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# Fluorogenic Bifunctional *Trans*-cyclooctenes as Efficient Tools for Investigating Click-to-Release Kinetics

Mark A. R. de Geus,[a][‡] Elmer Maurits,[a][‡] Alexi J.C. Sarris,[a][‡] Thomas Hansen,[a] Max S. Kloet,[a] Kiki Kamphorst,[a] Wolter ten Hoeve,[b] Marc S. Robillard,[c] Andrea Pannwitz,[a] Sylvestre A. Bonnet,[a] Jeroen D.C. Codée,[a] Dmitri V. Filippov,[a] Herman S. Overkleeft,[a] and Sander I. van Kasteren*[a]

**Abstract:** The inverse electron demand Diels-Alder pyridazine elimination reaction between tetrazines and allylic substituted *trans*-cyclooctenes (TCOs) is a key player in bioorthogonal bond cleavage reactions. Determining the rate of elimination on alkylamine substrates has so far proven difficult. Here, we report a fluorogenic tool consisting of a TCO-linked EDANS fluorophore and a DABCYL quencher for accurate detection of both the click and release rates for any tetrazine at physiologically relevant concentrations.

## Introduction

Bioorthogonal chemistry is broadening in scope to include bond cleavage reactions alongside known ligation methods,[1–4] with many new reactions steadily emerging in this arena.[5–12] Of these, the inverse electron demand Diels-Alder (IEDDA) pyridazine elimination,[13] being the first of the bioorthogonal processes known as "click-to-release" (Figure 1 A). In the IEDDA decaging sequence, reaction of a tetrazine and a *trans*-cyclooctene (TCO) bearing a leaving group at the allylic position[14,15] results in a 4,5-dihydropyridazine intermediate. This adduct tautomerizes into a 1,4-dihydropyridazine species, which induces elimination of the allylic payload. The biocompatibility of the tetrazine and TCO components, combined with their selectivity and overall deprotection rate have culminated in various applications, such as the activation of cytotoxic (pro)drugs,[16–18] proteins,[19,20] and immune cells.[21] The technique is also compatible with reagents that enable spatiotemporal control within biological systems.[16–18]

In order to successfully apply click-to-release in biology, the identification of optimized tetrazines is crucial. Altering tetrazine substituents can have drastic effects on both the rate and extent of release obtained.[13,22–24] Fluorogenic reporters, such as caged coumarin **1**[22] (Figure 1 B), are important tools to characterize tetrazine release-behavior. These are supported by NMR[13,25] and LC-MS[22–24] analyses. One difficulty associated with these studies is the profound effect of pH and buffer concentration on elimination.[23,25] Furthermore, Chen and co-workers[22] noted that **1** displayed a lower decaging rate compared to a caged compound in which the coumarin (aniline release) is substituted for Fmoc-lysine ε-amine (alkylamine release). Carlson *et al*.[23] described caged reporters which enabled the detection of primary amine release kinetics by LC-MS (e.g. Figure 1 B, **2**). Compound **2** was also evaluated in a quenched fluorescence assay[23] which employed a tetrazine bearing a black-hole-quencher. Click reaction of **2** with 1 mM black-hole-quencher-tetrazine gave rapid formation of a 4,5-dihydropyridazine adduct in which the caged MayaFluor is quenched. Detection of fluorescence thereby provided a direct measurement of $k_{release}$ (constituting the sum of $k_{tautomerization}$ and $k_{elimination}$) without the influence of cycloaddition ($k_{IEDDA}$). However, this method does not allow characterization of (unmodified) tetrazines at lower concentrations.

