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FULL PAPER

Fast and pH independent elimination of *trans*-cyclooctene using aminoethyl functionalized tetrazines

Alexi J.C. Sarris, Thomas Hansen, Mark A.R. de Geus, Elmer Maurits, Ward Doelman, Herman S. Overkleeft, Jeroen D.C. Codée, Dmitri V. Filippov,* and Sander I. van Kasteren*

Abstract: The inverse electron demand Diels-Alder/pyridazine elimination tandem reaction, in which the allylic substituent on *trans*-cyclooctene is eliminated following reaction with tetrazines, is gaining interest as a versatile bioorthogonal process. One potential shortcoming of such currently used reactions is their propensity to run faster and more efficient at lower pH, a feature caused by the nature of the tetrazines used. Here we present aminoethyl substituted tetrazines as the first pH independent reagents showing invariably fast elimination kinetics at all biologically relevant pH's.

Introduction

Bioorthogonal chemistry – the execution of selective chemical conversions within a biological sample – has provided a wealth of information on a wide variety of biological processes[1]. Initial work focused on the controlled ligation reactions within biological systems, used copper-catalyzed Huisgen cycloaddition[2], Staudinger ligation[3], inverse electron demand Diels-Alder (IEDDA)[4], and other chemistries[5] to introduce reporter groups, while minimally impacting the biological processes being studied. Recently, bioorthogonal reactions have also been used to unmask functional groups in living systems[6]. The IEDDA-pyridazine elimination reaction[7] – a "click-to-release" reaction by which an allylic substituent on *trans*-cyclooctene (2-TCO, axial (E)-cyclooct-2-en-1-ol) is eliminated upon rearrangement of the pyridazine intermediate – has proven particularly favorable in this regard. It has excellent biocompatibility[9] and low toxicity[10]. *In vivo* applications include chemical control of drug release[11], control over T-cell activation[8], release of drugs from hydrogels[12], as well as the control over kinase activity in mice[10].

Mechanistic insights into this reaction have provided a pathway to the improvement of this ligation-elimination reaction[13]. Chen and co-workers, for example, discovered that asymmetric tetrazines carrying both an electron-donating and electron-withdrawing substituent showed a significant improvement in the elimination rates compared to their symmetric counterparts, leading to improved results of their most prominent tetrazine **1** over 3,6-dimethyl tetrazine **2** (Figure 1)[13a]. Weissleder and co-workers used carboxy-functionalized tetrazine **3** (Figure 1) resulting in elimination rates much faster than tetrazine **2**[13b]. It was also discovered that, for tetrazines containing simple alkyl substituents, elimination rates are very sensitive to the pH of the reaction medium: while release is completed within an hour at acidic conditions (tetrazine **2**, pH 5.0), it takes several hours under physiological conditions (tetrazine **2**, pH 7.0)[13b], as the elimination step is subject to general acid catalysis.

What is currently lacking are tetrazines that maintain fast elimination kinetics over the whole biologically relevant pH range (pH 3.5 – pH 7.5). We postulated that tetrazines **4**, **5**, **6**, and **7** (Figure 1) could serve as pH-independent eliminating tetrazines. The amine on the aminoethyl substituent, as cationic ammonium functionality at and below physiological pH, would function as general acid during the elimination step, potentially improving both release rate and efficiency.

Here we describe the design, synthesis and characterization of a new family of tetrazines based on this premise. We show that, through intramolecular proton delivery, an increase in the elimination rate by 18-fold compared to the fastest reported literature tetrazines **1** and **3** could be attained. Furthermore, this intramolecular proton source renders the reaction pH independent with minimal change in the reaction rates from pH 3 to pH 7.4.

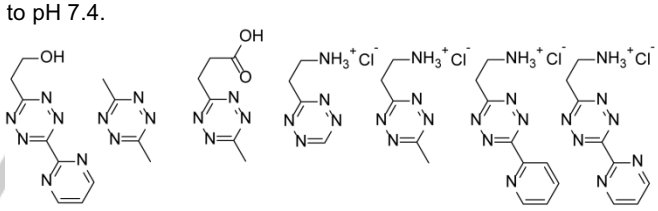

Figure 1. Known tetrazines (**1**, **2**, **3**) and aminoethyl tetrazines designed in this study (**4**, **5**, **6**, **7**)

Results and Discussion

Design

We hypothesized that the presence of an aminoethyl functionality could serve as intramolecular catalyst for both the 4,5- to 1,4-tautomerization and subsequent elimination process (Scheme 1). Tetrazines can add to 2-TCO in two ways with the aminoethyl functionality positioned at either the eliminating end (**A**, "head-to-head" adduct), or the non-eliminating end (**B**, "head-to-tail" adduct). In the "head-to-head" adduct a tautomerisation of the initial 4,5-tautomer may be promoted leading to either eliminating (**C**) 1,4-tautomer or non-eliminating (**D**) 2,5-tautomer. Elimination (**E**) may then be driven through the proximity of the intramolecular catalytic site near both the N-1 of the dihydropyridazine core and carbamate linkage.

