# Inverse electron demand Diels-Alder pyridazine elimination: synthetic tools for chemical immunology

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# 1

# Bioorthogonal bond-forming and breaking reactions in biology

## 1.1 Introduction to bioorthogonal chemistry

Chemical strategies have enabled the elucidation of new elements of biology by exacting precise control over processes in cells, and even in whole organisms. The twin pillars of this control have been the bioorthogonal bond forming, and bond breaking reactions, the first of which were invented by Bertozzi at the turn of the century.[1,2] The overarching property of bioorthogonal chemistry is that its transformations readily and selectively occur under physiological conditions without interfering with native biochemical processes.[3–7] This allows, for instance, the selective modification of proteins[8,9] or the characterization of active enzymes.[10,11] Reaction components must display a balanced profile of aqueous solubility, chemical stability, cellular toxicity, reaction kinetics and selectivity,[12] where the specific application dictates which properties are most influential.[13] Bioorthogonal reactions are developed using iterative modifications of known organic transformations,[14,15] such as the adaptation

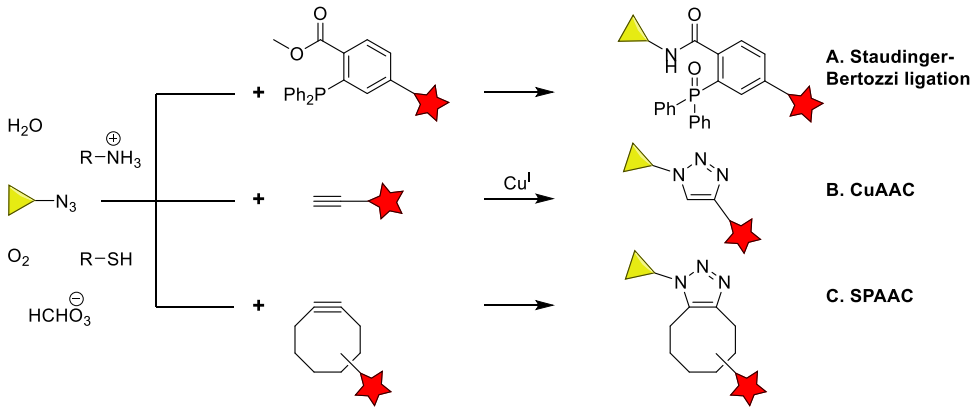


**Figure 1** Examples of bioorthogonal chemistry based on azide reactivity. A) Staudinger-Bertozzi ligation.[1-2] B) Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC).[19-22] C) Strain-promoted azide-alkyne cycloaddition (SPAAC).[24]

of the Staudinger reduction[16,17] to develop the Staudinger-Bertozzi ligation (Figure 1A).[1,2] Similarly, 1,3-dipolar cycloadditions[18] were revisited to develop the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC),[19–22] a transformation which has become the hallmark of 'click' chemistry[23] due to its reliability and simplicity (Figure 1B). Copper induced cytotoxicity, however, limited the applicability of CuAAC in living systems and therefore prompted the development of the strain-promoted azide-alkyne cycloaddition (SPAAC; Figure 1C).[24] Other bioorthogonal ligations include strain-promoted alkyne-nitrone cycloaddition (SPANC),[25] photoinduced tetrazole ligation,[26] the formation of oximes and hydrazones from aldehydes and ketones,[27] and transition metal catalysis under aqueous conditions.[28–31]

## 1.2 Inverse electron demand Diels-Alder cycloaddition

A recurring theme is that most bioorthogonal reactions suffer from at least one clear disadvantage, such as slow reaction kinetics, high cytotoxicity, insufficient stability, cross reactivity with other bioorthogonal moieties, undesired physicochemical characteristics of the reactants or improper tunability of the overall method.[13–15] Amidst the current scope of bioorthogonal chemistry, the inverse electron demand Diels-Alder (IEDDA) cycloaddition between 1,2,4,5-tetrazines and strained alkenes stands out as an exceptionally versatile method (Figure 2).[32,33] Initial studies by Carboni, Linsey[34] and Sauer[35–38] were adapted for bioorthogonal utilization by Fox[39] and Hilderbrand[40] using stabilized tetrazines as dienes and *trans*-cyclooctene (TCO) and norbonene as dienophiles, respectively. These were followed by numerous other dienophiles, including bicyclooctynes (BCN),[41,42] cyclopropenes,[43–45] vinylboronic acids (VBA),[46–49] and N-acyl azetines.[50] Depending on the strained alkene and

