Incorporation of 1,2,4,5-tetrazines into proteins: a potential method for targeted drug release

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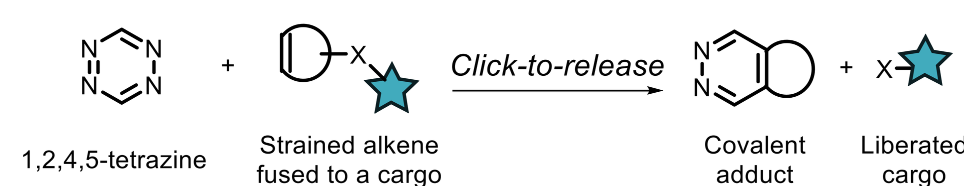
INTRODUCTION

Bioorthogonal Reactions

Bioorthogonal ligations are chemical reactions that can occur within a living system without interfering with its native biochemical processes. The participating functional groups must:[1]

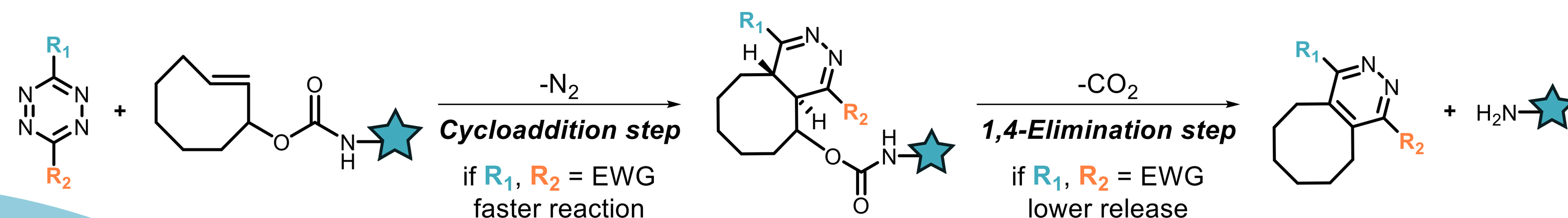
- Be inert to other biological moieties.
- Selectively react with each other under biocompatible conditions.
- Be non-toxic to cells and organisms.
- Present fast kinetics.

The **click-to-release reaction**, in which a **1,2,4,5-tetrazine** and a **strained alkene** react to form a covalent adduct and liberate a cargo of interest, is one of the bioorthogonal reactions with more applications in the development of new drug delivery approaches.



Click-to-release Parameters

Due to its kinetics and release percentage, the most widely used click-to-release reaction is the one involving a 1,2,4,5-tetrazine and a *transl*-cyclooct-2-en-1-yl carbamate:



The click-to-release reaction consists of **two steps**: an initial **cycloaddition step**, in which the 1,2,4,5-tetrazine fragment reacts with the dienophile, followed by a 1,4-**elimination step**, in which a cargo linked to the dienophile is released.

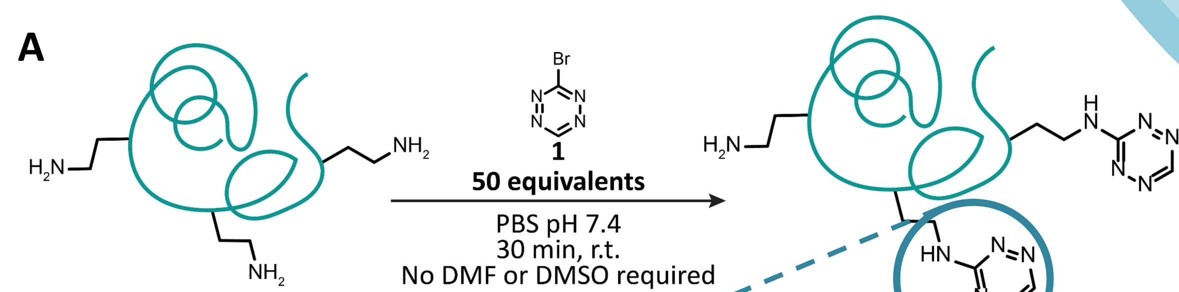
The presence of **electron-withdrawing substituents** (**EWG**) in the 1,2,4,5-tetrazine ring favours the first step, but also limit the degree of the second one. Therefore, a compromise in the substituents of the 1,2,4,5-tetrazine is important to increase the percentage of liberation of the cargo.[2]