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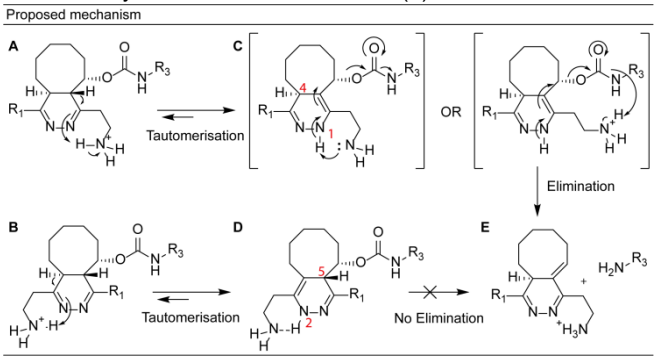
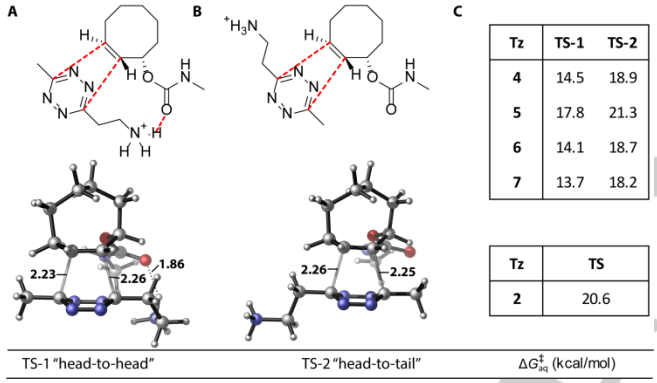
To investigate the behavior of tetrazines **4-7** density functional theory (DFT) calculations were performed (Figure S1-S4, Supporting Information)[14]. The transition state of the cycloaddition step was evaluated computationally using the reported transition states for initial guesses.[14] All structures were optimized with Gaussian 09 using the ωB97XD long-range corrected hybrid functional and 6-31+G(d) as basis set.



Scheme 1. The proposed mechanism for the tautomerisation and the elimination via intramolecular catalysis by aminoethyl functionality.



Tz	TS-1	TS-2
4	14.5	18.9
5	17.8	21.3
6	14.1	18.7
7	13.7	18.2

Tz	TS
2	20.6

Figure 2. ωB97XD/6-31+G(d)-optimized transition states for head-to-head (TS-1) and head-to-tail (TS-2) reactions of tetrazines with model axial (E)-cyclooct-2-ene. **A**) 3-ethylamine-6-methyltetrazine **5** head-to-head transition state. **B**) 3-ethylamine-6-methyltetrazine **5** head-to-tail transition state. **C**) Transition state energies of TS-1 and TS-2 for tetrazines **4**-**7** and **2**. $\Delta G^{\ddagger}_{aq}$ shown in kcal/mol.

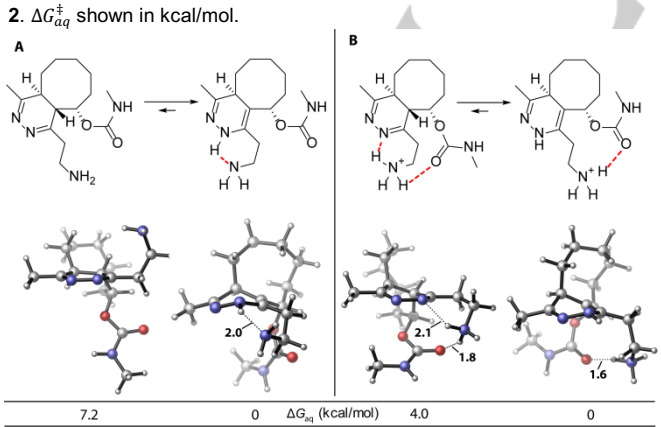


Figure 3. ωB97XD/6-311G(d)-optimized lowest energy geometries of dihydropyridazine adducts. Proton transfer is geometrically feasible (red lines). **A**) "Neutral" Initial 4,5-tautomer (left) and thermodynamically favorable 1,4-tautomer (right) **B**) "Cationic" Initial 4,5-tautomer (left) and thermodynamically favorable 1,4-tautomer (right).

Optimization was done in combination with a polarizable continuum model (PCM) using water as solvent parameter

The initial IEDDA reaction proved to favor the "head-to-head" adduct over the "head-to-tail" adduct for all studied tetrazines (Figure 2). For example, the "head-to-head" transition state (TS-1, Figure 2A) for tetrazine **5** proved to be 2.5 kcal/mol lower than the "head-to-tail" transition state (TS-2, Figure 2B). The thermodynamic preferences for the other aminoethyl tetrazines **4**, **6**, **7** appeared to be even greater at 4.4 - 4.6 kcal/mol (Figure 2C). For the "head-to-head" transition states the cationic ammonium functionality showed interaction with the carbamate linkage resulting in an energetically favorable approach.