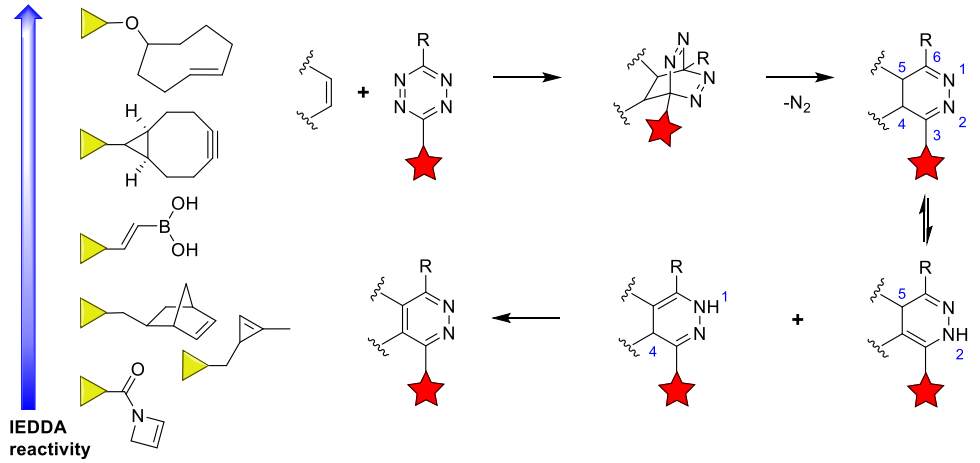


**Figure 2** Mechanism and scope of the inverse electron demand Diels-Alder (IEDDA) cycloaddition between 1,2,4,5-tetrazines and strained alkenes or alkynes, including several classes of dienophiles.

tetrazine employed, second order reaction rates ($k_2$) range between 1 – $10^6$ $M^{-1}s^{-1}$, which is superior compared to reaction rates for the Staudinger ligation ($10^{-3}$ $M^{-1}s^{-1}$), CuAAC (10 - 100 $M^{-1}s^{-1}$), SPAAC ($10^{-2}$ – 1 $M^{-1}s^{-1}$) and other commonly employed bioorthogonal reactions.[32] The reaction features an initial [4+2] cycloaddition between the tetrazine and alkene components, followed by a retro Diels-Alder reaction to give a 4,5-dihydropyridazine and $N_2$ as the only byproduct. Tautomerization affords 1,4- and 2,5-dihydropyridazines, whilst oxidation results in a pyridazine adduct. A catalyst is not required and reaction rates are accelerated under aqueous conditions due to hydrophobic interactions.[39,51]

Advanced bioorthogonal applications, such as live cell imaging[52,53] or *in vivo* tumor pre-targeting,[54,55] enforce stringent demands on reaction kinetics to ensure fast and complete bioconjugation at low dosage and sub micromolar concentrations. These requirements stimulated the choice of TCO as a dienophile for exceptionally fast tetrazine ligation ($k_2 \geq 10^3$ $M^{-1}s^{-1}$).[39,56–59] TCO's properties as a dienophile can be rationalized by high ring strain, which elevates the highest occupied molecular orbital (HOMO) of the olefin, and a crown conformation, which minimizes geometric distortion towards an IEDDA transition state.[60] *Cis* ring fusion can enforce a half chair conformation upon TCO, which exacerbates these effects to obtain even higher reactivity ($k_2$ up to $10^6$ $M^{-1}s^{-1}$) at the expense of stability.[61,62] Conversely, it was found that axially substituted TCOs were tenfold more reactive than equatorially substituted TCOs whilst being less susceptible towards *in vivo* isomerization to *cis*-cyclooctene (CCO) induced by copper-bound proteins.[63]

Synthetic advances towards both components of the TCO-tetrazine ligation have been of importance to enable their versatile application. For instance, the metal-catalyzed one-pot synthesis of tetrazines by Devaraj[64] expanded the precursor scope from aromatic nitriles to include alkyl nitriles and set the stage for the sustained emergence of improved methodologies towards functionalized tetrazines.[65–69] Furthermore, the direct, singlet sensitized photochemical transformation of functionalized CCOs to TCOs,[70–75] combined with selective complexation of the TCO product to Ag(I) in a flow based setup[76–78] has been a key method to simplify TCO synthesis. Modifications of this method can now synthesize TCOs in continuous flow[79] and have been applied to synthesize highly reactive *trans*-cycloheptene derivatives ($k_2$ = $10^7$ $M^{-1}s^{-1}$).[80] Taken together, these developments confirm the status of the tetrazine ligation as a 'state of the art' bioorthogonal reaction, with applications branching from chemical biology towards medicine and materials science.[32]