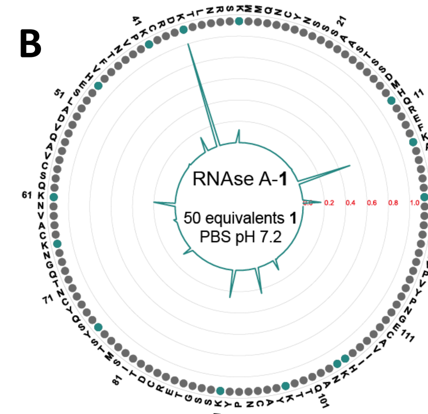
3-bromo-1,2,4,5-tetrazine (1)

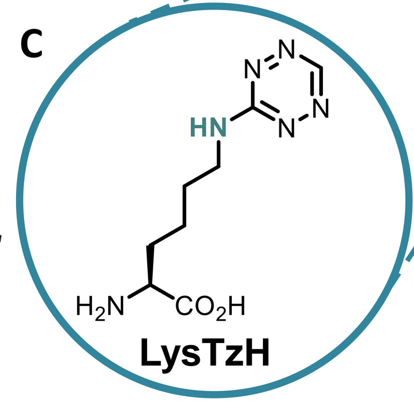
- New synthon useful for late-stage functionalization through nucleophilic aromatic substitutions (S_NAr)
- Metal- and oxidant-free synthesis
- Scalable (>1 g scale)
- Red crystalline solid
- Stable for storage

PROTEIN LABELING

Chemo-selective Labeling

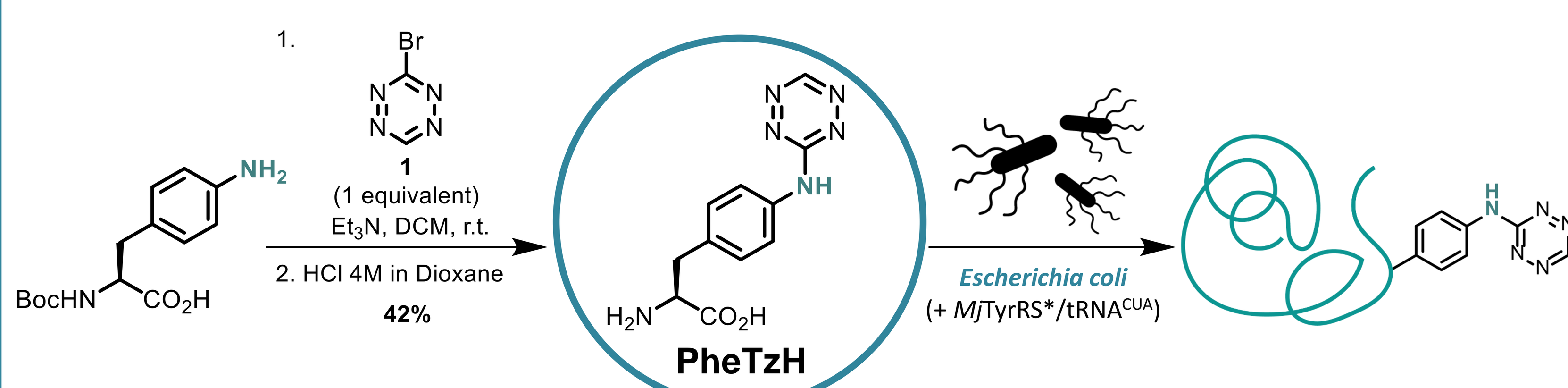






Chemo-selective protein labeling with 1: A) selective lysine labeling can be attained through the reaction of 50 equivalents of **1** dissolved in PBS pH 7.4 and added directly to the protein in solution. **B)** LC-MS/MS data on a labeled model protein (RNAse A) shows that >95% of the modified peptides were lysine-selective. **C)** Structure of the formed lysine-derived species (**LysTzH**) at the protein surface.