After establishing the theoretically favored geometries of the transition state in the IEDDA step, leading to the "head-to-head" adduct, we investigated the lowest energy geometries of the formed adducts with respect to the feasibility of the intramolecular proton transfer from the ammonium functionality. Towards this end we generated a conformer distribution, with Spartan 10 program using molecular mechanics with MMFF94 as force field, for the formed initial 4,5-tautomer and subsequent 1,4-tautomer after cycloaddition of a model TCO with tetrazines **2** and **5**. All generated structures were further optimized in the gas-phase with Gaussian 09 using the ωB97XD functional with 6-311G(d,p) basis set and optimized with PCM using water as solvent parameter, providing lowest energy geometries (Figure 3, S3, S4). The calculations show that tautomerization from initial 4-5-dihydropyridazine towards eliminating 1,4-dihydropyridazine appears energetically favorable and shows prominent interaction between the aminoethyl functionality and the N-1 of the dihydropyridazine core in both neutral (Figure 3A) and cationic (Figure 3B) state. The hypothetical proton transfer through a six-membered-cyclic-transition-state is geometrically feasible in the kinetically favored "head-to-head" adduct and likely to facilitate both tautomerization and elimination (Scheme 1). The design consideration described above, supported by the calculations, indicated that the tetrazines **4-7** would show fast and pH independent kinetics for the "click-to-release" reaction.

Synthesis

To test this hypothesis, we synthesized a library of amino functionalized tetrazines (**4-17**, Scheme 2). Readily accessible N-Boc-protected aminonitriles **18-20** were prepared in good (**20**) to quantitative yields (**18, 19**) by treatment of the corresponding aminoalkylated precursors with di-*tert*-butyl dicarbonate in presence of an appropriate base. Compounds **18**, **19** and **20** were subsequently converted to the N-Boc protected aminoalkyl tetrazines **4b-17b** according to the well-established method involving Lewis acid catalyzed condensation of nitriles with hydrazine followed by oxidation with sodium nitrite under acidic conditions.[15] Optimization of this two-step synthetic protocol was required to ensure successful synthesis of each tetrazine (Table 1). At the condensation stage five variables were altered (co-solvent, catalyst, reaction container, temperature and reaction time) to obtain the required individually tailored conditions for each tetrazine. Altering one of these variables generally led to poor or no product formation at all. As a co-solvent dioxane was

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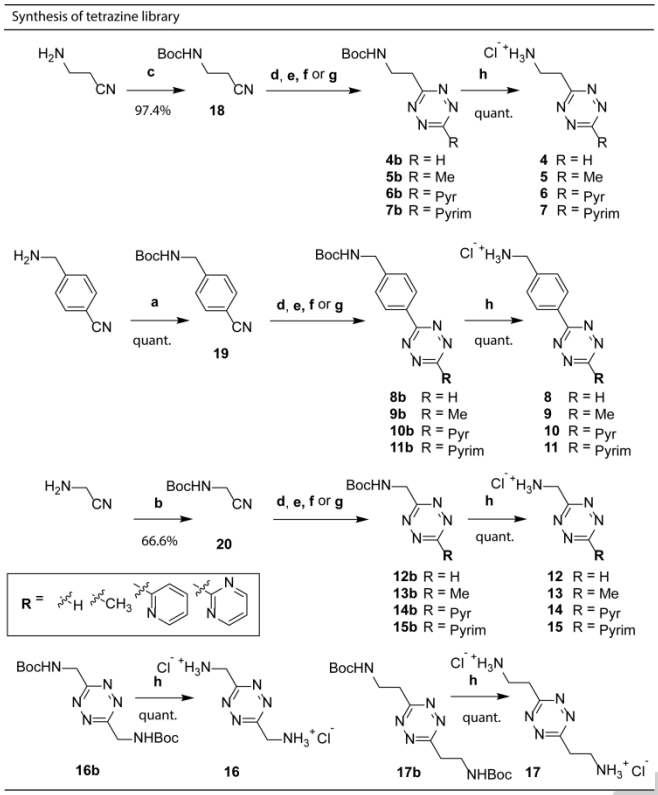
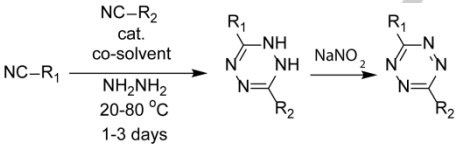


Scheme 2. Synthesis of tetrazine library. (**a**) Boc_2O, NaOH, H_2O (**b**) Boc_2O, TEA, DCM (**c**) Boc_2O, TEA, DCM. (**d**) NH_2NH_2, $Zn(OTf)_2$, formamidine acetate, then $NaNO_2$ oxidation. (**e**) NH_2NH_2, $Ni(OTf)_2$, acetonitrile, then $NaNO_2$ oxidation. (**f**) NH_2NH_2, $Zn(OTf)_2$, 2-cyanopyridine, then $NaNO_2$ oxidation. (**g**) NH_2NH_2, $Zn(OTf)_2$, 2-cyanopyrimidine, thzen $NaNO_2$ oxidation. (**h**) 4M HCl/Dioxane:DCM (1:1, v:v).