## 1.3 Dissociative bioorthogonal chemistry

The selective release of a protective moiety to expose functionality within a cellular system, known as bioorthogonal bond cleavage reactions[81] or dissociative bioorthogonal chemistry,[82] constitutes the second pillar of bioorthogonal chemistry, which has been of increasing interest.[15,32,81–87] The first example of such a 'decaging' reaction was reported by Meggers[88] in 2006, where a ruthenium catalyst was employed to intracellularly cleave an allylcarbamate. Deprotection strategies via other metals, such as palladium[31] and gold,[89] through organic transformations like the Staudinger reduction,[90,91] and by means of photochemistry[92] have steadily emerged ever since.

Dissociative bioorthogonal methods based on cycloadditions have been of particular relevance towards *in vivo* applications. The IEDDA pyridazine elimination, an adaptation of the TCO-tetrazine ligation[39] reported by Robillard and co-workers in 2013,[93] was the first example of what is now regarded as 'click to release' chemistry (Figure 3A). This deprotection method can be applied to induce selective release of cytotoxic agents, such as doxorubicin and monomethyl auristatin E (MMAE), using antibody-drug conjugates (ADCs),[94,95] tetrazine-modified hydrogels,[96–98] tetrazine carbon nanotubes,[99] or enzyme-instructed supramolecular self-assembly (EISA)[100] in preclinical *in vivo* models. The method has also been increasingly applied towards chemical biology, for instance to control the activity proteins,[101–104] the recognition of antigens,[105,106] imaging and profiling of proteomes,[107] or the purification of biomolecules.[108] While the first *in* vivo application was reported in 2014 by Chen and co-workers,[101] a phase I clinical trial has already surfaced as a result of the work described by Royzen and Oneto.[98]

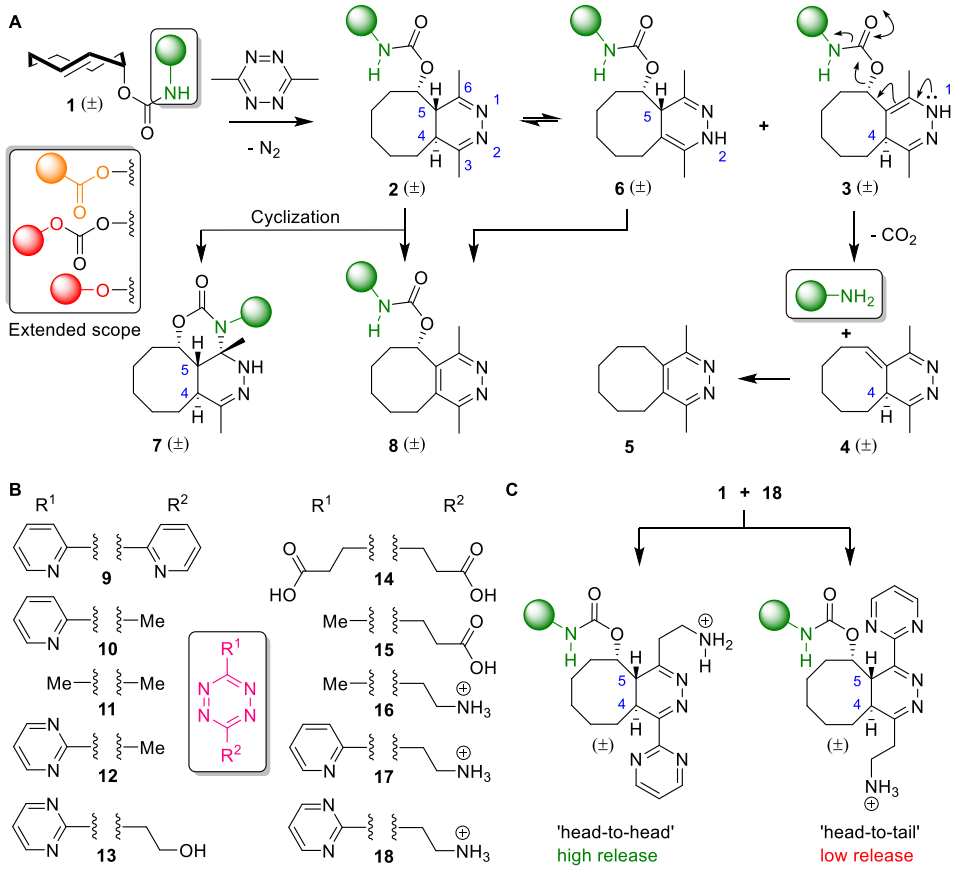


**Figure 3** A) Mechanistic overview of the IEDDA pyridazine elimination, a 'click to release' bioorthogonal decaging method. B) A panel of tetrazine activators selected from literature.[93, 109, 103, 114] C) 'head-to-head' versus 'head-to-tail' click orientation affects subsequent tautomerization and elimination for asymmetric tetrazines.