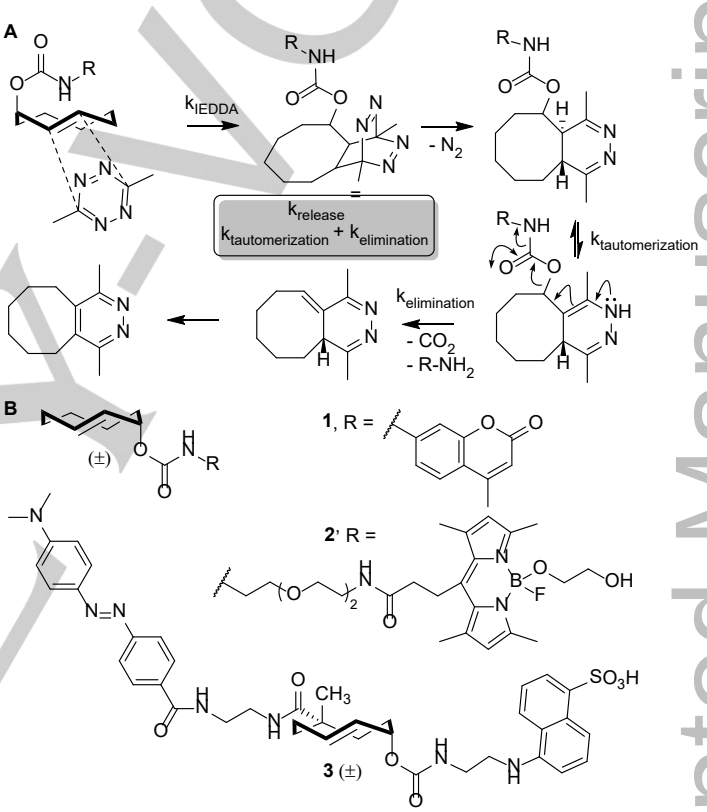


Figure 1. (A) Overview of the inverse electron demand Diels-Alder (IEDDA) pyridazine elimination reaction, which is also known as the "click-to-release"-reaction. (B) Fluorogenic TCOs to determine click-to-release kinetics: TCO-coumarin reporters **1** - **2** (by Fan *et al*.[22] and Carlston *et al*.[23], respectively) and bifunctional TCO-reporter-quencher pair reporter **3** designed in this study.

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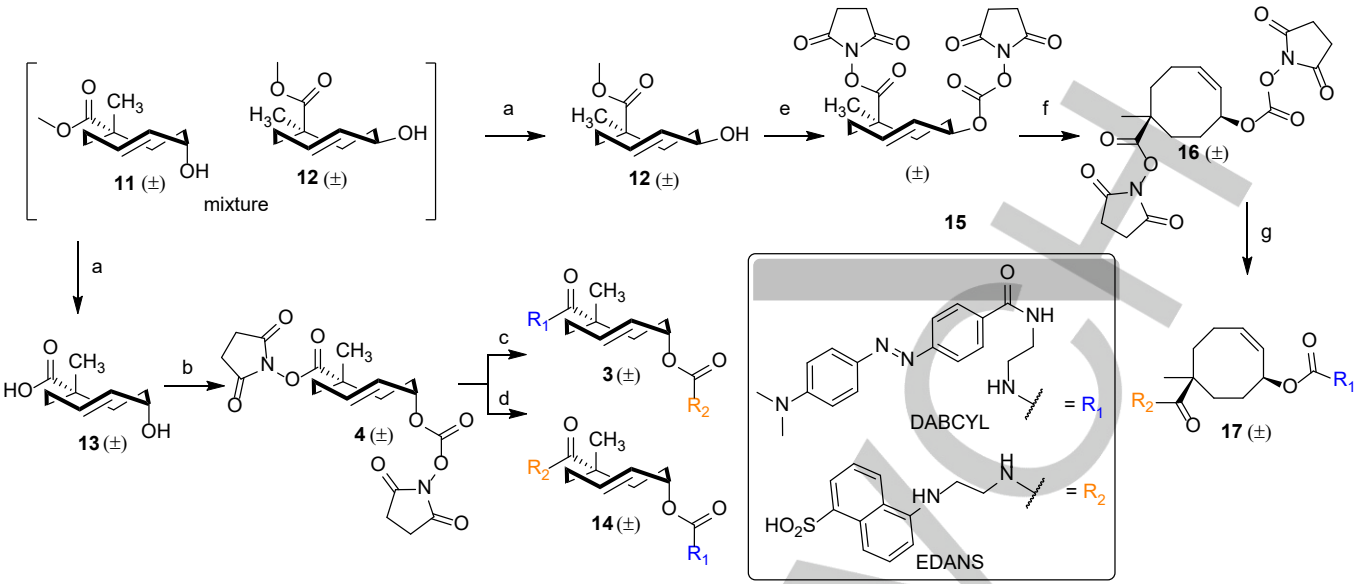