R_1	R_2	Co-solvent	Cat.	Container	T (°C)	Time	Yield	Tz
19	formamidine acetate (Form. Ac.)	dioxane	$Zn(OTf)_2$	Tube	60	o.n.	34%	**8b**
20		-	ZnI_2	Tube	30	3 days	14%	**12b**
18		dioxane	$Zn(OTf)_2$	Tube	20	3 days	6%	**4b**
19	Me-CN	-	$Zn(OTf)_2$	Flask	80	o.n.	31%	**9b**
20		dioxane	$Ni(OTf)_2$	Tube	60	o.n.	23%	**13b**
18		dioxane	$Ni(OTf)_2$	Tube	60	o.n.	16%	**5b**
19	Pyr-CN	-	$Zn(OTf)_2$	Flask	80	o.n.	53%	**10b**
20		-	$Zn(OTf)_2$	Flask	60	o.n.	49%	**14b**
18		-	$Zn(OTf)_2$	Flask	60	o.n.	13%	**6b**
19	Pyrim-CN	-	$Zn(OTf)_2$	Flask	80	o.n.	7%	**11b**
20		-	$Zn(OTf)_2$	Flask	60	o.n.	16%	**15b**
18		-	$Zn(OTf)_2$	Tube	60	o.n.	27%	**7b**
20	Form. Ac.	-	ZnI_2	Tube	30	3 days	18%	**16b**
18	Pyr-CN	dioxane	$Zn(OTf)_2$	Tube	60	o.n.	20%	**17b**

Table 1. Top: General procedure for the synthesis of N-Boc protected tetrazines **4b-17b** and carboxy functionalized tetrazines **3** and **21**. Bottom: Variations in five experimental parameters used to synthesize tetrazines **4b-17b**, **3**, and **21.**

required for the synthesis of **4b, 5b, 8b, 17b, 13b** to ensure efficient formation of the dihydrotetrazine intermediate. The used catalysts were limited to $Zn(OTf)_2$, $Ni(OTf)_2$ and ZnI_2 due to their successful use in literature.[15a] Overall $Zn(OTf)_2$ was the preferred catalyst, however $Ni(OTf)_2$ worked well with in reactions having Me-CN as the nitrile component (**5b**, **13b**). Condensation step towards compounds **6b**, **9b-11b**, **14b, 15b** was performed in an round-bottom flask under inert atmosphere ("Flask") while the other reactions were executed in a closed pressure resistant test-tube ("Tube"). A closed set up prevents the loss of ammonia that is formed as a side product in the condensation stage and apparently has a beneficial effect on the solubility of components present during this stage. Although we did not observe a consistent improvement of the yields for all tetrazines when executing the condensation stage in a closed vessel.

The reaction temperature and time were adjusted simultaneously, thus the condensations that required lower temperatures (20-30 °C) were left to react 3 days while those at higher temperatures (60-80 °C) were reacted overnight.[15] Oxidation of the formed dihydropyridazine intermediates was facilitated by transferring the reaction mixture to a 1:1 solution of AcOH and DCM followed by addition of solid $NaNO_2$.[15b] For the oxidation of dihydropyridazine intermediates of tetrazines **4b**, **6b** and **13b** a solution of $NaNO_2$ in aqueous HCl was used, a standard reagent described in the literature to oxidize dihydroterazines of different nature.[15a] N-Boc protected symmetric aminoalkyl tetrazines **16b** and **17b** were formed as side products in the synthesis of tetrazines **12b** and **6b** respectively.

The N-Boc protective group could be readily removed under anhydrous acidic conditions (4M HCl in dioxane) without decomposition of the tetrazine core yielding the target aminoalkyl tetrazines **4-17** in quantitative yields.

Determination of Elimination Kinetics

With the library of tetrazines (**4-17**) in hand we determined the elimination behavior as compared to the tetrazines from literature, either "releasing" (**1**, **2**, **25**) or "non-releasing" (**23**, **24**) (Figure 4A). To probe the importance of the intramolecular proton delivery by the aminoethyl functionality we included N-Boc protected tetrazines (**4b**-**17b**) and tetrazines (**8**-**16**) in which the amino group was expected to be unable to provide the intramolecular assistance due to its suboptimal position.

To assess the elimination properties of all tetrazines a direct fluorescence based assay[13a] was chosen in order to monitor the reaction mixture in real time during the elimination process. This method was preferred over LC-MS based analysis[13a][13b] as it allows a rapid measurement of several tetrazines simultaneously, acquisition of more data (at the initial stage of the reaction) and is not affected by possible "pseudo-release" artefacts caused by LC-MS analysis.[13b]

Tetrazine elimination properties were assessed through reaction with fluorogenic 2-TCO protected 7-amino-4-methylcoumarin **28** (2-TCO-AMC)[13a] and tracking the product AMC **27** by measuring its characteristic fluorescence at 450 nm (Figure 4B, S5, S6). Initially we tested the literature tetrazines in both

