Design and Synthesis of aza-Bicyclononene Dienophiles for Rapid Fluorogenic Ligations

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Abstract: Fluorogenic bioorthogonal reactions enable visualization of biomolecules under native conditions with excellent signal-to-noise ratio. Herein, we present the design and synthesis of conformationally-strained aziridine-fused *trans*-cyclooctene (aza-TCO) dienophiles, which lead to the formation of fluorescent products in tetrazine ligations without the need for attachment of an extra fluorophore moiety. The presented aza-TCOs adopt the highly strained 'half-chair' conformation, which was predicted computationally and confirmed by NMR measurements and X-Ray crystallography. Kinetic studies revealed that the aza-TCOs belong to the most reactive dienophiles known to date. The potential of the newly developed aza-TCO probes for bioimaging applications is demonstrated by protein labeling experiments, imaging of cellular glycoconjugates and peptidoglycan imaging of live bacteria.

Introduction

Fluorogenic bioorthogonal reactions have emerged as a powerful tool for bioimaging as they offer an excellent signal-to-noise ratio without the need to use extensive washing steps.^[1] Significant progress has been made in the development of probes which can be activated by means of a selective chemical reaction in a complex biological environment. These include Staudinger ligation,^[2] azide-alkyne cycloadditions,^[3] light-triggered nitrilimine-alkene cycloadditions^[4] and tetrazine ligations.^[5] In most cases, the fluorogenic probe contains an appropriately modified fluorophore moiety with its fluorescence being quenched via energy transfer and being restored upon the bioorthogonal chemical reaction. A much less common situation represents fluorogenic ligations where the formation of the fluorophore is an inherent result of the chemical reaction. One particular advantage of these types of probes is the absence of virtually any background fluorescence signal since no real pre-fluorophore (or any quenched fluorophore moiety) is present in the structure of reagents before the reaction takes place. One of the prominent examples of this type of reaction is the formation of pyrazolines upon cycloaddition of nitrilimines with alkenes.^[6] Shang et al. recently reported a conceptually similar styrene-tetrazine cycloaddition leading to fluorescent dihydropyridazines.^[7] In parallel to the later study, we have shown that the inverse electron-demand Diels–Alder reaction

(IEDDA) of 1,2,4,5-tetrazines with *trans*-cyclooctene (TCO) dienophiles can also directly lead to the formation of fluorescent products. In particular, we found that the axial *trans*-cyclooctene-5-ol (ax-TCO-ol) leads upon reaction with various tetrazines to the formation of fluorescent dihydropyridazines with tuneable photophysical properties.^[8] Although we could demonstrate the power of this chemistry for bioimaging, we also found that by modifying the hydroxyl group of the ax-TCO-ol, the fluorogenic properties of the reaction alter or even completely disappear. For many reasons it would be advantageous to maintain the fluorogenic nature of the reaction while enabling attachment of various useful functional groups to e.g. biomolecules. The aim of the present study was to design new TCO derivatives, which would fulfil these criteria. Here, we show that by *cis*-fusion of an aziridine ring to the *trans*-cyclooctene skeleton a new type of highly reactive dienophiles are formed, which produce fluorescent products in IEDDA reaction with tetrazines. In addition, our kinetic measurements show that these strained alkenes belong to the most reactive bioorthogonal probes known to date. Their excellent reactivity is a result of the highly strained 'half-chair' conformation, which was confirmed by NMR measurements and, to the best of our knowledge, for the first time also by X-Ray crystallography.

Results and Discussion

Rational design of aza-TCOs and computational methods

Our previous *in silico* conformational analysis and density functional theory (DFT) modelling of transition state structures of the reaction between ax-TCO-ol and diphenyl-*s*-tetrazine showed that the hydroxyl oxygen atom of TCO is very close to one of the bridgehead hydrogen atoms in a low energy conformer of the IEDDA product.^[8] These calculations show that the conformation of the intermediate 4,5-dihydropyridazine promotes hydrogen migration to the hydroxyl oxygen, which results in the formation of the final 1,4-dihydropyridazine fluorophore. This means that the presence of a heteroatom in the right conformation is responsible for the rapid tautomerization of the dihydropyridazine and for the formation of fluorescent products. We therefore speculated that fusion of an aziridine ring to *trans*-cyclooctene would ensure the fluorogenicity of the reaction by introducing the required heteroatom in the right position. In addition, this should also force the *trans*-cyclooctene ring to adopt the highly strained 'half-chair' conformation which is known to enhance the rate of the IEDDA reaction dramatically.^[9] With this hypothesis in mind we performed a computational study with two aims: 1) to predict the most favourable conformation of aza-TCO compound **1a** and 2) to predict the propensity of aza-TCO to form fluorescent products. The conformational analysis of compound **1a** was performed with 100 molecular-mechanics simulated annealings.