Scheme 1. Synthesis of bifunctional TCO-reporter-quencher pairs **3** – **4** and CCO-reporter-quencher pair **17** from cyclooctadiene **5**. Reagents/conditions for the synthesis of **11** and **12** cyclooctadiene (**5**) can be found in scheme S1; (a) KOH, MeOH, $H_2O$, 4°C, 43% (**12**), 42% (**13**); (b) *N,N'*-disuccinimidyl carbonate, DIPEA, DMAP, MeCN, rt, 72%; (c) i. EDANS-$NH_2$, DIPEA, DMF, rt, 79%; ii. DABCYL-$NH_2$, DIPEA, DMF, rt, 60%; (d) i. DABCYL-$NH_2$, DIPEA, DMF, rt, 96%; ii. EDANS-$NH_2$, DIPEA, DMF, rt, 88%; (e) i. KOH, MeOH, $H_2O$, 50°C, 79%; ii. *N,N'*-disuccinimidyl carbonate, DIPEA, MeCN, rt, 66%; (f) h*v* (CFL), $CDCl_3$, rt, 74%; (g) i. DABCYL-$NH_2$, DIPEA, DMF, rt, 60%; ii. EDANS-$NH_2$, DIPEA, DMF, rt, 50%.

A method based on fluorescence quenching to rapidly determine both the cycloaddition rate ($k_{IEDDA}$) and the alkylamine release rate ($k_{release}$) caused by tetrazines without stringent concentration requirements would be of great importance. We hereby report on the design, synthesis and evaluation of quenched reporter **3** (Figure 1 B) based on the bifunctional TCO introduced by Robillard and co-workers.[16] By linking a DABCYL quencher to an EDANS fluorophore via the bifunctional TCO scaffold, cycloaddition and alkylamine release rates can now be quantified in a 96-well plate reader format. Pseudo-first order rate constants were determined for a series of tetrazines and DFT calculations were employed to probe the differences for the initial cycloaddition step between monofunctional and bifunctional TCOs.

## Results and Discussion

The synthesis of bifunctional TCO reagent **4** was based on the published route (Scheme 1).[16] However, to both simplify and accelerate the procedure, the initial functionalization of cyclooctadiene **5** into the α-methylated carboxylic acid **6** (bromination, cyanide substitution, oxidation, basic hydrolysis and methylation) was carried out using crude reaction mixtures obtained after aqueous workup. Crystallization of iodolactone **7** from EtOH resulted in a yield of 23% over five steps from **5** at 500 mmol scale, without the need for the previously reported distillations.[16]

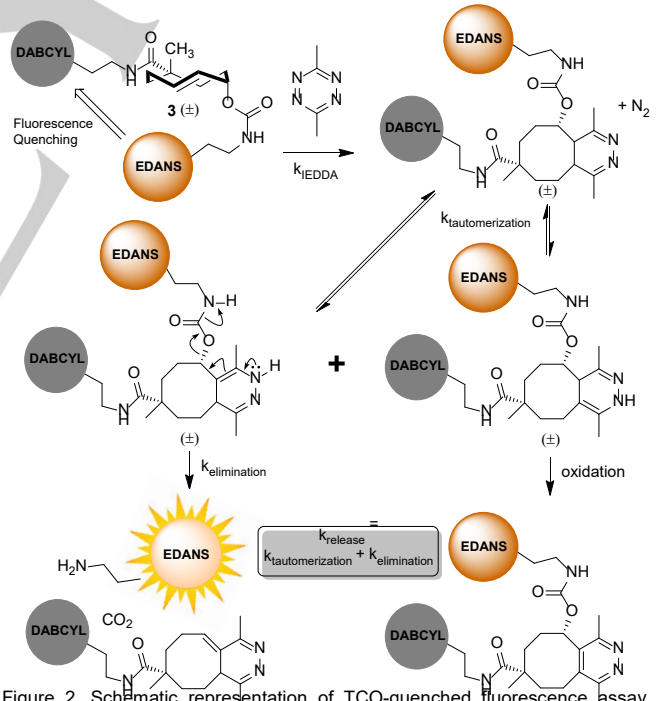


Figure 2. Schematic representation of TCO-quenched fluorescence assay. Bifunctional TCO-reporter-quencher pair **3** does not display fluorescence in its native state due to fluorescence quenching between the EDANS fluorophore and DABCYL quencher. Upon cycloaddition of a tetrazine, the 4,5-dihydropyridazine adduct may tautomerize into the 1,4-dihydropydazine intermediate. This species can eliminate $CO_2$ and the EDANS fluorophore, thereby disabling the fluorescence quenching and enabling a fluorescent readout for the elimination step. Alternatively, the 2,5-dihydropyridazine intermediate is formed which may tautomerize back to the 4,5-dihydropyridazine adduct or undergo oxidation.