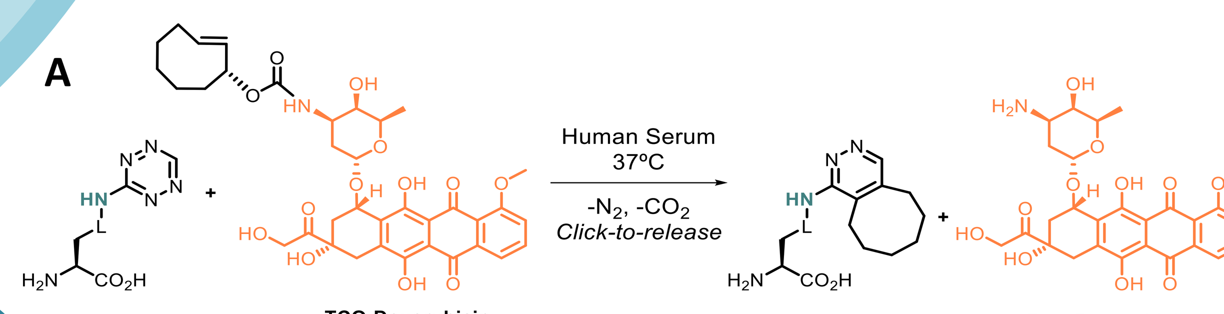
Site-selective Labeling

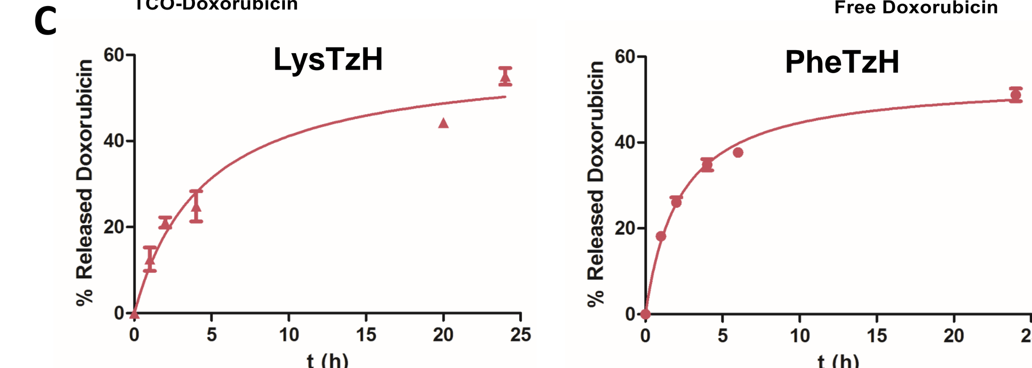


Site-selective protein labeling with 1: labeling with **1** at a specific protein position can be achieved through the genetic incorporation of the phenylalanine derived amino acid (**PheTzH**). *E. coli* DH10β cells transformed with a mutant *Methanocaldococcus janaschii* Tyrosyl-tRNA synthetase and its cognate tRNA with a suppressor anticodon (*Mj*TyrRS*/tRNA^{CUA})[3] are able to recognize **PheTzH** and selectively incorporate it into a protein position, which then can be purified.

