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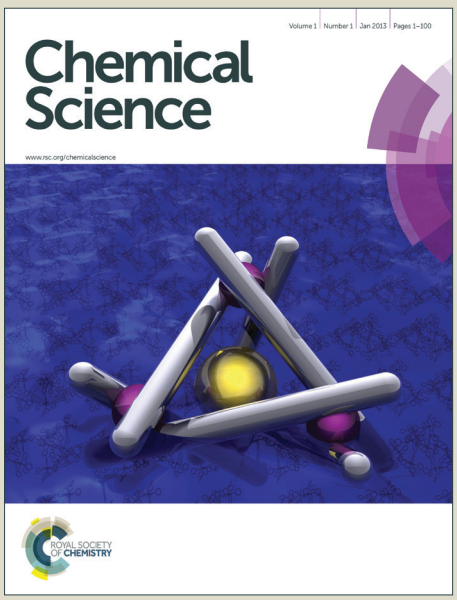

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ARTICLE

Conformationally Strained *trans*-Cyclooctene with Improved Stability and Excellent Reactivity in Tetrazine Ligation

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Computation has guided the design of conformationally-strained dioxolane-fused *trans*-cyclooctene (d-TCO) derivatives that display excellent reactivity in the tetrazine ligation. A water soluble derivative of 3,6-dipyridyl-*s*-tetrazine reacts with d-TCO with a second order rate k_2 366,000 (+/– 15,000) $M^{-1}s^{-1}$ at 25 °C in pure water. Furthermore, d-TCO derivatives can be prepared easily, are accessed through diastereoselective synthesis, and are typically crystalline bench-stable solids that are stable in aqueous solution, blood serum, or in the presence of thiols in buffered solution. GFP with a genetically encoded tetrazine-containing amino acid was site-specifically labelled *in vivo* by a d-TCO derivative. The fastest bioorthogonal reaction reported to date [k_2 3,300,000 (+/– 40,000) $M^{-1}s^{-1}$ in H_2O at 25 °C] is described herein with a cyclopropane-fused *trans*-cyclooctene. d-TCO derivatives display rates within an order of magnitude of these fastest *trans*-cyclooctene reagents, and also display enhanced stability and aqueous solubility.