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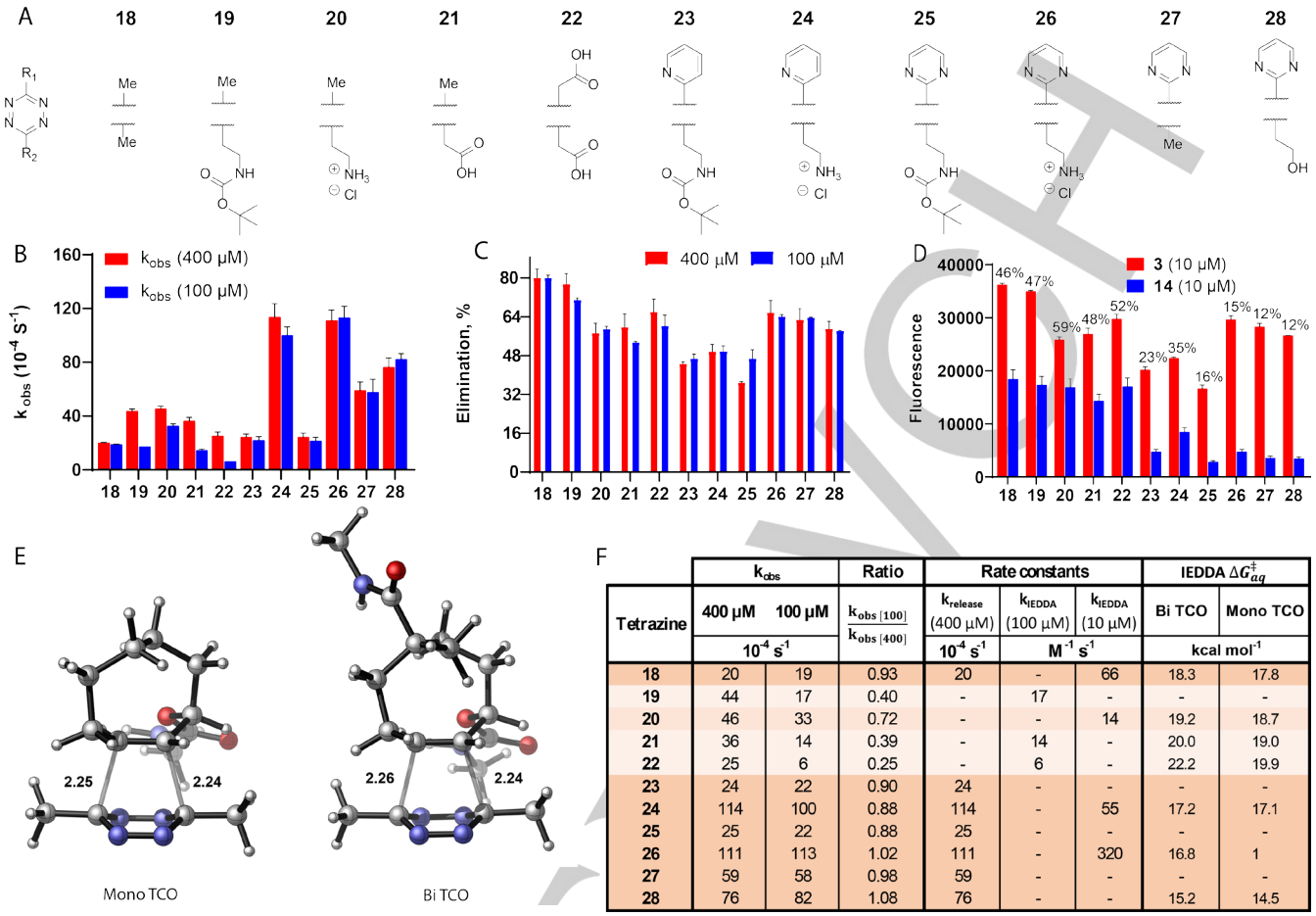