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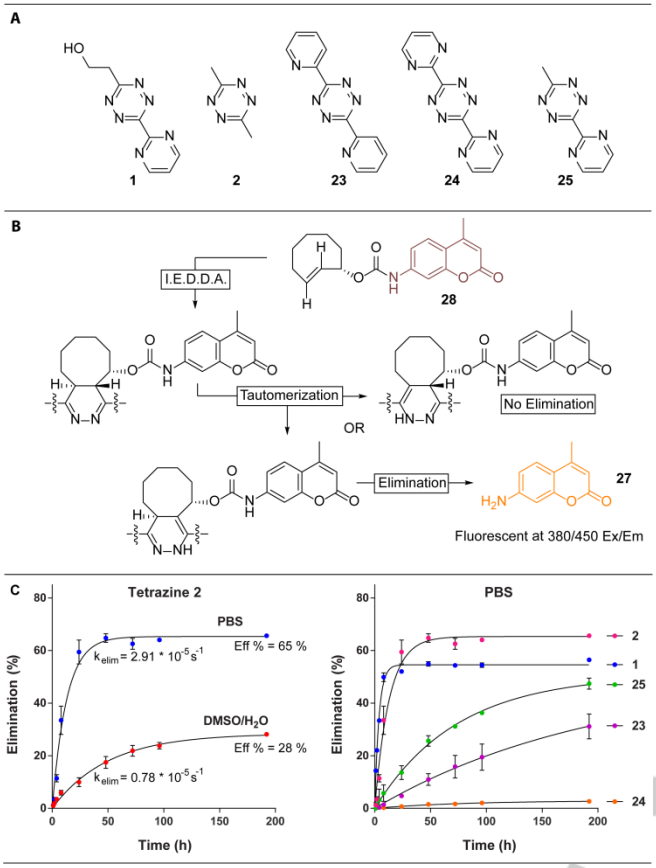

Figure 4. A) Tetrazines **1**, **2**, **23-25** used as literature references. B) Fluorescence based assay: Fluorogenic 2-TCO-AMC **28** is used as a measurement tool to track the release of fluorescent AMC **27** upon reaction with tetrazines. C) Data obtained in DMSO/H_2O (**2**, left panel) or PBS (**1**, **2**, **23-25**, right panel) as solvent during the fluorescence based assay.

DMSO/H_2O (1:1, v:v) and PBS (0.25% DMSO) (Figure 4C, right panel, S8). The elimination properties in PBS were vastly different compared to DMSO/H_2O. Tetrazine **2**, for example, was both faster and more efficient in PBS (Figure 4C, left panel). As a result, the further focus was on the experiments conducted in PBS. The elimination efficiency (Eff %) and rate (k_{elim}) of the tetrazine library was determined by exposing 2-TCO-AMC **28** (5 μM) to 4 equivalents (20 μM) of a tetrazine in PBS (pH 7.4) at 37 °C for 8 days (Figure S7-S13). At various time points, the fluorescence intensity relative to 2-TCO-AMC **28** (0%) and AMC **27** (100%) was quantified (Figure S14-S15). The solvent and concentrations were chosen to be relevant to experiments conducted in biological systems.

The data obtained was analyzed and the rate (k_{elim}) and efficiency (Eff%) was determined for each tetrazine. The values for all tetrazines were plotted as dots in an XY-scatterplot as k_{elim} (logarithmic scale) vs Eff% (linear scale) (Figure 5A, XY-scatterplot). Three classes of tetrazines were most conspicious: **14b**, **15b**, **23**, **11** caused slow elimination rates (k_{elim} < 0.20 * 10^{-5} s^{-1}; Figure 5B). **11b**, **2**, **5**, **17** all displayed intermediate elimination rates (k_{elim} between 1.5 and 3.0 * 10^{-5} s^{-1}; Figure 5C). Tetrazines **1**, **4**, **6**, **7** formed a third, most interesting, class of tetrazines defined by their very fast release kinetics (Figure 5D). While reference tetrazine **1** is fast (k_{elim} = 8.86 * 10^{-5} s^{-1}), it is still significantly slower compared to **4**, **6**, and **7** (k_{elim} = 15.3, 23.7, 35.9 10^{-5} s^{-1} respectively). Release rates for tetrazines **4**, **6** and **7** were too fast to be accurately determined by our initial assay and have therefore been displayed as their recorded minimum rates. It is noteworthy, that the tetrazines with the protected amino group (**14b**, **15b**) and the tetrazines with a suboptimal positioned amino group (**11**) showed slow elimination rates (Figure 5B). This behavior proved to be general across the whole test set (Figure S7-S13) and underscores the importance of the intramolecular proton delivery for the rate of the "click-to-release" process.

