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PROJECT FINAL REPORT

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4.1 Final publishable summary report

Chemical tailoring of proteins is of outmost importance to understand biological processes. Ligation techniques, mostly Native Chemical Ligation (NCL), allow chemical access to medium-sized proteins including Post-Translational Modifications (PTM) at specific residues, a hallmark that could not be reached with expression methods. However, NCL demands a low-abundant Cysteine residue appropriately placed in the protein sequence and therefore extended approaches are required to ensure a wider applicability of the method. The **CycLATTR** project aims at broadening the scope of medium-sized proteins accessible by NCL, thereby allowing the synthesis and engineering of Cys-deficient proteins such as Transthyretin (TTR), involved in rare amyloidogenic diseases like Familial Amyloid Polyneuropathy (FAP).

The auxiliary design focused in overcoming drawbacks associated with previous benzyl and phenylethyl scaffolds such as tedious synthetic routes, slow ligation kinetics and harsh acidic removal. Based on the properties of the Dcpm backbone protecting group, the substitution of the phenyl ring by a cyclopropyl moiety in the phenylethyl family of ancillaries was envisaged to radically improve its performance (*Fig.1*). We anticipated that this novel auxiliary would accelerate ligation in sterically hindered sites and that it would be afterwards released under mild conditions, features that could be compatible with proteins bearing sensitive PTM. Priority was given to this template over others initially proposed, in view of its chemical stability and ease of preparation. In fact, one of the advantages of the cyclopropylmethyl auxiliary lies on a fast, facile synthetic route from the commercially available starting material. In a simple one-pot procedure, the desired ligation auxiliary is isolated in 52% yield from cyclopropylmethylketone via bromination in presence of NBS and Se-catalysis, followed by nucleophilic substitution with *tert*-butyl mercaptan. *S*-*tert*-butyl protection ensures faster introduction into the desired *N*-terminus of the peptide than *S*-trityl and can be removed prior to ligation.

In order to prove our hypothesis, the cyclopropylmethyl auxiliary was appended to several analogues of test peptide LYRA**XY**RAEYSGLG (where **X**=Gly, Ala, Ser, His, Leu, Val, Ile and **Y**=Gly, Ala). Ligation junction on this peptide model spanned from the most reactive Gly-Gly to the strongly hindered Ile-Gly. Initial attempts of introducing the auxiliary via reductive amination to *C*-protected Gly in solution suffered from low conversion but gave insight into the high acid-stability of the building block when treated with TFA-d. In contrast, the auxiliary was more efficient attached to the peptide in solid-phase although optimization of the acidic additives and solvent was necessary to minimize acetylation and formylation. Using a combination of 0.1M Oxyma and NMP/MeOH, auxiliary-peptide was obtained in high conversion, followed by thiol deprotection in Hg^{2+} after peptide cleavage from the resin in 35% overall yield. The synthesis of the corresponding LYRAX counterparts took place via Boc SPPS featuring alkyl (mercaptopropionic acid-leucine) thioesters in 16-45% isolated yield.

The ability of the cyclopropyl-based auxiliary to assist ligation was tested in TCEP-containing buffers at pH=7.5. Noteworthy, only in TFA-quenched samples transthioesterificated intermediate was observed, suggesting that a reverse N-->S acyl shift is favored at pH=1. Ligation took place quantitatively in Gly-Gly junctions in 1 hour, whereas Ala-Gly, Ser-Gly, His-Gly and Leu-Gly were completed in only 2-4 hours, a dramatic reduction of the time required for full conversion in comparison to previous TFA-labile auxiliaries. Additionally, the inclusion of the cyclopropyl moiety in the auxiliary structure allowed the possibility of reaching sterically demanding sites such as Val-Gly and Leu-Gly (in 70-88% conversion) that were not accessible to date in auxiliary-mediated NCL (*Fig.2*). The presence of 3% thiophenol did not result in rate acceleration. Preparative purification afforded isolated ligation products in 29-49% yield. Even though ligation at Gly-Ala junctions is completed at 37ºC in 4 hours, Ala-Ala

remains the bottleneck of this method due to the extremely hampered S-->N shift at α-branched auxiliary-peptides. Contrary to our expectations, ligation products displayed unexpected stability towards TFA-treatment and only under TFMSA exposure partial auxiliary removal was observed. However, since the cyclopropylmethyl unit stabilizes carbon-centered radicals through an homoallylic-cyclobutyl-cyclopropylmethyl tautomery, mild radical conditions allowed auxiliary removal (*Fig. 2*). Slight basic medium, high peptide dilution and low TCEP concentration reduced the extent of side reactions (peptide bond cleavage and desulfurization), thereby rendering the native peptide as major compound in 27-31% isolated yield. In a nutshell, the novel cyclopropylmethyl auxiliary promotes faster NCL and extends the scope of accessible ligation junctions to hindered X-Gly sites. Milder removal conditions than originally devised open perspectives for application of PTM-containing proteins bearing acid-sensitive glycans or sulfoTyr moieties, and will be studied in the near future (*Fig. 3*). CycLATTR may grant access to a vast number of previously chemically unexplored proteins and derived diseases.

A practical example of the socioeconomical impact of improved ligation technologies would be the chemical synthesis of Transthyretin (involved in amyloidogenic-like diseases), which can not be accomplished neither by standard NCL nor by SPPS, in spite of remarkable efforts by Craik et. al. A synthetic approach to a 127-residue protein is a complex, time-consuming endeavor that in exchange will provide valuable insight into its malfunction mechanism. In order to guarantee the use of optimal ligation tools for such a demanding synthesis, we decided to rather employ a β-branched 2-phenyl-2-mercaptoethyl auxiliary, which was separately developed in the host group and that presented slight slower kinetics but cleaner removal (*Fig. 4*).

A first step on assessing the feasibility of the method consisted in synthesizing the full TTR amino acid sequence, divided in five different fragments. Unoptimized coupling protocols afforded 20-70% pure segments, which encouraged us to keep forward. Such relative straight-forward synthesis prompted us to redesign the original 5-fragment strategy and to consider 3- and 4-fragment alternatives. The latter approach relies on a 2+2 convergent ligation and involves an *N*-terminal peptide featuring a masked bis(2-sulfanylethyl)amido (SEA) thioester, whereas the former one comprises only one auxiliary-thioester bivalent building block. Preference was given to the 3-fragment approach given the significant reduction of ligation/removal/purification steps (*Fig. 4*). A critical retrosynthetic aspect is *S*-protection of the central ambivalent fragment, which ideally should resist peptide cleavage and ligation and be released upon mild acid. Therefore, an auxiliary-Glycine building block was *S*,*N*-masked via a thiazolidine moiety in solution, since the active auxiliary can be selectively released at pH=4. In a similar manner, Cys^{10} was orthogonally protected as Cys(PhAcm), masking the thiol during synthesis/desulfurization and allowing selective removal with a PGA-enzyme.

At this stage, solid-phase synthesis of *N*-t, *C*-t and central fragments has been optimized with various pseudoprolines and backbone-protecting groups to increase purity and avoid aspartimides. The *C*-terminal fragment has been successfully modified with the desired auxiliary. In the near future, thioester formation and subsequent fragment ligation will follow in order to achieve the full length protein and modified versions at Val^{30}. In conclusion, the second generation of ligation auxiliaries met the initial expectations and allowed to reach an unprecedented advanced stage on the synthesis of a Cys-defficient protein like TTR, a high risk-high reward endeavor, which promises to translate into publication in high-impact journals in the near future.