F

| Tetrazine | $k_{obs}$ 400 µM | $k_{obs}$ 100 µM | Ratio $\frac{k_{obs}[100]}{k_{obs}[400]}$ | Rate constants $k_{release}$ (400 µM) | $k_{IEDDA}$ (100 µM) | $k_{IEDDA}$ (10 µM) | IEDDA $\Delta G^{\ddagger}_{aq}$ Bi TCO | IEDDA $\Delta G^{\ddagger}_{aq}$ Mono TCO |
|---|---|---|---|---|---|---|---|---|
| | $10^{-4}$ $s^{-1}$ | | | $10^{-4}$ $s^{-1}$ | $M^{-1}$ $s^{-1}$ | | kcal $mol^{-1}$ | |
| **18** | 20 | 19 | 0.93 | 20 | - | 66 | 18.3 | 17.8 |
| **19** | 44 | 17 | 0.40 | - | 17 | - | - | - |
| **20** | 46 | 33 | 0.72 | - | - | 14 | 19.2 | 18.7 |
| **21** | 36 | 14 | 0.39 | - | 14 | - | 20.0 | 19.0 |
| **22** | 25 | 6 | 0.25 | - | 6 | - | 22.2 | 19.9 |
| **23** | 24 | 22 | 0.90 | 24 | - | - | - | - |
| **24** | 114 | 100 | 0.88 | 114 | - | 55 | 17.2 | 17.1 |
| **25** | 25 | 22 | 0.88 | 25 | - | - | - | - |
| **26** | 111 | 113 | 1.02 | 111 | - | 320 | 16.8 | 1 |
| **27** | 59 | 58 | 0.98 | 59 | - | - | - | - |
| **28** | 76 | 82 | 1.08 | 76 | - | - | 15.2 | 14.5 |

Figure 3. (A) Literature tetrazines **18** – **28** investigated in this study. (B) Pseudo-first order reaction rate constants ($k_{obs}$) determined by treating TCO-reporter-quencher pair **3** (10 µM) with tetrazines **18** – **28** (100 µM and 400 µM, N = 2) and measuring the fluorescence intensity of released EDANS (ex = 340 nm, em = 495 nm). (C) Elimination efficiency observed for TCO-reporter-quencher pair **3** (10 µM) using tetrazines **18** – **28** (100 µM and 400 µM, N = 2, t = 4 h), normalized to tetrazine **18** (100 µM, t = 1 h, 80%, Figure S5). (D) Fluorescence observed for TCO-reporter-quencher pairs **3** (10 µM) and **14** (10 µM) using tetrazines **18** – **28** (100 µM, N = 2, t = 4 h). The fluorescence detected for **14** relative to the corresponding result for **3** is given in percentages. (E) Representative example of a PCM($H_2O$)-M06-2X/6-31+G(d)-optimized transition state for the reaction of tetrazines (**18** in figure) and model axial bifunctional TCO. Bond lengths are in Å. (F) Summary of kinetic investigations with TCO-reporter-quencher pair **3**, including pseudo-first order reaction rate constants ($k_{obs}$, for 10 µM **3** and 400/100 µM tetrazine, Figure 3B), ratio of $k_{obs}$ values ($k_{obs}$ 100µM / $k_{obs}$ 400µM ≈ 1, orange; $k_{obs}$ 100µM / $k_{obs}$ 400µM < 1, transparency increases), estimated rate constants ($k_{release}$ and $k_{IEDDA}$, based on the ratio of $k_{obs}$ values or on a separate experiment at 100 nM **3** with 10 - 100 µM tetrazine, Figure S6) and calculated transition states between tetrazines **18** - **28** and model mono and bifunctional TCO model compounds.