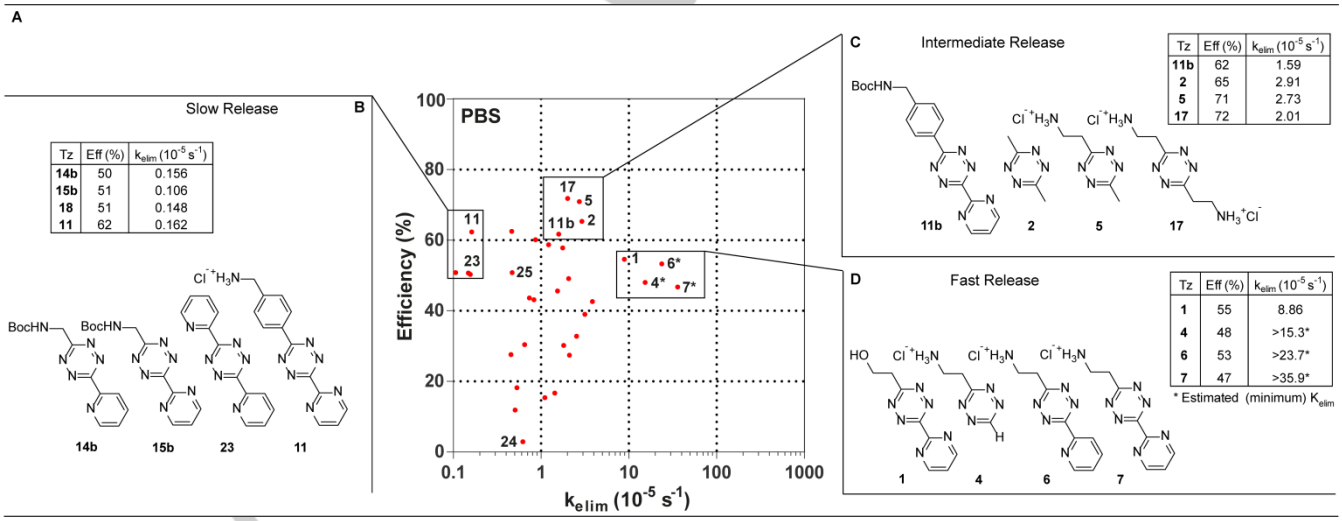

Figure 5. A) XY-scatterplot of all data obtained in PBS as solvent during the fluorescence based assay. Each result (tetrazines **1**, **2**, **23-25**, **4b-17b**, **4-17**) is depicted as a dot based on their elimination efficiency and rate. B) Tetrazines **14b**, **15b**, **23** and **11** with slow release properties k_{elim} < 0.2 * 10^{-5} s^{-1}. C) Tetrazines **11b, 2**, **5** and **17** with intermediate release properties: Eff% > 60%. D) Tetrazines **1, 4**, **6** and **7** with fast release properties: k_{elim} > 8 * 10^{-5} s^{-1}.

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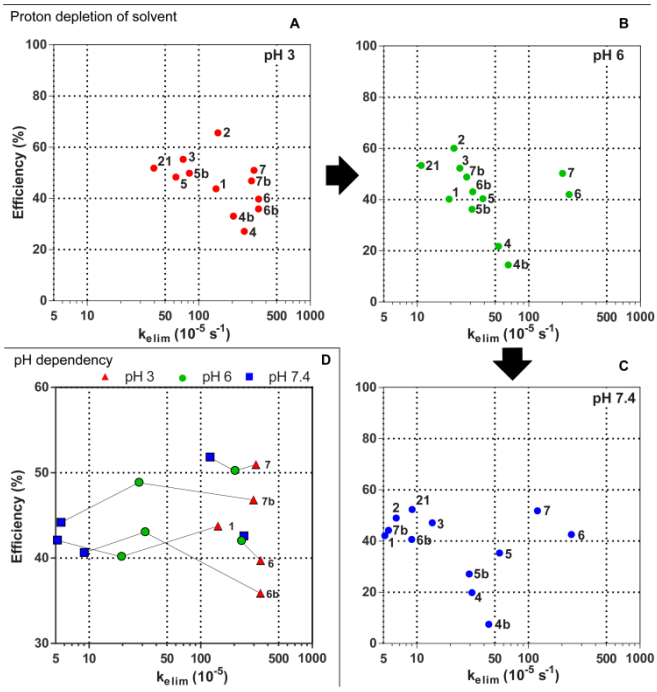

Figure 6. Proton depletion of solvent. A) Scatter plot of tetrazines at pH = 3. B) Scatter plot of tetrazines at pH = 6. C) Scatter plot of tetrazines at pH = 7.4. D) Scatter plot of pH dependent tetrazines (**6**, **7**) and pH independent tetrazines(**6b**, **7b**, **1**).

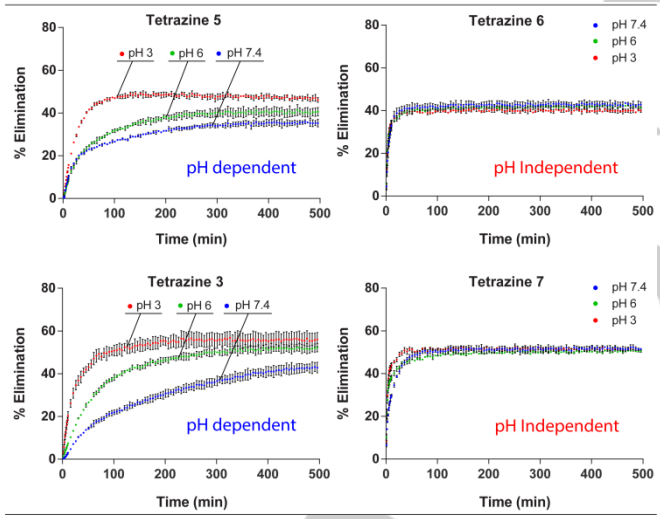

Figure 7. Elimination graphs of tetrazines **5**, **3**, **6** and **7** showing pH dependent and pH independent behavior.