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In 97 out of the 100 simulations, the resulting structure had the desired 'half-chair' conformation depicted in Figure 1A. The 'half-chair' conformation of **1a** was also retained after geometry optimization by the DFT method (Figure 1A and ESI).

Figure 1. A) Lowest-energy conformation found by molecular modelling together with the most important experimental NOE contacts (indicated by arrows) confirming the half-chair conformation of **1a** B) Selected experimental and calculated (B3LYP/6-31+g(d,p)/PCM) vicinal coupling constants (Hz) between hydrogen atoms in **1a** C) The proposed reaction pathway for the reaction of **1a** with diphenyl-s-tetrazine leading to fluorescent product **1a-III** and the transition-state structure.

The expected first step in the reaction sequence of compound **1a** with 1,2,4,5-tetrazines is the IEDDA reaction followed by nitrogen elimination, which provides 4,5-dihydropyridazine intermediate **1a-II** (Figure 1C). The transition state structures were predicted by DFT computations for both of these reactions starting from compound **1a** and diphenyl-s-tetrazine and the energy barriers toward intermediates **1a-I** and **1a-II** were found to be 12.1 and 8.3 kcal/mol, respectively. The key reaction step found previously for the formation of a 1,4-dihydropyridazine fluorophore involved a hydrogen atom transfer from one of the bridgehead carbon atoms to a heteroatom. This hydrogen atom transfer was only possible for conformations with short distances between the hydrogen atom and the heteroatom. Therefore, we performed conformational analysis of compound **1a-II** by simulated annealing followed by DFT geometry optimization, which revealed that a low-energy conformer of **1a-II** has the bridgehead hydrogen–aziridine nitrogen distance of 2.29 Å, which is suitable for the hydrogen atom migration to take place. The transition-state structure and a modest energy barrier (10 kcal/mol from the suitable conformation, 16 kcal/mol from

the lowest-energy conformation of **1a-II**) for the hydrogen atom migration to the nitrogen atom were found by DFT calculations. Compound **1a-II** protonated at one of the pyridazine nitrogen atoms was considered in the transition-state search. The hydrogen atom migration step is possible also for neutral compound **1a-II**; however, the reaction barrier is higher by 18 kcal/mol (see details in ESI). The computational study thus corroborates our presumption of both, the 'half-chair' conformation of **1a** and its tendency to promote the formation of fluorescent 1,4-dihydropyridazines in reaction with 1,2,4,5-tetrazines. For comparison, we also performed the transition-state search for the hydrogen atom migration reaction for dioxolane fused TCO (d-TCO), which also contains a heteroatom and adopts the highly strained 'half-chair' conformation as reported previously.^[9d] The calculated reaction barrier was found to be significantly higher (28 kcal/mol) than for compound **1a-II** (See ESI) showing that it is the unique structure of aza-TCOs, which leads to the formation of fluorescent products by promoting the dihydropyridazine tautomerization. This unique behavior of aza-TCO was confirmed also experimentally (Fig. S5).

