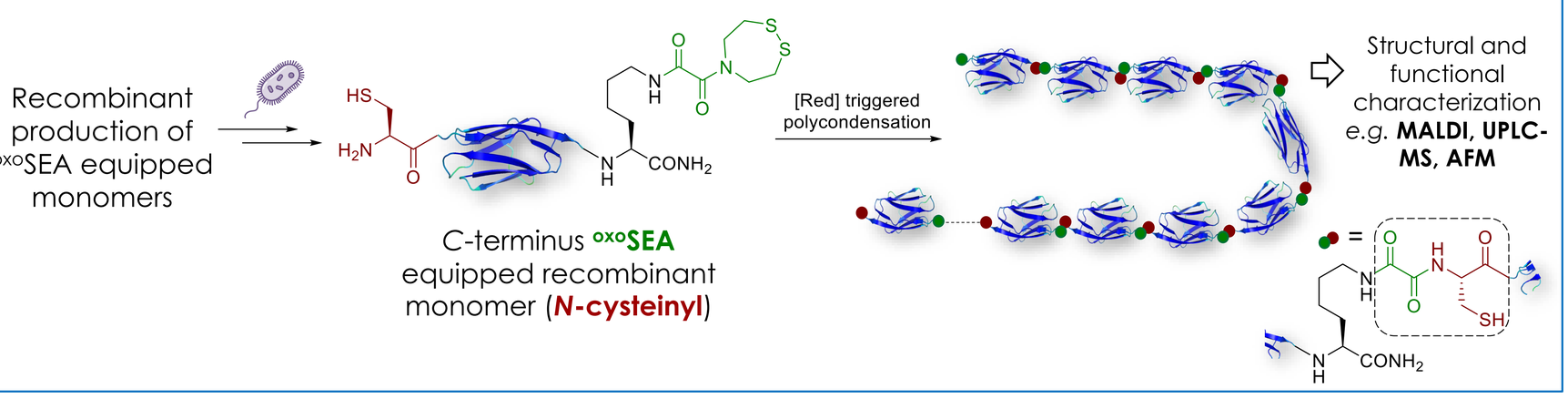
Introduction and Aim

In recent years, the **production of genetically engineered protein biopolymers** has been the subject of intense research with promising applications in biomedical or materials science.[1] Despite significant advances, the access to tailored biopolymers and thus, the development of versatile and efficient approaches, remain major challenges. We recently documented the potential of **oxalyl thioester precursors (ᵒˣᵒSEA)** as reactive handles for the modification of peptides and proteins [2] (Scheme A). Upon **activation by a reductant**, they can react in an **NCL-fashion**[3] with an *N*-terminal cysteine residue to provide a ligated peptide. Notably, the reaction has been reported to be **highly chemoselective**, **triggered on-demand**, **fast (< 30 M^{-1} s^{-1})** and efficient in the high **nanomolar range**. The goal of this project is to build on the tremendous reactivity of oxalyl thioesters in order to access **high-molecular weight biopolymers by polycondensation of bifunctional protein-derived monomers** (Scheme B). This will be done by setting up a fully integrated approach from synthetic biology to chemistry, involving the **recombinant production** of monomeric units equipped with an *N*-terminal cysteine residue and a genetically encoded ᵒˣᵒSEA-amino acid derivative close to the *C*-terminus.

Scheme A: ᵒˣᵒSEA based ligation chemistry developed in our group



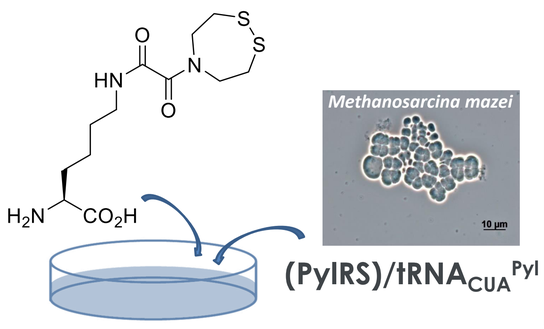
Scheme B: Reductant-triggered polycondensation from the recombinant production of *N*-cysteinyl ᵒˣᵒSEA monomers



Genetic encoding of ᵒˣᵒSEA ncAAs: preliminary results

Up to now more than 150 non-canonical amino acids have been successfully incorporated ribosomally into proteins using the **amber stop codon approach**. The **pyrrolysyl-tRNA synthetase (PylRS)/$tRNA_{CUA}^{Pyl}$ pair** from the archaeon *Methanosarcina mazei* is known to be an efficient tool for the incorporation of lysine derived compounds presenting sterically hindered side chain similar to the ᵒˣᵒSEA fuctional group.[4]

Incorporation of **ᵒˣᵒSEA Lysine** as a first attempt



Despite structural similarities between previously incorporated lysine derived ncAAs and the ᵒˣᵒSEA lysine, initial results showed **no incorporation into the target protein sequence**

We identified **3 possible reasons** and we designed a **library of ᵒˣᵒSEA analogs** to help understand and to improve the incorporation of Lys(ᵒˣᵒSEA)