pH Dependency of Elimination Rates

Following these results we set out to determine whether the fast releasing aminoethyl functionalized tetrazines retained these properties over a wide pH-range. To this end, tetrazines **1**-**3**, **21**, **4b**-**7b** and **4**-**7** were exposed to phosphate buffered solutions at various pH (0.2M [PO_4^{2-}], 10% DMSO, pH = 3.0 (red lines), 6.0 (green lines), and 7.4(blue lines)). At pH 3.0 (Figure 6A, S16-S18) all tetrazines showed elimination rates between 30 * 10^{-5} s^{-1} and 500 * 10^{-5} s^{-1}, indicating that the

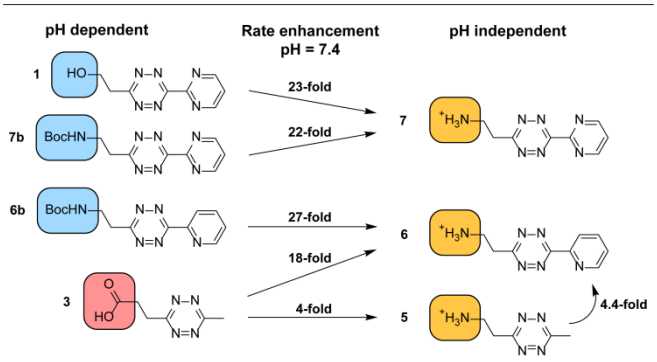

Figure 8. A) Rate enhancement of pH independent functional groups depicted in orange over pH dependent functional groups depicted in blue and red.

availability of protons in solution enhances the elimination rate. Lowering the proton concentration by raising pH to 6.0 or 7.4 resulted in large decreases in the elimination rates observed for most tetrazines (Figure 6B, 6C, S16-S18). At pH 7.4 tetrazines **1**-**3**, **21**, **6b**, and **7b** showed much slower elimination rates (5-15 * 10^{-5} s^{-1}, Figure 6C). Aminoethyl tetrazines **6** (k_{elim} = 241 * 10^{-5} s^{-1}) and **7** (k_{elim} = 120 * 10^{-5} s^{-1}) on the other hand showed a minimal reduction in rate at increased solvent pH (Figure 7), resulting in a rate 18-27-fold faster at pH 7.4. Substituting amino (**6**, **7**) for alcohol (**1**) or carbamate (**6b, 7b**) functionality reintroduced pH-dependency in the elimination process (Figure 8). A carboxy functionality, as seen in tetrazine **3**, provided an overall moderate elimination rate and moderate pH-dependency (Figure 8). Substitution of carboxy (**3**) for amino (**5**) functionality improved both properties. Furthermore, the fastest tetrazine **6** outperforms tetrazine **3** by 18-fold in terms of elimination rate. Finally, when comparing tetrazines **4-7**, the data also shows that the elimination rate is positively affected by electron-withdrawing substituents (pyridine/pyrimidine) explaining the differences between the tetrazines, in line with previous findings.[13b]

These results strongly support our hypothesis that the elimination process is dependent on proton availability and can be catalyzed at biologically relevant pH (3-7.4) through careful placement of cationic ammonium functionality acting as an intramolecular catalyst.

LC-MS analysis

In order to gain additional insight in the course of the "click-to-release" reaction with the newly developed aminoalkyl tetrazines and to study the intermediates and possible side products we also assessed the elimination rates of the key compounds **5 - 7** using reported LC-MS based approach that uses an ammonium formate buffered water-acetonitrile eluent (2.5mM $NH_4^+HCOO^-$, pH = 8.4) and taking the first analysis point at t = 5 min.[13b] Known tetrazines **1**-**3** were analyzed by this method for comparison. The elimination rates of tetrazines **2** and **3** showed results corresponding to data reported by Weissleder et al[13b], where tetrazine **3** gives 48% release at t = 5 min, going up to 72% after 16 hours (Figure 9).

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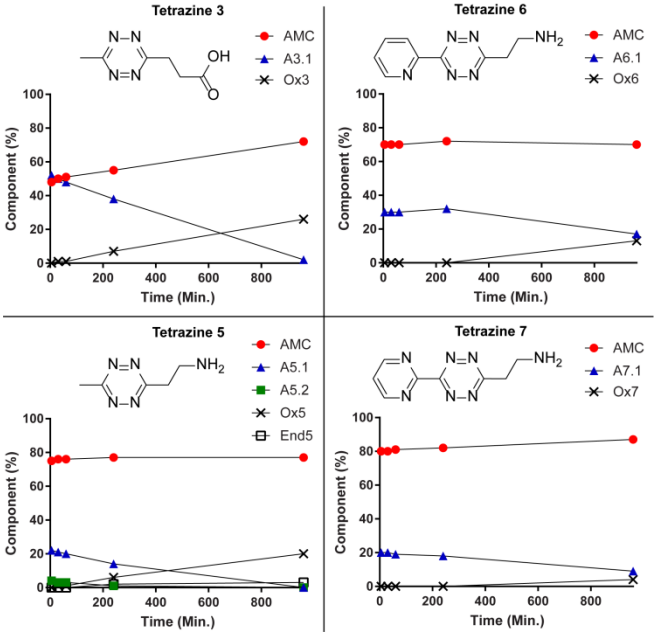

Figure 9. Time dependent LC-MS analysis of 2-TCO-AMC **28** with tetrazines **3**, **5**, **6** and **7** in PBS.