Synthesis of aza-TCOs

Encouraged by these computational results, we next focused on the synthesis of the aziridine-fused *trans*-cyclooctene. In our initial synthesis, we utilized Sharpless aziridination,^[10] which yielded the desired *N*-tosyl-protected aziridine **3** in a single step (Scheme 1). Compound **3** was then conveniently converted to the corresponding *trans*-isomer **4** by the continuous flow-photoisomerization protocol developed by Fox and coworkers.^[11] Unfortunately, our attempts to efficiently remove the tosyl group using sodium naphthalenide^[12] were unsuccessful and led only to concomitant *trans*-to-*cis* isomerization of (1*R*,8*S*,*E*)-9-azabicyclo[6.1.0]non-4-ene **5**. We therefore used a different strategy and started the synthesis from known (*Z*)-9-oxa bicyclo[6.1.0]non-4-ene **6**. Opening of the epoxide afforded azido alcohol **7**, which was converted to the corresponding aziridine **8** via the Staudinger reduction-cyclization reaction using triphenylphosphine.^[13] This sequence of reaction steps leads to *cis*-ring fusion which is known to induce the 'half-chair' conformation in bicyclononenes.^[9a, 9c] The next crucial step in the synthesis was the protection of the aziridine nitrogen atom. After extensive experimentation, we found that the trimethylsilylethoxycarbonyl (Teoc) protecting group enables efficient photoisomerization and subsequent mild deprotection to yield compound **5**. Alkylation of the aziridine nitrogen afforded ethyl ester **11**, which was reduced by LiAlH₄ to the desired 2-((1*R*,8*S*,*E*)-9-azabicyclo[6.1.0]non-4-en-9-yl)ethan-1-ol **1a**. Since the conformation of the *trans*-cyclooctene ring is crucial for its reactivity, we performed conformational analysis of compound **1a** by NMR spectroscopy. All proton and carbon NMR signals were assigned by a combination of 1D and 2D NMR experiments and all observable proton–proton spin–spin coupling constants were determined. The experimental coupling constants are in excellent agreement with those calculated by using the DFT method for the lowest-energy (half-chair)

Scheme 1. A) Synthesis of aza-TCOs. B) An ORTEP view of the major enantiomer of compound **4** (displacement ellipsoids shown with 50% probability). CCDC 1553867.

conformer of **1a** (Figure 1B). The 'half-chair' conformation of **1a** was further confirmed by Nuclear Overhauser Effect Spectroscopy (NOESY); the most important NOE contacts for the determination of the conformation are depicted in Figure 1A. In addition, *N*-tosylated bicyclononene **4** was a crystalline compound which afforded crystals suitable for X-Ray analysis and further confirmed the 'half-chair' conformation. The compound crystallized as a mixture of two enantiomers in a 6:4 ratio (Scheme 1B and Fig. S2).

Reactivity of aza-TCOs and photophysical properties of the click products after reaction with 1,2,4,5-tetrazines

As already predicted by the computations, the new aza-TCO dienophile **1a** yields fluorescent products in the IEDDA reaction with tetrazines similarly to the previously reported ax-TCO-ol.^[8] A series of experiments and calculations confirmed the identity of 1,4-dihydropyridazines as the actual fluorescent species formed during the reaction (see ESI). We determined the photophysical properties of the corresponding click products, which are summarized in Table 1. The dihydropyridazine products **13a-13e** have large Stokes shifts and tunable emission maxima depending on the substitution pattern of the starting tetrazine (Fig. S7). The fluorescence enhancement upon the reaction ranges from 20- to an excellent 70-fold. The fluorescence quantum yields are relatively low when compared to the more commonly used fluorophores (e.g. fluorescein ϕ_f = ca. 0.9 in 0.1 M NaOH)^[14] however, still sufficient for bioimaging applications as we show below.

The second order rate constants of the reaction between **1a** and tetrazines **12a-12e** were determined. The kinetic measurements were performed under pseudo first-order conditions using an excess of the dienophile. The decrease in concentration of the starting tetrazine was followed in time by

measuring the decrease in its absorbance by UV-Vis spectroscopy. All measurements were carried out at least in triplicate and the average rate constants are summarized in Table 1 (for details see ESI). The second order rate constants range from $250 \text{ M}^{-1} \text{ s}^{-1}$ to more than $6\,000 \text{ M}^{-1} \text{ s}^{-1}$ (in MeOH at 21°C), highlighting the exceptional reactivity of **1a**. The aza-TCO showed good reactivity toward 1,2,4-triazines as well, which were recently shown to be a good alternative to the more commonly used 1,2,4,5-tetrazines (Table S10).^[15]

Furthermore, we used some of the known TCO derivatives to directly compare their reactivity with **1a**. We used diphenyl-*s*-tetrazine as the heterodiene in this case (Figure 2). Our data show, that **1a** reacts about 20 times faster than simple ax-TCO-ol (Table S9), which we have used previously in fluorogenic cell labeling experiments.^[8]

Figure 2. Comparison of the reactivity of various TCOs in reaction with diphenyl-*s*-tetrazine measured in MeOH at 21°C using manual stopped-flow device connected to UV-Vis spectrophotometer.