Reason 1: Poor cellular penetration of Lys(ᵒˣᵒSEA) trough *E. Coli* membranes

R = CH_3 ⇨ *C*-terminus esterified = better intracellular uptake

Reason 2: H-Lys(ᵒˣᵒSEA)-OH could be degraded due to the reducing environment of *E. Coli* cytoplasm

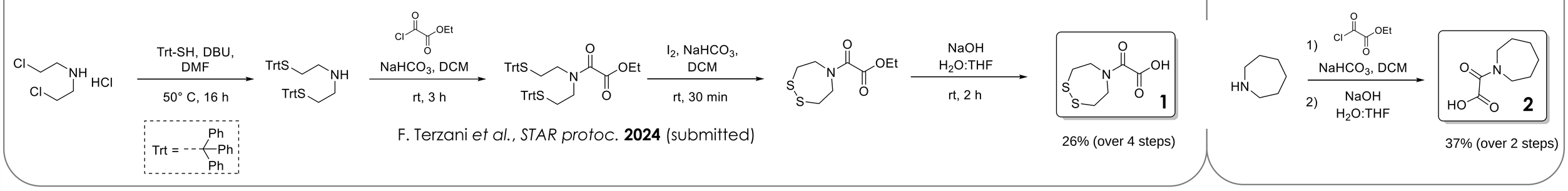
X = CH_2, Se ⇨ Unaffected/more resistant to possible intracellular reduction

Reason 3: (PylRS)/$tRNA_{CUA}^{Pyl}$ pair is not the appropriate one to load Lys(ᵒˣᵒSEA) on the tRNA

n = 1 Dap(ᵒˣᵒSEA)-OH
n = 2 Dab(ᵒˣᵒSEA)-OH
n = 3 Orn(ᵒˣᵒSEA)-OH
⇨ Possibility to screen different orthogonal pairs

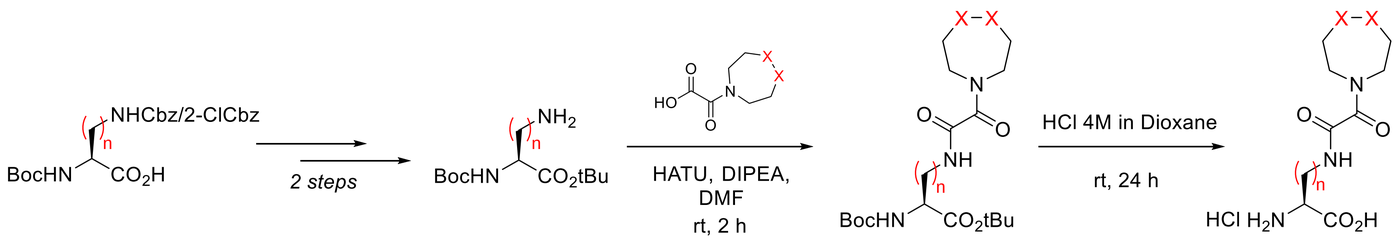
Synthesis of ᵒˣᵒSEA ncAAs

Synthesis of the ᵒˣᵒSEA building block 1 and the corresponding non-reactive version 2



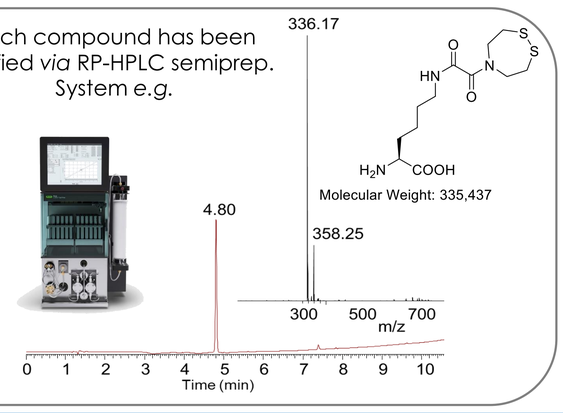
F. Terzani *et al.*, *STAR protoc.* **2024** (submitted)

Coupling of the ᵒˣᵒSEA building blocks with Lys,Orn, Dab and Dap



n	X	yield
4	S	20% (4 steps)
4	CH_2	18% (4 steps)
3	S	16% (4 steps)
2	S	38% (2 steps)
1	S	32% (2 steps)

Each compound has been purified *via* RP-HPLC semiprep. System *e.g.*



Conclusion

The library of ᵒˣᵒSEA ncAAs synthetized will help us understand the problems encountered during the first attempts of incorporation of the ᵒˣᵒSEA lysine and will hopefully allow us to achieve the ambitious challenge of genetically encode the latter in a target protein. Once this objective will be achieved we will be able to exploit the features of the ᵒˣᵒSEA based ligation chemistry for the development of a polycondensation method for the synthesis of tailored biopolymers.

(1) Werten, M. *et al*, *Biotechnology Advances*, **2019**, 642–666.
(2) a) Snella, B. *et al*, *Angew. Chem. Int. Ed*. **2022**, 61. b) Grain, B. *et al*, *Org. Lett*. **2023**, 25, 5117-5122.
(3) a) Dawson, P. E. *et al*, *Science* **1994**, 266, 776. b) Agouridas, V. *et al*, *Chem. Rev*. **2019**, 12, 7328.
(4) Dumas, A. *et al*, *Chem. Sci.* **2015**, 6, 50.

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