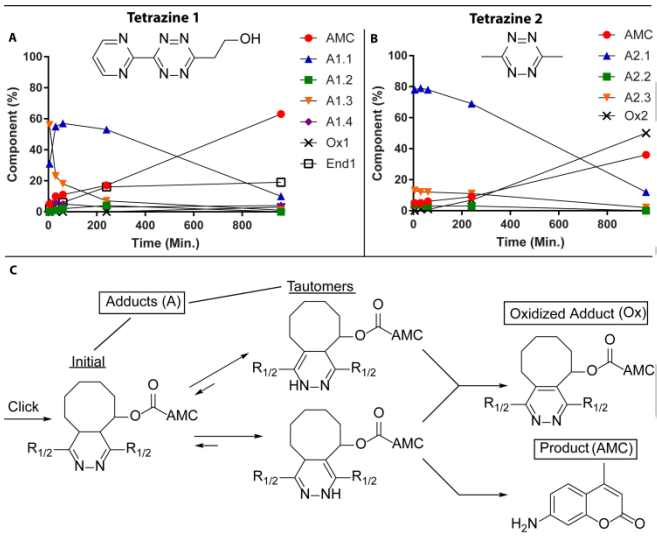

Figure 10. Time dependent LC-MS analysis of 2-TCO-AMC **28** with tetrazines in PBS. Aminomethyl coumarin containing molecules: product "AMC", adduct "A", oxidized adduct "Ox" and dead-end adduct "End". A) Reaction of **28** with tetrazine **1**. B) Reaction of **28** with tetrazine **2**. C) Elimination pathway with possible adducts and products.

The explanation offered by the authors invokes rapidly releasing "head-to-head" adduct and slowly releasing "head-to-tail" adduct which are formed in approximately equal amounts.[13b] The data showed much faster initial release than measured in the fluorescence assay, and this is likely due to additional "pseudo-release" or concentration effects during the LC-MS analysis. In turn, aminoethyl tetrazines **5**, **6** and **7**, showed 78%, 80% and 70% initial release respectively at t = 5 minutes with only a small further increase in release over time (Figure 9). This would correspond to the preferential formation of the rapidly releasing "head-to-head" adduct as predicted by our calculations.

It is noteworthy that although we do observe a multitude of different intermediates, including putative "head-to-tail" and "head-to-head" adducts, in the reaction of 2-TCO-AMC **28** with comparatively slow eliminating tetrazines **1** and **2** (Figure 10, S19, S20), as well as with tetrazines **3, 5, 6** and **7** (Figure 9, S21-S24), we do not consistently see formation of dead-end adduct (End) as reported by others[13b]. Furthermore the amount of oxidized adduct (Ox) is reduced for tetrazines **1**, **6-7** compared to tetrazines **2**, **3**, and **5**. The results of the LC-MS analysis of the "click-to-release" reaction of 2-TCO-AMC **28** with tetrazines **1** and **2** are similar to what is published by Robillard and co-workers in terms of observed intermediates and side products (Figure 9A-B).[13c] To summarize, the results obtained from the LC-MS analysis of the key tetrazine derivatives demonstrate that the method shows reproducible results for the known tetrazines **2** and **3** and that the aminoethyl tetrazines **5-7** do perform as designed in the "click-to-release" IEDDA reaction.

Conclusions

The full kinetic profiling of a focused tetrazine library clearly shows that the presence of a properly placed cationic ammonium functionality acting as an intramolecular proton donor gives the tetrazine mediated "click-to-release" elimination a pH independent character. Asymmetric tetrazines that contain both this aminoethyl substituent and an electron-withdrawing substituent on the tetrazine core show unprecedented release rates combined with nearly complete pH independence over the whole biologically relevant pH range.

Experimental Section

Full experimental details can be found in the online supporting information.

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Keywords: inverse electron demand Diels-Alder • *trans*-cyclooctene elimination • click-to-release • pH independent • bioorthogonal deprotection

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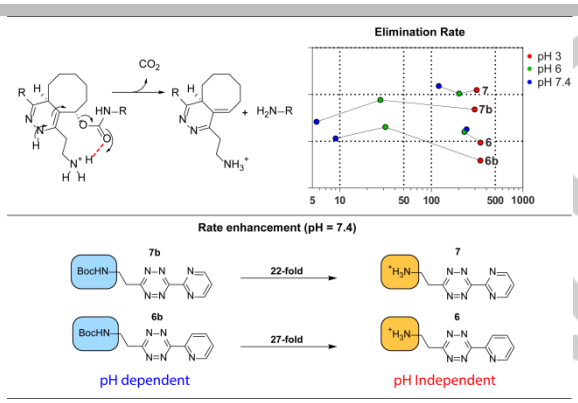

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Fast and pH independent elimination of *trans*-cyclooctene using aminoethyl functionalized tetrazines