Table 1. Photophysical properties of click products and second-order rate constants of the reaction between 1,2,4,5-tetrazines and aza-TCO **1a**.

Entry	Tetrazine	Click product	R ¹	R ²	Fluorescence of the click product ^[a]	$\lambda_{\text{Abs}}/\lambda_{\text{Em}}$ ^[b]	Stokes shift ^[b]	$\phi_f/\epsilon_{\text{max}}$ ^[c]	Intensity increase	k_2 (M ⁻¹ s ⁻¹) ^[d]
1	12a	13a				317/478	161	0.06/12	20-fold	6360 ± 90
2	12b	13b				333/501	168	0.07/21	60-fold	318 ± 3
3	12c	13c				321/475	154	0.15/16	55-fold	255 ± 7
4	12d	13d				337/540	203	0.04/23	40-fold	316 ± 2
5	12e	13e				335/489	154	0.11/17	70-fold	740 ± 25

[a] Pictures were captured in quartz cuvettes using a hand-held UV lamp (365 nm), [b] given in nm, [c] extinction coefficients ϵ_{max} are in 10³ M⁻¹ cm⁻¹, quantum yields were determined using quinine sulfate in 0.5 M H₂SO₄ as standard, [d] kinetic measurements were performed in MeOH at room temperature under pseudo first-order conditions using an excess of **1a** (for details see ESI).

Compound **1a** reacts about 1.5 times faster than the dioxolane-fused TCO (d-TCO) reported by Fox and coworkers.^[9d] The only derivative, which exceeded **1a** in reactivity (about 3 times), was the cyclopropyl-fused *trans*-cyclooctene (s-TCO) developed by the same group.^[9c] These data clearly show that fusion of an aziridine ring to *trans*-cyclooctenes yields highly reactive dienophiles belonging to the fastest reacting bioorthogonal probes known to date.

Stability of aza-TCOs and of the click products under biologically relevant conditions

We then studied the stability of the newly developed dienophile under various conditions. Compound **1a** showed excellent stability when stored in the dark at room temperature as a solution in CD₃OD (Fig. S9). The stability of the compound in the presence of L-cysteine, which is known to promote the *trans*-to-*cis* isomerization of TCOs,^[9d, 16] was also very good. A 1:1 mixture of *cis* and *trans* isomers was obtained in 7 days after subjecting compound **1a** to 3.5 equivalents of L-cysteine at neutral pH in deuterated phosphate buffer (Fig. S10). We also examined the stability of the click products. The 1,4-dihydropyridazines formed upon reaction of **1a** with diphenyl-*s*-tetrazine or dipyrindyl-*s*-tetrazine are stable under various conditions (e.g. phosphate buffered saline, fetal bovine serum) for several days. HPLC-MS analysis showed that the

corresponding fully aromatic pyridazines are formed as major 'side' products in this case. Our data show that the click product of diphenyl-*s*-tetrazine oxidizes faster than the click product of dipyrindyl-*s*-tetrazine (Fig. S11).

Fluorogenic labeling of peptides

To evaluate the performance of **1a** as a bioorthogonal handle for fluorogenic biomolecule tagging, we first performed labeling experiments on a model peptide. Our attempts to prepare the active ester directly from **1a** failed, giving under various reaction conditions a mixture of products.

Scheme 2. Synthesis of aza-TCO active ester **1b**.

Figure 3. The fluorogenic click reaction was performed on a model peptide modified with aza-TCO **1b** using various tetrazine probes. Pictures were captured under a fluorescence stereomicroscope at 350 nm irradiation using CoolLED pE-300 light source and the following setup in each case: Exposure time 300 ms, IL light intensity 1000 and objective 1x/0.08. Colors were finally adjusted using LAS AF Lite program.