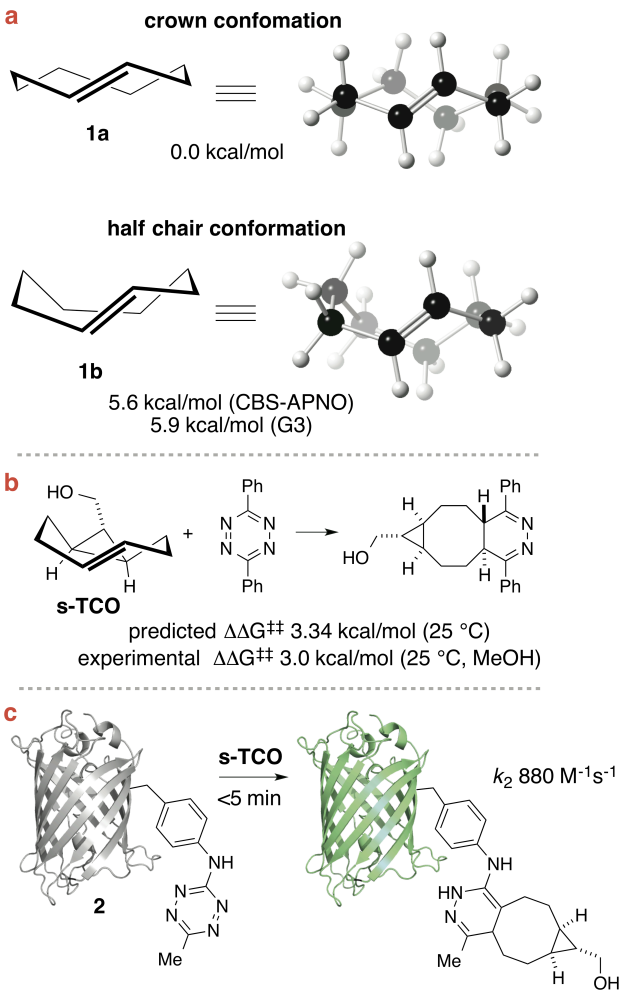
Introduction

The use of strain energy to enable molecular reactivity is a venerable concept in the field of organic synthesis, and has in recent years become an increasingly important tool for enabling bioorthogonal reactions—those transformations that retain selectivity in a biological context.[1-12] In 2004, Bertozzi and co-workers first described the use of strain to accelerate the rate of the bioorthogonal cycloaddition between alkynes and azides[13]— a transformation that otherwise requires transition metal catalysis.[14-16] Subsequently, strained molecules have been employed as reactive cycloaddition partners across a range of applications where efficient bioorthogonal labeling is required.[1, 8, 9, 17-21]

In 2008, our group developed a photochemical flow-method for producing functionalized *trans*-cyclooctenes (TCO's),[22] and subsequently demonstrated that tetrazines combine with *trans*-cyclooctenes with unusually fast rates (k_2 $>10^3$ $M^{-1}s^{-1}$) in bioorthogonal reactions.[23] Contemporaneous with our work on TCO, bioorthogonal reactions of tetrazines with norbornenes[24] and the Reppe Anhydride[25] were described. More recently, cyclopropenes,[26-31] cyclooctynes[32-35] and simple terminal alkenes[36] have been used as dienophiles in bioorthogonal reactions with tetrazines. Each of these dienophiles offers unique advantages. Still, there remains a high level of interest in *trans*-cyclooctene dienophiles due to their high reactivity, and as such *trans*-cyclooctenes have been used in a range of applications including cellular imaging and nuclear medicine.[37-42]

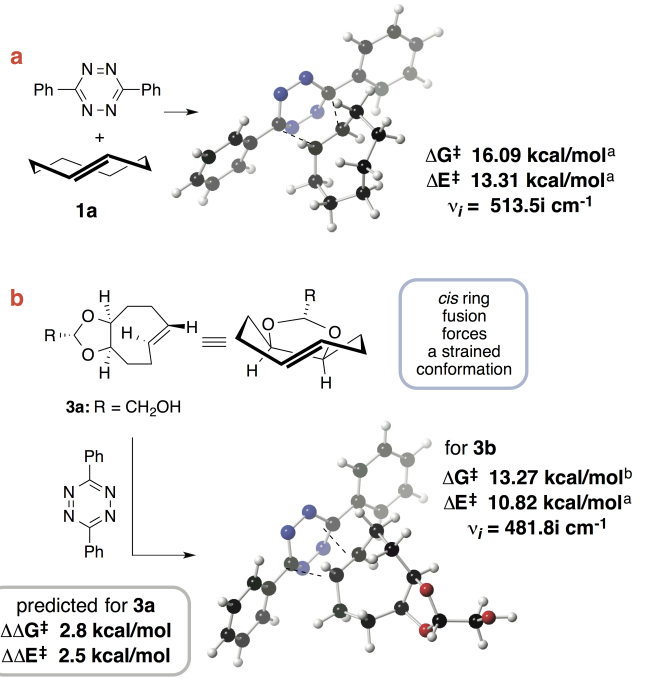
While simple *trans*-cyclooctene derivatives combine with *s*-tetrazines with very fast rates, several situations have arisen where even faster rates are necessary. Reaction rate is always a premium consideration for applications to nuclear medicine due to the low concentrations and short half-lives intrinsic to radiochemistry.[40, 43-51] Fast bioorthogonal reativity is especially important for pretargeted imaging and radioimmunotherapy where the bioconjugation event takes place *in vivo*.[43-48] The development of more highly reactive TCO probes has also proven necessary for applications to intracellular labeling,[35, 52-57] as tetrazines that display the best long term *in vivo* stability also tend to be less reactive in Diels-Alder reactions. For example, it was possible to genetically encode an electron-rich tetrazine-containing amino acid into proteins such as GFP (**2**,