The transesterification procedure starting from bicyclic lactone **8** (64 hours, 48% yield)[16] was replaced by a one pot, - two step procedure to improve overall conversion and reaction time. Saponification afforded monocyclic carboxylic acid **9**, which was directly methylated to obtain methyl ester **10** in 77% yield over two steps. Photoisomerization[26] resulted in a 1 : 1.4 mixture of axial isomer **11** (axial hydroxyl, equatorial methyl ester) and equatorial isomer **12** (equatorial hydroxyl, axial methyl ester), respectively. The isomeric mixture was treated with potassium hydroxide at 4°C to selectively hydrolyze axial isomer **11**, followed by acid-base extraction to separately obtain carboxylic acid **13** and ester **12**. The bis-NHS functionalization of **13** into **4** (three days, 46% yield)[16] was accelerated using nucleophilic catalysis (DMAP) and hydrolysis of the obtained product during chromatographic purification was prevented by employing neutralized silica gel, resulting in a yield of 72%. Compound **4** reacted with EDANS (fluorophore) or DABCYL (quencher) moieties with complete regioselectivity towards the carbonate due to the steric hindrance induced by the methyl group. This was followed by functionalization with the complementary moiety, obtaining axial TCO-reporter-quencher pairs **3** and **14**. We also synthesized a bifunctional *cis*-cyclooctene (CCO) reporter-quencher pair. Attempts to functionalize carboxylic acid **9** resulted in formation of cyclic lactone **8**. Instead, equatorial isomer **12** was hydrolyzed and functionalized to obtain equatorial TCO reagent **15**, followed by *trans* to *cis* isomerization in the presence of visible light (26 W CFL bulb, see Figure S1-S3) to obtain bifunctional CCO reagent **16**. Functionalization with EDANS and DABCYL occurred with the same degree of regioselectivity, obtaining CCO-reporter-quencher pair **17**.

We used **3** to assess a series of tetrazines for their ability to induce IEDDA pyridazine elimination. Axial TCO-reporter-quencher pair **3** is fluorogenic by fluorescence quenching until

the EDANS fluorophore is released from the post-ligation construct (Figure 2) and could thus serve to determine the overall properties of the deprotection reaction. This was confirmed by treating **3** and **17** with 3,6-dimethyltetrazine (**18**, Figure S4). The reaction between **3** and **18** was further characterized by quantifying the fluorescence emitted from the liberated EDANS fluorophore (Figure S5). This experiment gave an EDANS release yield of 80% after 1 hour, which corresponds with other reports.[13,23,25] A panel of tetrazines was selected from literature (Figure 3 A, **18** - **28**), including key entries reported by Chen,[22] Weissleder[23] and our own recent investigations.[24] The release of EDANS from TCO-reporter-quencher pair **3** (10 μM) was measured over time using an excess of tetrazine (100 and 400 μM) in a phosphate buffered solution (0.2 M $PO_4^{2-}$, 10% DMSO in $H_2O$, Figure S6). The functional groups of (asymmetric) tetrazines direct the regiochemistry of the initial Diels-Alder ligation reaction to form the 4,5-dihydropyridazine adduct. Further tautomerization towards the 1,4-dihydropyridazine enables fast release whereas tautomerization to the 2,5-dihydropyridazine results in a slow releasing tautomer (which proceeds via a tautomerization back to the 4,5-dihydropyidazine). Part of the 2,5-dihydropyridazine population can also convert to the oxidation product without releasing the allylic EDANS.[23,25] The release rate $k_{obs}$ is therefore the sum of the rate of these processes, which we fitted with a biphasic decay trend line. The pseudo-first order reaction rate constants for the "fast-releasing" adduct ($k_{obs}$, Figure 3B) and elimination at 4 hours (Figure 3C, normalized for [**18**] = 400 μM) were determined. For several tetrazines (**18**, **20**, **24** and **26**) with comparable $k_{obs}$ values at 100 and 400 μM, the measurements were repeated at 100 nM **3** and 10 – 100 μM of tetrazine (Figure S7). We repeated the initial measurements for TCO-reporter-quencher pair **14** to determine the release of DABCYL over time (Figure 3D, Figure S8-9). In this case, the formed pyridazine adduct appeared to act as a fluorescence quencher after IEDDA, limiting the fluorescence emitted by EDANS compared to the results obtained for **3**. These results render **14** unfit for elimination efficiency analysis.