Similarly, the hydrolysis of ethyl ester **11** also did not afford the desired carboxylic acid derivative. Fortunately, the structural features of aza-bicyclononene **5** enable a straightforward synthesis of its derivatives by simple alkylation of the free aziridine nitrogen atom. After optimization, we prepared the active ester of aza-TCO using bromomethyl benzoate as the alkylating agent followed by reduction of the ester to the corresponding alcohol and finally by reaction with *N,N'*-disuccinimidyl carbonate (Scheme 2 and ESI). Active ester **1b** was first reacted with the model peptide (KYHWYGYTPQNVI) containing an N-terminal lysine. The final aza-TCO-modified peptide, still attached to the resin (Tentagel-OH), was treated with various tetrazines (**12b**, **12c** and **12e**) and the formation of fluorescent products was verified using fluorescence microscopy (Figure 3 and Fig. S12). We observed the successful formation of the desired click products in all cases, which was also confirmed by HPLC-MS analysis (Fig. S13-S17). Our previous experiments with the ax-TCO-ol showed that the fluorogenic properties of the reaction under these conditions alter or even completely disappear.^[8] Aza-TCOs are therefore superior compounds for fluorescent peptide labeling applications.

Fluorogenic labeling of proteins

The active ester-mediated protein modification still belongs to the most frequently applied methodology for covalent protein modification.^[17] Alternatively, a cysteine-maleimide reaction can be used for this purpose.^[18] We performed a series of experiments to probe aza-TCO derivatives for the fluorogenic labeling of proteins. We first modified human carbonic anhydrase II (hCAII) with the active ester **1b** under standard conditions and added tetrazine **12c** to the protein (Figure 4 and Fig. S18). We also used a biotin-conjugated tetrazine probe **12f**, which enables double detection after the reaction. Namely by spectrophotometry, based on the formation of the fluorescent dihydropyridazine, and by western blot analysis due to the

presence of the biotin moiety. SDS-PAGE analysis of the reaction mixtures showed that the reaction proceeded efficiently and the fluorescently-labeled products were clearly visible after UV irradiation of the gel (Figure 4A). The presence of the biotin was successfully confirmed by western blot analysis using fluorescein-conjugated streptavidin. The fluorogenicity, which is an intrinsic property of the chemical reaction, thus facilitates straightforward detection of the reaction products and should enable the development of e.g. multimodal detection probes similar to **12f**.

We then used ADP-ribosylation factor 1 protein (Arf1), which was prepared using standard recombinant techniques.^[19] Arf1 contains a single cysteine residue, which was used for the attachment of aza-TCO using maleimide **1c**. The success of the reaction after addition of **12c** was evident from the shift of the protein band on SDS-PAGE gel as well as after spectrophotometric imaging (Figure 4B and Fig. S19). These experiments manifest that aza-TCOs are useful bioconjugation reagents enabling efficient fluorogenic tagging of proteins.

Figure 4. Fluorogenic labeling of proteins. A) Labeling of aza-TCO-modified hCAII using tetrazine **12c** and **12f**, B) labeling of Arf1 protein via maleimide and subsequent reaction with tetrazine **12c**.

Fluorogenic labeling of glycoconjugates on U2OS cancer cells

We next turned our attention to cell labeling experiments to further demonstrate the potential of aza-TCOs for bioimaging applications. First, we attached the aza-TCO moiety to concanavalin A (ConA). ConA is a lectin which shows high specificity for α -D-mannose- and α -D-glucose-containing glycoconjugates and is useful in carbohydrate studies.^[20] To compare the performance of aza-TCO with the previously reported ax-TCO-ol, we also conjugated two different axial

TCOs to ConA. Derivative **1d** is attached to ConA via a carbamate linker while **1e** via a peptide bond. We next separately added the three TCO-modified ConAs to fixed and permeabilized U2OS cancer cells to enable intracellular glycoconjugate staining after addition of **12c**.

Figure 5. A) Structures of active esters used to modify ConA and structure of tetrazines **12c** and **12g** used for labeling. B) Confocal microscope images of fluorogenic U2OS cell labeling using ConA modified with TCOs **1b**, **1d**, **1e** and tetrazine **12c** or FITC-conjugated tetrazine **12g**. Conditions: The cells were fixed, permeabilized and incubated for 1 hour with the respective TCO-modified ConA (0.25 $\mu\text{g}/\mu\text{L}$). After washing, the cells were incubated with 0.25 mM tetrazine and 5 μM DRAQ5 for 10 min and imaged under confocal microscope. Click product (ex.: 405 nm, em.: 560-666 nm); FITC channel (ex.: 458 nm, em.: 490-552 nm); the nucleus was stained with DRAQ5 (ex.: 633 nm, em.: 653-732 nm). Scale bar: 20 μm .