Scheme 1c); however subsequent labeling by 5-hydroxy-*trans*-cyclooctene was limited by sluggish reactivity.[58]



Scheme 1. (a) Conformation analysis of ground states show that the half-chair conformation of TCO is higher in energy by 5.6–5.9 kcal/mol relative to the lowest energy crown conformation. (b) As predicted computationally, the conformationally constrained s-TCO (**1b**) combines with 3,6-diphenyl-*s*-tetrazine in with a rate that is 160 times faster than *trans*-cyclooctene itself. (c) For GFP with a genetically encoded tetrazine-containing amino acid, s-TCO enables rapid fluorogenic labeling inside living bacteria.

Computation has emerged as an important tool for understanding and predicting the reactivity of bioorthogonal reaction partners.[29, 34, 59-63] In parallel with studies by van Delft on the strained cycloalkyne, bicyclo[6.1.0]non-4-yn-9-ylmethanol,[18] we described how the reactivity of TCO could be increased through the introduction of conformational strain. (Scheme 1a).[62] *Ab initio* calculations predicted the lowest energy 'crown' conformation (**1a**) of *trans*-cyclooctene to be 5.6–5.9 kcal/mol lower in energy than the 'half-chair' conformation (**1b**). Recognizing that *trans*-cyclooctenes with *cis*-ring fusions would be forced to adopt strained conformations similar to **1b** (Scheme 1b), [60, 62] computation was used to design a strained *trans*-cyclooctene (termed "s-TCO") with a *cis*-fused cyclopropane ring that enforces a



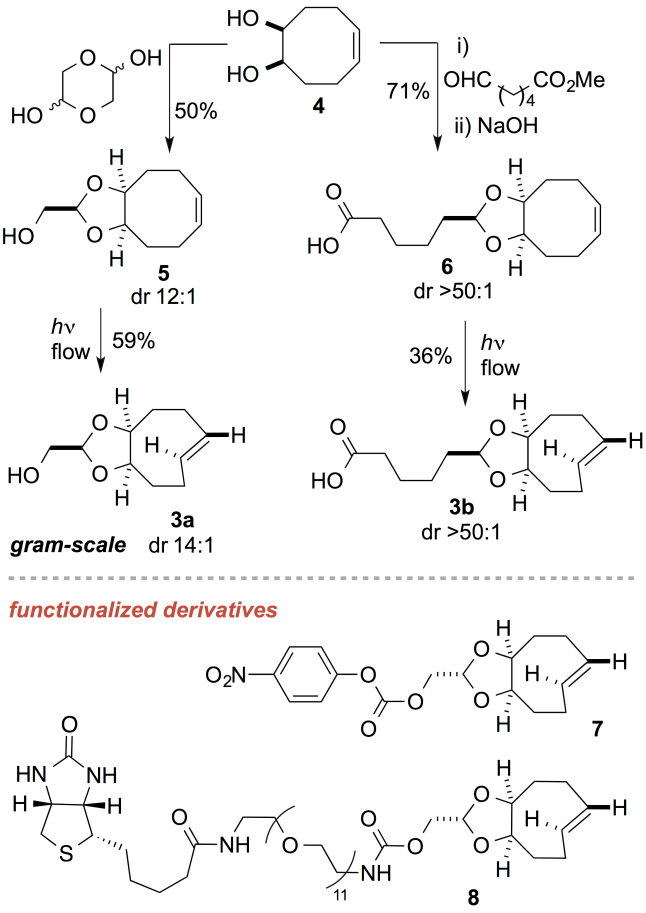
Scheme 2 Transition state calculations (level) for 3,6-diphenyl-*s*-tetrazine with (a) *trans*-cyclooctene and (b) dioxolane-fused *trans*-cyclooctene **3a**. The *cis*-ring fusion of **3a** confines the 8-membered ring to a strained half-chair conformation, resulting in a significantly lower transition state barrier for the cycloaddition.