Mechanistically, the IEDDA pyridazine elimination employs an axially positioned carbamate substituent, containing a 'caged' amine moiety, on the allylic position of TCO (Figure 3A, **1**). Tautomerization of the 4,5-tautomer (**2**) obtained after tetrazine ligation can therefore result in elimination from the 1,4-tautomer (**3**) to release the free amine, $CO_2$ and **4**, which can aromatize to form pyridazine **5**.[93] Subsequent studies by Weissleder[109] and Robillard[110] confirmed these observations and revealed the 2,5-tautomer (**6**) can contribute to additional formation of **3** by tautomerization back to **2**. Competing side reactions include 'dead end' cyclization of **2** to form **7**[109] and aromatization of **2** and **6** to form pyridazine **8**. The fast releasing **3** and the slow releasing **6** constitute a biphasic release profile which relies on formation of **3** and less

on the nature of the leaving group, thereby enabling the scope of the method to be extended to include release of esters, carbonates, and even ethers.[110–113]

Initial IEDDA ligation rate and subsequent tautomerization/elimination rate and yield are heavily affected by tetrazine substituents (Figure 3B).[93,103,109,114,115] Electron withdrawing groups (EWGs), such as 2-pyridine or pyrimidine substituents increase tetrazine ligation rates but can decrease release performance when used excessively. For instance, the poor release induced by **9** (12%) could be gradually increased to 46% (**10**) and 75% (**11**) by adding electron donating methyl substituents.[93] Chen and co-workers subsequently reported asymmetric tetrazines which combined a pyrimidine substituent for rapid tetrazine ligation with methyl (**12**) and ethanol (**13**) substituents to induce elimination.[103] Due to the profound acceleration and augmentation of release at reduced pH,[109,110] tetrazines which carry intramolecular proton sources, such as carboxylic acids (**14** and **15**)[109] and amines (**16** – **18**),[114] were developed to render the IEDDA pyridazine elimination pH independent. In this regard, the orientation of the initial IEDDA ligation for asymmetric tetrazines directs subsequent tautomerization: 'head-to-head' is desired over 'head-to-tail' (shown for **18** in Figure 3C). Notably, Mikula and co-workers[116] developed $C_2$-symmetric TCOs carrying double payloads to obtain fast and complete release irrespective of click orientation.

In addition to further developments concerning photodecaging[117–121] and metal induced deprotections,[122–124] various other cycloaddition-based decaging strategies have emerged in the wake of the IEDDA pyridazine elimination. For instance, Gamble and co-workers[125–127] utilized TCOs to trigger release from an azido derivatized *para*-aminobenzyloxycarbonyl (PABC) system (Figure 4A), whereas Taran and co-workers[128–134] developed a strain-promoted iminosydnone-cycloalkyne cycloaddition (SPICC) release strategy (Figure 4B). Furthermore, several additional tetrazine-induced release systems have recently surfaced. Devaraj,[135] Bernardes,[136] and Bradley[137] reported vinyl ether decaging (Figure 4C), which was extended towards vinylboronic acids by Bonger,[138] whereas Franzini reported release systems based on benzonorbornadienes[139,140] and isonitriles (Figure 4D).[141–145] Finally, reverse decaging methods, where tetrazine is the caging moiety, have been reported by Robillard[146] (Figure 4E) and Franzini[147] (Figure 4F) using highly reactive TCOs and isonitriles as triggers, respectively.