We used fluorescein-tagged tetrazine **12g** as a positive control in this experiment as this compound yields fluorescent products in each case. The formation of fluorescent products was verified by inspecting the cells under a confocal microscope (Figure 5 and Fig. S21). Our experiment confirmed the superior performance of the newly developed aza-TCO. ConA modified with **1b** gave the most intensive labeling when compared to ConA modified with either **1d** or **1e**, although a weak staining of glycoconjugates was visible in these cases as well. On the other hand, equal staining of the cells was observed when we used FITC-tetrazine derivative **12g** under the same conditions (Figure 5B and Fig. S21).

Fluorogenic imaging of peptidoglycans in live bacteria

Various derivatives of D-amino acids can be incorporated into the peptidoglycans (PGs) of diverse bacterial species and can be used as valuable chemical probes providing information

about dynamics, synthesis and maturation of PGs.^[21] In order to test if our aza-TCOs could serve as a reporter molecule for similar application, we prepared the aza-TCO modified D-amino acid **1f** (See ESI). The gram-positive *Bacillus subtilis* were first grown in the presence of **1f** for 2 hours. Similarly, gram-negative *E. coli* cells were grown in the presence of **1f** for different period of time (2, 6 or 12 hours). Incorporation of the amino acid into PGs was subsequently validated under confocal microscope after labeling with the fluorogenic BODIPY-tetrazine probe **12h**. We observed a fluorescent signal on the surface of the bacteria after incubation with **1f** and labeling with **12h**, while no fluorescence formed in cells incubated only with the amino acid **1f** or tetrazine **12h** under the same conditions (Figure 6B-C and Fig. S22-23). Weak fluorescent signal formed in *E. coli* cells even after 12 hours of incubation with **1f** and subsequent labeling with **12h** showing that at least part of the modified amino acid remains in the highly reactive *trans*-conformation within this time period. These experiments further demonstrate that the aza-TCO moiety can serve as valuable reporter group for various bioimaging applications. The high reactivity accompanied with good stability favors aza-TCOs for experiments where both of these attributes are important and desirable.

Figure 6. A) Structure of D-amino acid **1f** and Tetrazine-bodipy **12h**. B) and C) Confocal microscope images of fluorogenic bacterial cell wall labeling using **1f** and **12h**. Conditions: Live *B. subtilis* or *E. coli* cells were incubated with **1f** (1 mM) for the indicated time. After washing, the cells were incubated for 15 min with **12h** (25 μM) and 50 μM DRAQ5. The cell were then inspected under confocal microscope using the following set up: nucleus was stained with DRAQ5 (ex.: 633 nm, em.: 653-732 nm) and click product resulting from the reaction (ex.: 458 nm, em.: 499-569 nm). 5x and 10x zoom for *B. subtilis*, 10x zoom for *E. coli*.

Conclusions

In this study, we present the design and synthesis of aziridine-fused *trans*-cyclooctene derivatives, which produce fluorescent products in reaction with 1,2,4,5-tetrazines. The newly developed dienophiles adopt the 'half-chair' conformation as a result of the *cis*-ring fusion of the aziridine moiety. This highly strained conformation leads to remarkable reactivity in the IEDDA reactions making these compounds one of the most reactive bioorthogonal probes known to date. Despite their high reactivity, these compounds show good stability under various conditions. Stability of the corresponding click products was found to be excellent as well. We demonstrate the potential of aza-TCOs in fluorogenic ligation reactions by the labeling of model biomolecules and by cell imaging experiments. The exceptional reactivity together with the intrinsic formation of fluorescent products upon reaction with simple tetrazines make aza-TCO dienophiles attractive candidates for fluorogenic biomolecule tagging and bioimaging applications.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: cell labeling • Diels-Alder reaction • fluorogenic • *trans*-cyclooctene • tetrazines