highly strained half-chair conformation for the eight-membered ring. Transition state calculations with 3,6-diphenyl-*s*-tetrazine predicted that s-TCO would react much faster than the crown conformation of TCO (**1a**) ($\Delta\Delta G^{‡‡}$ = 3.34 kcal/mol at 25 °C). Experimentally, s-TCO combined with 3,6-diphenyl-*s*-tetrazine in MeOH at 25 °C with a rate of 3100 M^{-1}s^{-1}— 160 times faster than *trans*-cyclooctene (Scheme 1b). The experimentally measured $\Delta\Delta G^{‡}$ (3.0 kcal/mol) correlated closely with this predicted value. With more reactive tetrazines under aqueous conditions, rates can exceed 10^6 M^{-1}s^{-1}, making s-TCO the most reactive dienophile reported to date for bioorthogonal applications.[44, 62, 64] With GFP derivative **2**, fluorogenic labeling by s-TCO takes place rapidly inside living bacteria (Scheme 1c).[58]

While the reactivity of s-TCO in tetrazine ligation is desirable, there are some limitations that also emerge from its high reactivity. As we have discussed previously, s-TCO was found to isomerize in the presence of high thiol concentrations (30 mM), presumably via a free radical mediated pathway.[62] Furthermore, many s-TCO derivatives tend to be only moderately stable to prolonged storage, and must be kept in cold solution to avoid polymerization and isomerization.[65]

Additionally, s-TCO is hydrophobic, and its synthesis is not stereoselective, necessitating chromatographic separation of *syn*- and *anti*- product diastereomers.

For the reasons outlined above, we considered the design of new conformationally strained *trans*-cyclooctene derivatives that would maintain high reactivity toward *s*-tetrazines but display enhanced stability and be more easily prepared. To this end, we considered *cis*-dioxolane-fused *trans*-cyclooctenes (d-TCO) derivatives of structure **3** (Schemes 2 and 3). It was expected that the *cis*-fused ring would enforce a strained half-chair conformation of the cyclooctene, imparting high reactivity toward tetrazines. However, it was also reasoned that the alkene of d-TCO would be less electron rich (and hence more stable) than that of s-TCO as a result of the inductively electron withdrawing oxygens. The hope for d-TCO was that a modest compromise in reactivity toward tetrazines would be compensated by enhanced stability. We also projected that the dioxolane functionality would impart improved aqueous solubility for d-TCO.