TARGETED DRUG RELEASE

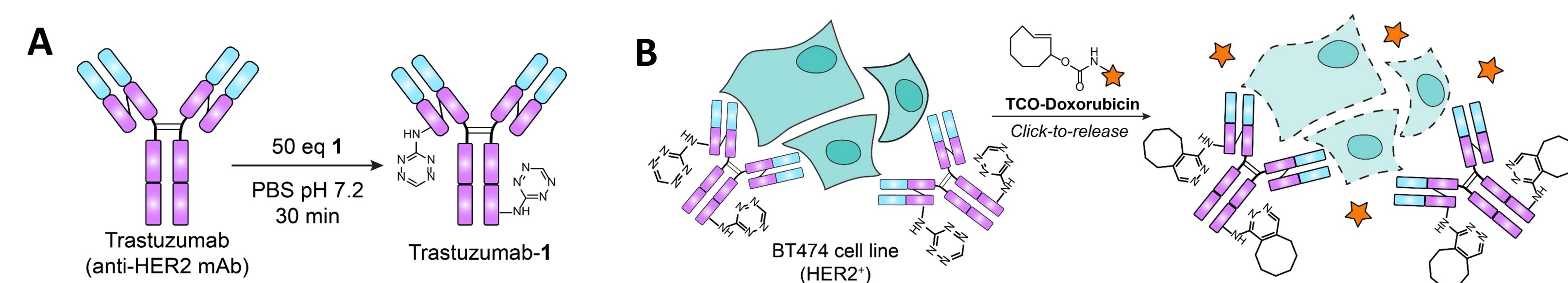
Click-to-release *in vitro*

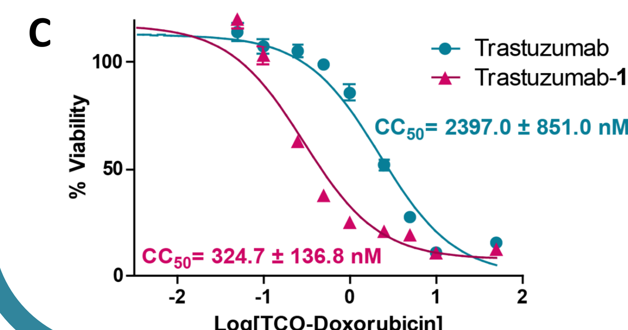




In vitro **characterization of the click-to-release reaction: A)** Reaction scheme between a 1,2,4,5-tetrazine-containing amino acid (**LysTzH** or **PheTzH**) and **TCO-Doxorubicin**[4] acting as a prodrug of doxorubicin. **B)** Chemical properties of **LysTzH** and **PheTzH** to trigger Click-to-release reactions. **C)** Liberation of free doxorubicin by **LysTzH** and **PheTzH** in physiological conditions (human serum at 37ºC) by UPLC after protein precipitation.

Click-to-release in cells





Click-to-release reaction of Trastuzumab-1 and TCO-Doxorubicin in cell culture: A) lysine-labeling scheme of the anti-HER2 monoclonal antibody (mAb) Trastuzumab to generate Trastuzumab-1. **B)** Release of free doxorubicin (orange star) by the reaction of Trastuzumab-**1** and **TCO-Doxorubicin** in BT474 (HER2$^+$) cell culture. **C)** Comparison of the cytotoxic concentration 50 (CC_{50}) of **TCO-Dox** with the labeled/unlabeled Trastuzumab.

CONCLUSIONS

- 3-bromo-1,2,4,5-tetrazine (**1**) is a new compound useful for protein labeling.
- **1** can be used to selectively label surface lysines (**Chemo-selective Labeling**).
- **1** can be used to generate an unnatural amino acid (**PheTzH**) recognized by a mutant aminoacyl-tRNA synthethase.
- **PheTzH** can be incorporated at a specific position on a protein of interest expressed in *E. coli* (**Site-selective Labeling**).
- **LysTzH** and **PheTzH** have been used as model compounds to characterize the click-to-release reaction between the labeled proteins and a liberating prodrug (**TCO-Doxorubicin**).
- In both cases, the reaction takes place in physiological conditions, reaching release levels of ca. 50% free doxorubicin.
- A labeled monoclonal antibody (Trastuzumab-**1**) can be used to liberate free doxorubicin from **TCO-Doxorubicin** in cell culture, thereby resulting in cell death.
- This approach can serve as a potential alternative to achieve targeted prodrug activation specifically at the site of action.

Funding

References

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