Within the panel of tetrazines we experimentally examined at 10 μM **3**, tetrazines **19**, **21** and **22** displayed reduced $k_{obs}$ values when switching from 400 μM to 100 μM, indicating that for these tetrazines the initial cycloaddition step plays a significant role in overall reaction rate ($k_{obs} \approx k_{IEDDA}$* [tetrazine], Figure 3F). For tetrazines **18**, **23** – **28**, the $k_{obs}$ values remained similar for both concentrations examined, demonstrating that the overall reaction rate is controlled by the release rate ($k_{obs} \approx k_{release}$, Figure 3F). Additionally, the experiments performed at 100 nM **3** with tetrazines **18**, **20**, **24** and **26** (10 - 100 μM) allowed us to determine $k_{IEDDA}$ for these tetrazines as well, because in this concentration range the $k_{obs}$ values decreased when reducing tetrazine concentrations ($k_{obs} \approx k_{IEDDA}$* [tetrazine], Figure 3F). The total elimination yield for a given tetrazine is difficult to predict based on the functional groups present and no clear correlation between release rate and elimination yield can be determined, highlighting the importance of the kinetic quantification described here. It should be noted that the 4 hour timepoint doesn't always show the absolute endpoint of a given reaction. In order to rationalize IEDDA cycloaddition rates determined with **3**, density functional theory (DFT) calculations were performed. The transition state of the IEDDA cycloaddition step was studied for two model TCOs (mono TCO and bi TCO) with tetrazines **18, 20** – **22**, **24**, **26** and **28** (Figure 3E) by the use of PCM($H_2O$)-M06-2X/6-31+G(d) (See the SI). The results reveal a slight destabilization of the transition state for the cycloaddition of the bifunctional TCO compared to the monofunctional TCO for all tetrazines, resulting in a minor increase of the reaction barrier (Figure 3F). Transition state structures for both model TCOs were highly similar in terms of geometry (see the SI) and reactivity trends between tetrazines were maintained. These results suggest the steric bulk introduced on the bifunctional TCO does not significantly hamper the initial cycloaddition step.

## Conclusions

In conclusion, we developed a new method based on fluorescence quenching to determine both the cycloaddition and alkylamine release rates of the IEDDA pyridazine elimination in a 96-well plate reader format. TCO-reporter-quencher pair **3** was synthesized by functionalization of bifunctional NHS TCO reagent **4** with an EDANS fluorophore and a DABCYL quencher. The new method was used to determine click-to-release kinetics and yields for **3** with tetrazines **18** – **28**. For tetrazines **19** – **22**, the results indicated a greater concentration dependence compared to the other tetrazines measured. We were able to determine both rate parameters ($k_{IEDDA}$ and $k_{release}$) for tetrazines **18**, **24** and **26**. The DFT-calculations described here, also indicate similar IEDDA reactivity of both mono and bifunctional TCOs, suggesting the observations made with probe **3**, are translatable to the deprotection of mono-functionalized TCOs. Predicting tetrazine behavior in IEDDA pyridazine elimination remains challenging. We therefore recommend this new fluorescence assay to rapidly screen tetrazines for click-to-release potential.

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## Conflict of interest

Wolter ten Hoeve: employment at Syncom. Marc Robillard: cofounder of Tagworks Pharmaceuticals.

**Keywords:** biorthogonal chemistry • click-to-release • *trans*-cyclooctenes • tetrazines • quenched fluorescence assay

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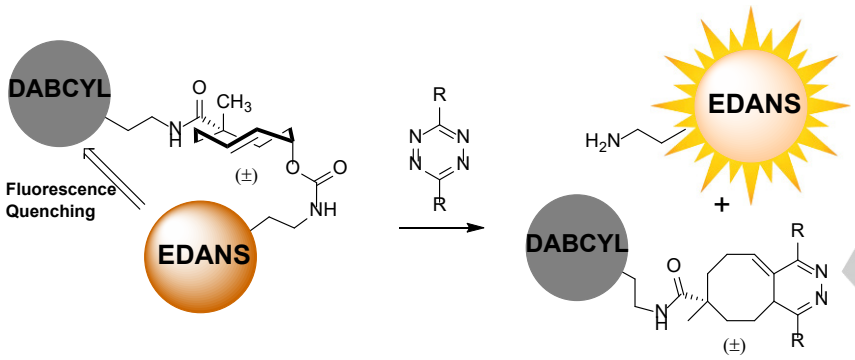


A new fluorogenic *trans*-cyclooctene probe was designed and synthesized to determine elimination kinetics of the click-to-release reaction. This reagent enables accurate detection of the decaging potential for any tetrazine at physiologically relevant concentrations.

**Click-to-release kinetics**

*Mark A. R. de Geus, Elmer Maurits, Alexi J.C. Sarris, Thomas Hansen, Max S. Kloet, Kiki Kamphorst, Wolter ten Hoeve, Marc S. Robillard, Andrea Pannwitz, Sylvestre A. Bonnet, Jeroen D.C. Codée, Dmitri V. Filippov, Herman S. Overkleeft, and Sander I. van Kasteren**

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