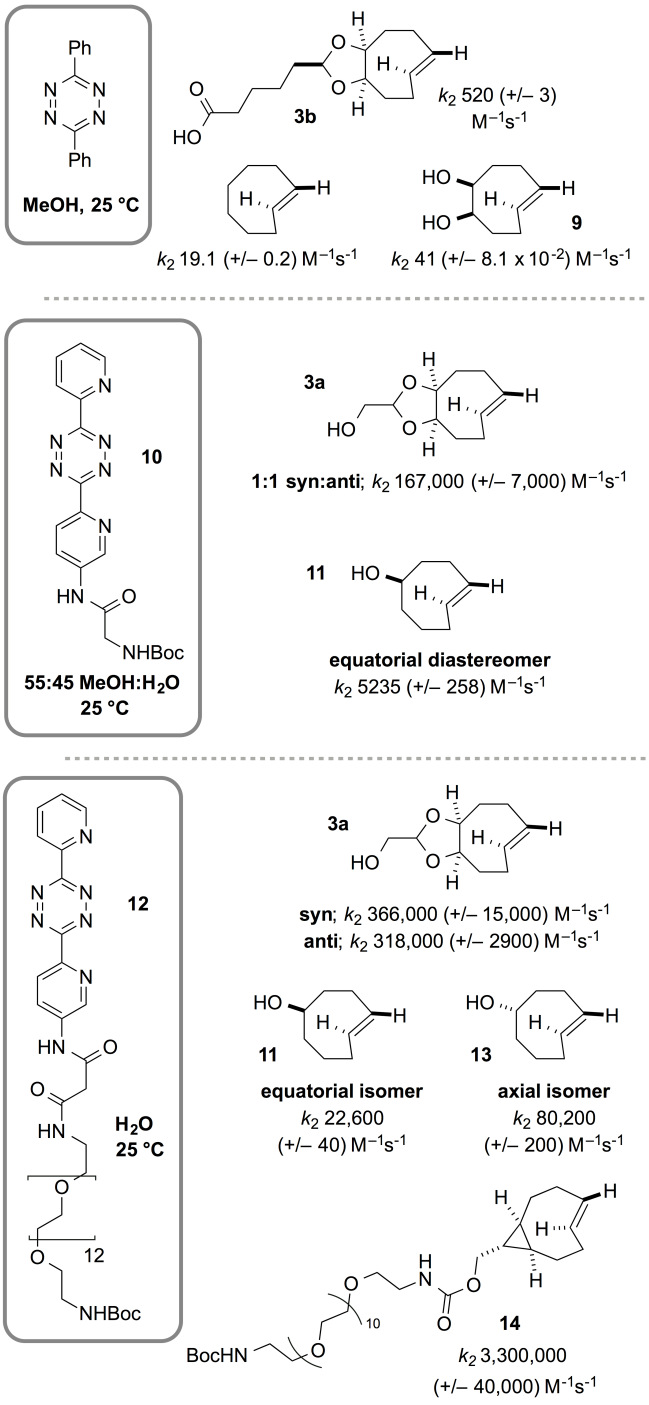
Scheme 3. Synthesis of d-TCO derivatives

Our studies began with a computational comparison of the reactions of 3,6-diphenyl-*s*-tetrazine with the crown conformation of *trans*-cyclooctene (**1a**) and that of d-TCO derivative **3a**, which is locked in the half-chair conformation. Transition state calculations in the gas phase between **1a** and 3,6-diphenyl-*s*-tetrazine at the M06L/6(311)+G(d,p) level were described previously, and shown to proceed with a barrier of $\Delta E^{\ddagger}$ = 13.31 kcal/mol and $\Delta G^{\ddagger}$ = 16.09 kcal/mol (Scheme 2a). By comparison, the computed reaction of 3,6-diphenyl-*s*-tetrazine and d-TCO **3a** proceeds with a significantly lower barrier ($\Delta E^{\ddagger}$ = 10.82 kcal/mol and $\Delta G^{\ddagger}$ = 13.27 kcal/mol, Scheme 2b). These calculations predict that the reactions of d-TCO would be considerably faster than that of the parent TCO **1**.

Encouraged by this computational prediction, we prepared derivatives of **3a** and **3b** as shown in Scheme 3. The diol **4** can be prepared in multigram quantity from 1,5-cyclooctadiene by Upjohn dihydroxylation.[66] Diol **4** combined with glycolaldehyde dimer at room temperature to give **5** as a 12:1 mixture in favor of the *syn*-diastereomer. When dioxolane formation was conducted at 80 °C, both *syn*- and *anti*-diasteromers of **5** were produced in a 1:1 ratio. This kinetic preference for the *syn*-diastereomer is known for cyclic acetals,[67-69] and has been ascribed to the relief of 1,3 allylic strain[70] in the cyclization of the oxonium intermediate. Photoisomerization using the closed-loop flow reactor[22, 71] developed in our labs gave d-TCO **3a** in 59% yield. Likewise, acetal **6** was prepared from adipic semialdehyde methyl ester, and photoisomerized to give d-TCO **3b** in 36% yield, predominantly as the *syn*-diastereomer (>50:1). The brief and diastereoselective nature of the syntheses of d-TCO derivatives greatly facilitates material throughput, as illustrated by a gram-scale preparation of d-TCO **3a**. A conjugatable derivative of **3a** could be prepared by treatment with *p*-nitrophenylchloroformate to give the activated carbonate **7** in 56% yield, and can be readily conjugated to amines under standard conditions using Hunig's base, as demonstrated by the synthesis of biotin-mini-PEG derivative **8** (Scheme 3).