- [1] a) A. Nadler, C. Schultz, *Angew. Chem. Int. Ed.* **2013**, *52*, 2408-2410; b) G. B. Cserép, A. Herner, P. Kele, *Methods Appl. Fluoresc.* **2015**, *3*, 042001.
- [2] a) M. J. Hangauer, C. R. Bertozzi, *Angew. Chem. Int. Ed.* **2008**, *47*, 2394-2397; b) G. A. Lemieux, C. L. De Graffenried, C. R. Bertozzi, *J. Am. Chem. Soc.* **2003**, *125*, 4708-4709.
- [3] a) F. Friscourt, C. J. Fahrni, G. J. Boons, *J. Am. Chem. Soc.* **2012**, *134*, 18809-18815; b) A. Herner, I. Nikic, M. Kallay, E. A. Lemke, P. Kele, *Org. Biomol. Chem.* **2013**, *11*, 3297-3306; c) C. Le Droumaguet, C. Wang, Q. Wang, *Chem. Soc. Rev.* **2010**, *39*, 1233-1239; d) J. Li, M. Hu, S. Q. Yao, *Org. Lett.* **2009**, *11*, 3008-3011; e) J. Qi, M. S. Han, Y. C. Chang, C. H. Tung, *Bioconjugate Chem.* **2011**, *22*, 1758-1762; f) P. Shieh, M. S. Siegrist, A. J. Cullen, C. R. Bertozzi, *Proc. Natl. Acad. Sci.* **2014**, *111*, 5456-5461; g) K. Sivakumar, F. Xie, B. M. Cash, S. Long, H. N. Barnhill, Q. Wang, *Org. Lett.* **2004**, *6*, 4603-4606; h) Z. Zhou, C. J. Fahmi, *J. Am. Chem. Soc.* **2004**, *126*, 8862-8863; i) O. Demeter, A. Kormos, C. Koehler, G. Mezö, K. Németh, E. Kozma, L. B. Takács, E. A. Lemke, P. Kele, *Bioconjugate Chem.* **2017**, *28*, 1552-1559.
- [4] a) Y. Wang, C. I. Vera, Q. Lin, *Org. Lett.* **2007**, *9*, 4155-4158; b) W. Song, Y. Wang, J. Qu, Q. Lin, *J. Am. Chem. Soc.* **2008**, *130*, 9654-9655; c) W. Song, Y. Wang, J. Qu, M. M. Madden, Q. Lin, *Angew. Chem. Int. Ed.* **2008**, *47*, 2832-2835; d) Z. Yu, L. Y. Ho, Q. Lin, *J. Am. Chem. Soc.* **2011**, *133*, 11912-11915; e) E. Kaya, M. Vrabel, C. Deiml, S. Prill, V. S. Fluxa, T. Carell, *Angew. Chem. Int. Ed.* **2012**, *51*, 4466-4469; f) Z. Yu, T. Y. Ohulchanskyy, P. An, P. N. Prasad, Q. Lin, *J. Am. Chem. Soc.* **2013**, *135*, 16766-16769; g) X. S. Wang, Y. J. Lee, W. R. Liu, *Chem. Commun.* **2014**, *50*, 3176-3179.
- [5] a) N. K. Devaraj, S. Hilderbrand, R. Upadhyay, R. Mazitschek, R. Weissleder, *Angew. Chem. Int. Ed.* **2010**, *49*, 2869-2872; b) J. Yang, J. Seckute, C. M. Cole, N. K. Devaraj, *Angew. Chem. Int. Ed.* **2012**, *51*, 7476-7479; c) L. G. Meimetis, J. C. Carlson, R. J. Giedt, R. H. Kohler, R. Weissleder, *Angew. Chem. Int. Ed.* **2014**, *53*, 7531-7534; d) A. Wieczorek, T. Buckup, R. Wombacher, *Org. Biomol. Chem.* **2014**, *12*, 4177-4185; e) H. Wu, J. Yang, J. Seckute, N. K. Devaraj, *Angew. Chem. Int. Ed.* **2014**, *53*, 5805-5809; f) E. Kozma, O. Demeter, P. Kele, *ChemBioChem* **2017**, *18*, 486-501; g) A. Wieczorek, P. Werther, J. Eüchner, R. Wombacher, *Chem. Sci.* **2017**, *8*, 1506-1510; h) E. Kozma, G. Estrada Girona, G. Pací, E. A. Lemke, P. Kele, *Chem. Commun.* **2017**, *53*, 6696-6699.
- [6] C. P. Ramil, Q. Lin, *Curr. Opin. Chem. Biol.* **2014**, *21*, 89-95.
- [7] X. Shang, X. Song, C. Faller, R. Lai, H. Li, R. Cerny, W. Niu, J. Guo, *Chem. Sci.* **2017**, *8*, 1141-1145.