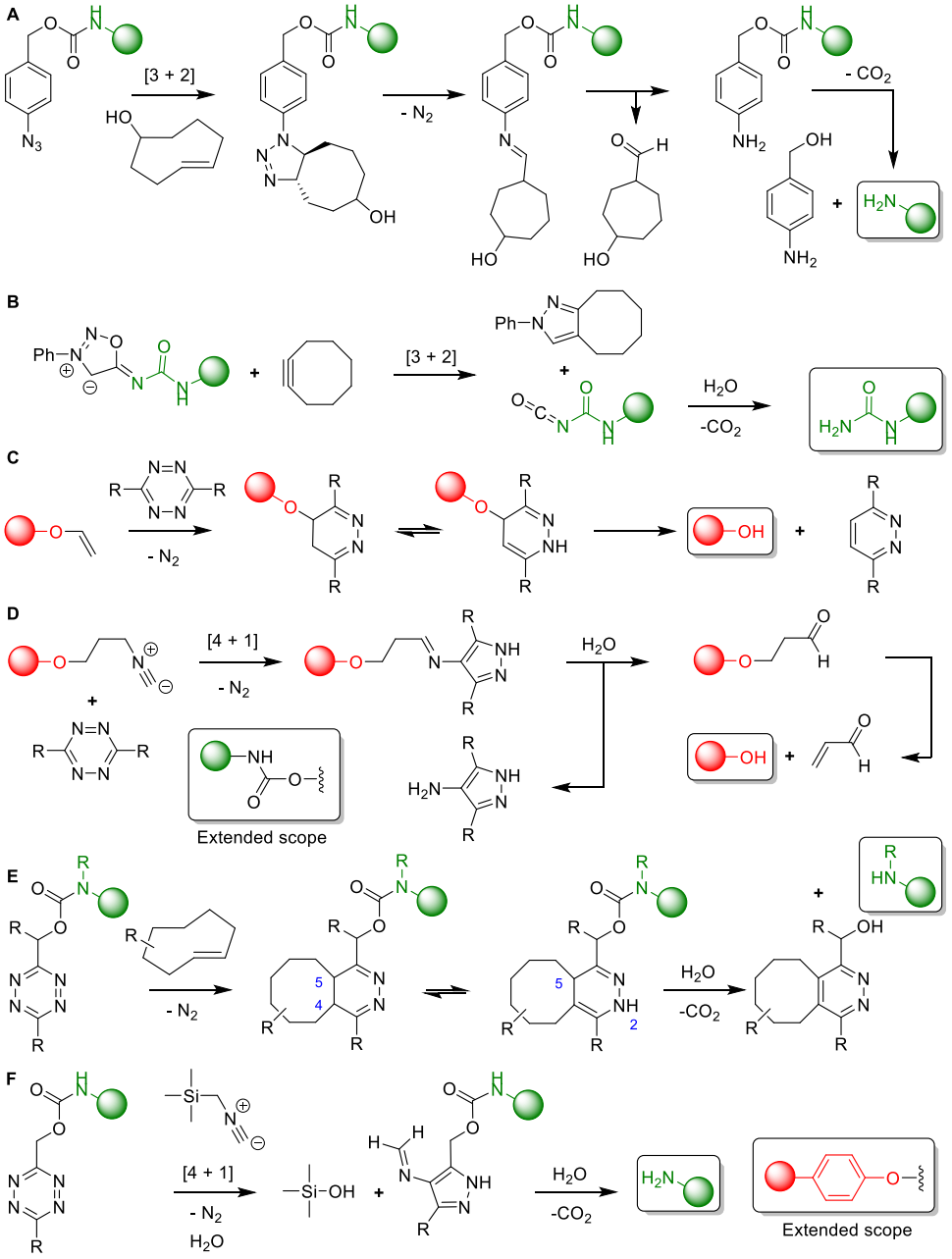


**Figure 4** An overview of alternative dissociative bioorthogonal strategies based on cycloaddition chemistry A) TCO triggered release from azido derivatized *para*-aminobenzyloxycarbonyl (PABC) systems.[125-127] B) Strain-promoted iminosydnone-cycloalkyne cyloaddition (SPICC) release strategy.[128-134] C) Tetrazine-induced release from vinyl ethers.[135-137] D) Tetrazine induced release from isonitriles.[141-145] E/F) Reverse decaging methods to liberate tetrazine as the caging moiety using TCO[146] or isonitrile triggers.[147]

## 1.4 Outline of this Thesis

This research described in this Thesis is aimed to develop new synthetic strategies centered around the IEDDA pyridazine elimination to enable its application in chemical biology. **Chapter 2** presents an improved synthesis towards a frequently employed bifunctional TCO scaffold. **Chapter 3** describes attempts to develop an Fmoc SPPS-based strategy for TCO-modified peptide synthesis. **Chapter 4** reports an unprecedented method for *in vivo* chemical control over T-cell activation based on deprotection of TCO-modified peptide antigens. **Chapter 5** describes the design and synthesis of TCO caged glycolipid antigens to obtain chemical control over iNKT cells. **Chapter 6** reports synthetic methodology towards allylic TCO-ethers which is subsequently applied to control carbohydrate-induced protein expression in *E. coli*. **Chapter 7** summarizes this Thesis and provides future prospects based on the research conducted thus far.

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