Rate constants for the reaction of d-TCO derivatives with tetrazine derivatives were measured under several conditions (Scheme 4). Diphenyl-*s*-tetrazine with d-TCO **3b** in MeOH (25 °C) reacts with a rate of 520 (+/– 3) $M^{-1}s^{-1}$. The same tetrazine was found to react with the parent *trans*-cyclooctene with a rate of 19.1 (+/– 1) $M^{-1}s^{-1}$.[62] The observed 27-fold rate enhancement ($\Delta\Delta G^{\ddagger}$ = 2.0 kcal/mol) for d-TCO is in good alignment with the computationally predicted value ($\Delta\Delta G^{\ddagger}$ = 2.8 kcal/mol; ($\Delta\Delta E^{\ddagger}$ = 2.5 kcal/mol) described above. To ensure that the rate acceleration for d-TCO was due to the ring fusion, and not due to the electronic influence of the hydroxyl groups, we compared the rate of diphenyl-*s*-tetrazine with diol **9**, which was prepared by photoisomerizing diol **4**. The observed rate constant k_2 = 41 (+/– 8.1 x 10^{-2}) $M^{-1}s^{-1}$ was significantly slower than the rate of the d-TCO **3b**. This observation provides strong

evidence that the conformational strain of the 8-membered ring is indeed responsible for the observed rate acceleration.



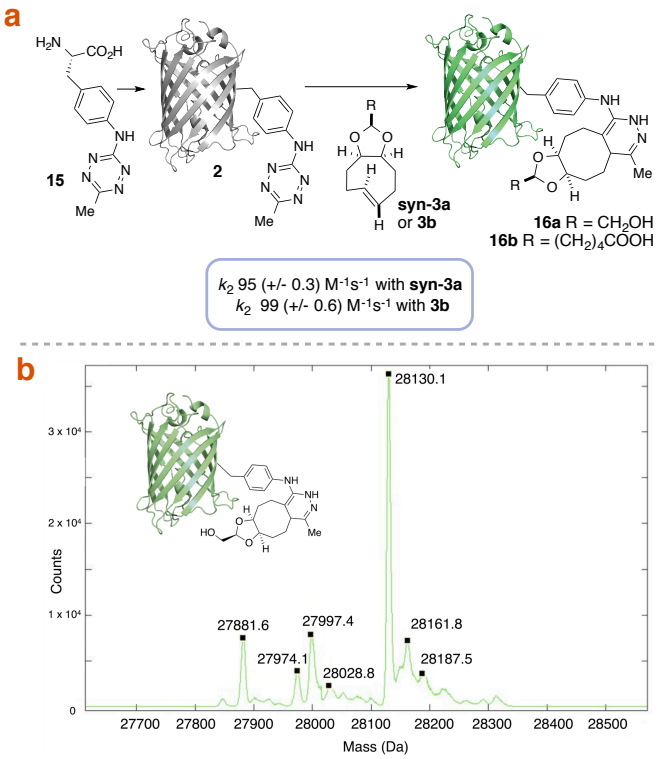
Scheme 4 Comparison of second order rate constants