- [8] A. Vazquez, R. Dzajak, M. Dracinsky, R. Rampmaier, S. J. Siegl, M. Vrabel, *Angew. Chem. Int. Ed.* **2017**, *56*, 1334-1337.
- [9] a) R. D. Bach, *J. Am. Chem. Soc.* **2009**, *131*, 5233-5243; b) J. Dommerholt, S. Schmidt, R. Temming, L. J. Hendriks, F. P. Rutjes, J. C. van Hest, D. J. Lefeber, P. Friedl, F. L. van Delft, *Angew. Chem. Int. Ed.* **2010**, *49*, 9422-9425; c) M. T. Taylor, M. L. Blackman, O. Dmitrenko, J. M. Fox, *J. Am. Chem. Soc.* **2011**, *133*, 9646-9649; d) A. Darko, S. Wallace, O. Dmitrenko, M. M. Machovina, R. A. Mehl, J. W. Chin, J. M. Fox, *Chem. Sci.* **2014**, *5*, 3770-3776.
- [10] J. U. Jeong, B. Tao, I. Sagasser, H. Henniges, K. B. Sharpless, *J. Am. Chem. Soc.* **1998**, *120*, 6844-6845.
- [11] M. Royzen, G. P. Yap, J. M. Fox, *J. Am. Chem. Soc.* **2008**, *130*, 3760-3761.
- [12] S. C. Bergmeier, P. P. Seth, *Tetrahedron Lett.* **1999**, *40*, 6181-6184.
- [13] Y. Ittah, Y. Sasson, I. Shahak, S. Tsaroom, J. Blum, *J. Org. Chem.* **1978**, *43*, 4271-4273.
- [14] A. M. Brouwer, *Pure Appl. Chem.* **2011**, *83*, 2213-2228.
- [15] a) K. A. Horner, N. M. Valette, M. E. Webb, *Chem. Eur. J.* **2015**, *21*, 14376-14381; b) D. N. Kamber, Y. Liang, R. J. Blizzard, F. Liu, R. A. Mehl, K. N. Houk, J. A. Prescher, *J. Am. Chem. Soc.* **2015**, *137*, 8388-8391; c) S. J. Siegl, R. Dzajak, A. Vazquez, R. Pohl, M. Vrabel, *Chem. Sci.* **2017**, *8*, 3593-3598.
- [16] R. Rossin, S. M. van den Bosch, W. Ten Hoeve, M. Carvelli, R. M. Versteegen, J. Lub, M. S. Robillard, *Bioconjugate Chem.* **2013**, *24*, 1210-1217.
- [17] a) E. Basle, N. Joubert, M. Pucheault, *Chem. Biol.* **2010**, *17*, 213-227; b) O. Koniev, A. Wagner, *Chem. Soc. Rev.* **2015**, *44*, 5495-5551.
- [18] S. B. Gunnoo, A. Madder, *ChemBioChem* **2016**, *17*, 529-553.
- [19] A. Baumlova, D. Chalupska, B. Rózycki, M. Jovic, E. Wisniewski, M. Klima, A. Dubankova, D. P. Kloer, R. Nencka, T. Balla, E. Boura, *EMBO Rep.* **2014**, *15*, 1085-1092.
- [20] I. J. Goldstein, R. D. Poretz, in *The Lectins. Properties, Functions, and Applications in Biology and Medicine.*, Vol. 32 (Eds.: I. E. Liener, N. Sharon, I. J. Goldstein), Elsevier, **1986**, pp. 33-247.
- [21] a) M. S. Siegrist, S. Whiteside, J. C. Jewett, A. Aditham, F. Cava, C. R. Bertozzi, *ACS Chem. Biol.* **2013**, *8*, 500-505; b) E. Kuru, S. Tekkam, E. Hall, Y. V. Brun, M. S. Van Nieuwenhze, *Nat. Protoc.* **2015**, *10*, 33-52.

FULL PAPER

Super-reactive dienophiles: Fusion of an aziridine ring to *trans*-cyclooctenes was found to enhance their reactivity while still maintaining good stability under biological conditions. These aza-TCOs lead to the formation of fluorescent products upon reaction with 1,2,4,5-tetrazines which is useful for bioimaging applications.

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