Under aqueous conditions, reactions are significantly faster due to acceleration by the hydrophobic effect, and as such a stopped-flow technique was used to measure rate constants. Previously, a rate of k_2 5235 (+/– 258) $M^{-1}s^{-1}$ was measured for the equatorial diastereomer of 5-hydroxy-*trans*-cyclooctene (**11**) with tetrazine **10** at 25 °C in 45:55 H_2O:MeOH.[35] Under similar conditions, d-TCO **3a** reacts with tetrazine **10** with a rate k_2 167,000 (+/– 7000) $M^{-1}s^{-1}$. As expected, the fastest rates are measured under purely aqueous conditions. Improved aqueous solubility of the d-TCO derivates made kinetic measurements in aqueous conditions possible. At 25 °C in water, the *syn*-diastereomer of d-TCO (*syn*-**3a**) combines with the freely water soluble derivative **12** with a rate k_2 366,000 (+/– 15,000) $M^{-1}s^{-1}$. The *anti*-diastereomer (*anti*-**3a**) combines with a rate k_2 318,000 (+/– 2900) $M^{-1}s^{-1}$. In a recent study, Robillard reported that the axial diastereomer of 5-hydroxy-*trans*-cyclooctene is more reactive than the equatorial diastereomer.[44] Under similar conditions, d-TCO derivatives **3a** and **3b** combine with **12** with a rate that is significantly faster than either diastereomer of 5-hydroxy-*trans*-cyclooctene: the axial diastereomer **13** reacts with k_2 80,200 (+/– 200) $M^{-1}s^{-1}$, and the equatorial diastereomer **11** reacts with k_2 22,600 (+/– 40) $M^{-1}s^{-1}$. As expected, faster rates were observed with s-TCO derivatives. Water soluble s-TCO **14** reacts with **12** at a rate of k_2 3,300,000 (+/– 40,000) $M^{-1}s^{-1}$, which to the best of our knowledge, makes this the fastest conjugation rate measurement to date,

While d-TCO derivatives are extremely reactive toward tetrazines, they also display excellent stability. While s-TCO derivatives can be handled as neat materials, for storage we find it is necessary to keep most[72] s-TCO compounds in solution at freezer temperatures. By contrast, the stability of d-TCO derivatives are greatly improved. Both **3a** and **3b** are crystalline solids that can be stored uneventfully on the bench and are soluble (log*P* = 0.94 for *syn*-**3a**) and stable in aqueous solutions. As a safeguard for longterm storage, these compounds are kept as solids in the freezer (–20 °C), where they are stable for at least 14 months. In phosphate buffered D_2O (pD 7.4), no isomerization or decomposition of *syn*-**3a** (20 mM) was noted after 14 days. In concentrated solution (0.5 M in CD_3OD), *syn*-**3a** and *anti*-**3a** isomerizes very slowly at room temperature, with 5% isomerization after three days. For an 5 mM solution of *syn*-**3a** in human serum at room temperature, no isomerization or decomposition was observed after 24 hours, and the compound was >97% *trans* after 4 days. In the presence of rabbit reticulocyte lysate (10%) in buffered D_2O (pD 7.4), compound *syn*-**3a** isomerized slowly with 12%, 22% and 41% isomerization after 10, 24 and 96 hours, respectively. The isomerization of *syn*-**3a** in the presence of reticulocyte lysate is possibly a thiol-radical promoted process, as both glutathione and mercaptoethanol were found to promote d-TCO isomerization. Thus, mercaptoethanol (30 mM) promoted isomerization of *syn*-**3a** slowly at pH 6.8 (44% isomerization after 48 hours), and more rapidly at pH 7.4 (43% isomerization after 5 hours). Previously, we noted that s-TCO (Scheme 1b) is susceptible toward isomerization in the presence of thiols,[62] presumbably by a radical mediated process. Robillard has also noted the higher propensity of s-TCO to isomerize *in vivo*.[73] While d-TCO appears more robust than s-TCO toward thiol-

promoted isomerization, both of these strained TCOs are less resilient than conformationally unstrained TCOs such as **9**.[74, 75] Full details of isomerization studies are provided in the Supplementary Information.

In a prior study, it was shown that the tetrazine derived amino acid **15** could be site-specifically encoded into proteins such as GFP (**2**) by an evolved *Methanococcus jannaschii* (*Mj*) tyrosyl-tRNA synthetase/tRNA$_{(CUA)}$ pair (Scheme 5).[58] When genetically encoded into GFP, this amino acid could be tagged *in vivo* by reactive s-TCO derivatives. Given the advantages of d-TCO in terms of synthesis and stability, we investigated the ability of this dienophile to label protein **2**. As described previously, the tetrazine of **2** quenches the fluorescence of the attached GFP. After tetrazine ligation the fluorescence is restored, thus providing a convenient handle for monitoring the reaction. With d-TCO *syn*-**3a** and **3b**, *in vitro* labeling takes place rapidly with a rate constant of k_2 95 (+/– 0.3) $M^{-1}s^{-1}$ and k_2 99 (+/– 0.6) $M^{-1}s^{-1}$ to give conjugates **16a** and **16b**, respectively (Scheme 5a). As expected, labeling by the d-TCOs were much faster than labeling by 5-hydroxy-*trans*-cyclooctene (**13**), and within an order of magnitude of the more reactive s-TCO (Scheme 1b). *In vivo* labeling of **2** with d-TCO *syn*-**3a** was confirmed by mass spectroscopy**.** Thus, *E. coli* containing expressed **2** were washed and incubated with *syn*-**3a** for 2 hrs minutes in PBS buffer at room temperature. After washing the cells to remove excess *syn*-**3a**, purification was carried out using His-tagged protein affinity chromatography. Q-TOF MS of the purified protein confirmed the molecular mass of **16a**, thereby successfully demonstrating site-specific labelling *in vivo* (Scheme 5b).



Scheme 5 Characterization of tetrazine d-TCO reaction on GFP (a) *in vitro* labelling rates of d-TCOs with tetrazine unnatural amino acid derived GFP **2** (b) Q-TOF mass spectrum from the *in vivo* labelling of *syn*-**3a** and **2.** Near quantitative conversion of **2** with ***syn*-3a** demonstrating specific conversion to **16a** (expected 28130 Da; observed 28130 ± 1 Da). Each sample did show a small peak at -131 ±1 Da indicating minor amounts of peptidase-based removal of N-terminal methionines and +22 sodium adducts.

Conclusions

Computation was used to design of conformationally-strained dioxolane-fused *trans*-cyclooctene (d-TCO) derivatives that also display excellent reactivity in the tetrazine ligation. The *cis*-dioxolane ring introduces conformational strain and improved aqueous solubility to the cyclooctene derivatives. A water soluble derivative of 3,6-dipyridyl-*s*-tetrazine reacts with d-TCO with a second order rate k_2 366,000 (+/– 15,000) $M^{-1}s^{-1}$ at 25 °C in pure water. d-TCO derivatives can be prepared easily through diastereoselective synthesis, and are typically crystalline bench-stable solids that are stable in aqueous solution, blood serum, or in the presence thiols in buffered solution. GFP with a genetically encoded tetrazine-containing amino acid was site specifically labeled *in vivo* by a d-TCO derivative. This combination of reactivity and stability make d-TCOs attractive for bioorthogonal applications where fast rates and stability are of importance.

Acknowledgements

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Notes and references

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† Electronic Supplementary Information (ESI) available: Full experimental details, copies of spectral data, kinetic plots, and computational details. See DOI: 10.1039/b000000x/

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75. At pH 7.4 in D_2O in the presence of glutathione (10 mM), only 12% isomerization of compound 9 (10 mM) was observed after 8 hours. A similar experiment with *syn*–**3a** showed 53% isomerization after 4 hours, and 92% isomerization after 8 hours.

TOC graphic