

Design and Synthesis of Highly Reactive Dienophiles for the Tetrazine–*trans*-Cyclooctene Ligation

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Supporting Information

ABSTRACT: Computation was used to design a *trans*-cyclooctene derivative that displays enhanced reactivity in the tetrazine–*trans*-cyclooctene ligation. The optimized derivative is an (*E*)-bicyclo[6.1.0]non-4-ene with a *cis*-ring fusion, in which the eight-membered ring is forced to adopt a highly strained ‘half-chair’ conformation. Toward 3,6-dipyridyl-*s*-tetrazine in MeOH at 25 °C, the strained derivative is 19 and 27 times more reactive than the parent *trans*-cyclooctene and 4*E*-cyclooct-4-enol, respectively. Toward 3,6-diphenyl-*s*-tetrazine in MeOH at 25 °C, the strained derivative is 160 times more reactive than the parent *trans*-cyclooctene.

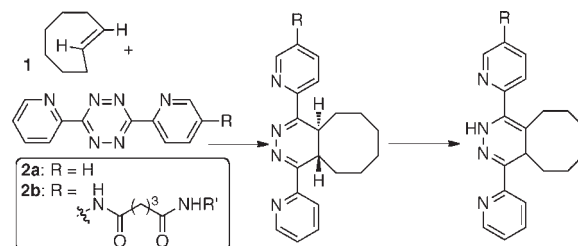


Figure 1. Tetrazine–*trans*-cyclooctene ligation.

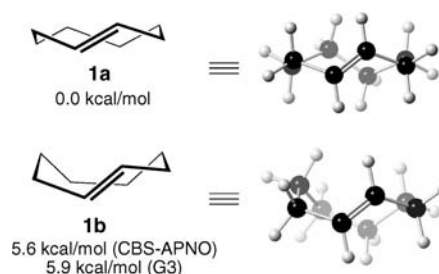


Figure 2. Calculated relative energies (0 K) for two conformations of *trans*-cyclooctene at the CBS-APNO and G3 levels of theory.

Reactions which proceed efficiently in the presence of biological functionality have broad reaching applications that span chemistry, biology, and materials science.¹ The Cu-catalyzed azide–alkyne cycloaddition, the archetypical ‘click reaction’, finds broad use and application² but can be limited by the cytotoxicity of Cu.³ Accordingly, a number of bioorthogonal methodologies have been advanced that proceed efficiently without the need for catalysis.^{3–5} In 2004, Bertozzi made a seminal advance through the development of a strain-assisted reaction between cyclooctyne and organic azides.⁴ This methodology has found significant applications as a tool for *in vivo* labeling,^{4,5} and efforts to improve reaction rates and substrate accessibility have been under continual development.^{4,5}

Recently, our group introduced the tetrazine–*trans*-cyclooctene ligation (Figure 1), a bioorthogonal reaction with unusually fast rates that is based on the cycloaddition of tetrazines and *trans*-cyclooctene.⁶ The development of this bioorthogonal reaction was enabled by a photochemical flow-reaction developed by our group for the efficient preparation of *trans*-cyclooctenes.⁷ A variety of *s*-tetrazine derivatives were known to react with strained alkenes,⁸ and we have found that 3,6-diaryl-*s*-tetrazines offer an excellent combination of fast reaction rates and stability for both the starting material and conjugation products.⁷ Thus, 3,6-di(2-pyridyl)-*s*-tetrazine (**2a**) reacts with *trans*-cyclooctene (**1**) in 9:1 MeOH/water with $k_2 = 2000 \text{ M}^{-1} \text{ s}^{-1}$.⁸ Amido substituted 3,6-di(2-pyridyl)-*s*-tetrazines (**2b**) are readily synthesized⁶ and display excellent stability toward water and biological nucleophiles.⁹ Derivatives of **2b** ($R' = \text{DOTA}^{10}$ or cyclic RGD peptide¹¹) have been used by Robillard¹⁰ and our group¹¹ for radiochemical imaging and shown to participate in the tetrazine–*trans*-cyclooctene ligation with excellent rates. After we described the use of *trans*-cyclooctene for tetrazine

ligations, the groups of Hilderbrand and Weissleder¹² and Pipkorn and Braun¹³ described ligations between tetrazines and less reactive strained alkenes. Yet, the use of *trans*-cyclooctene derivatives is necessary for fast rates of reactivity. Recently, the tetrazine–*trans*-cyclooctene ligation has been used in applications by a number of groups,^{10,14} including our own.¹¹

The lowest energy, ‘crown’ conformation of *trans*-cyclooctene¹⁵ (**1a**, Figure 2) bears structural analogy to the chair conformation of cyclohexane, as the methylenes in both conformations display an alternating arrangement of axial and equatorial hydrogens. Alternate conformations of *trans*-cyclooctene are significantly higher in energy.^{15a,b} In a recent *ab initio* study, Bach calculated the ‘half chair’ conformation (**1b**, Figure 2) to be 5.9 kcal/mol higher in energy than the crown conformation **1a**.^{15a} Calculations at the CBS-APNO level of theory (see Supporting Information) are in close agreement and find **1b** to be 5.6 kcal/mol higher in energy than **1a**.

We speculated that the increase in strain energy associated with noncrown conformations of *trans*-cyclooctene could be used to accelerate the reactivity toward tetrazines. Previously,

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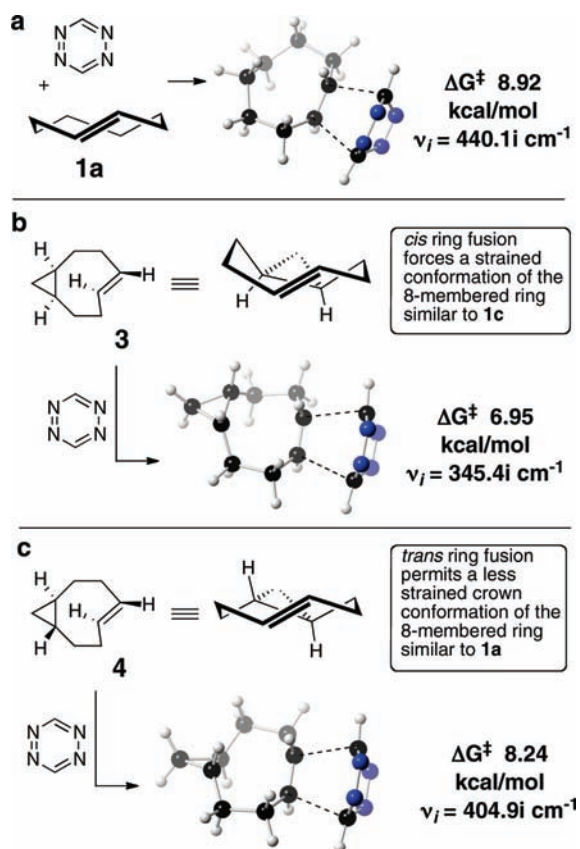


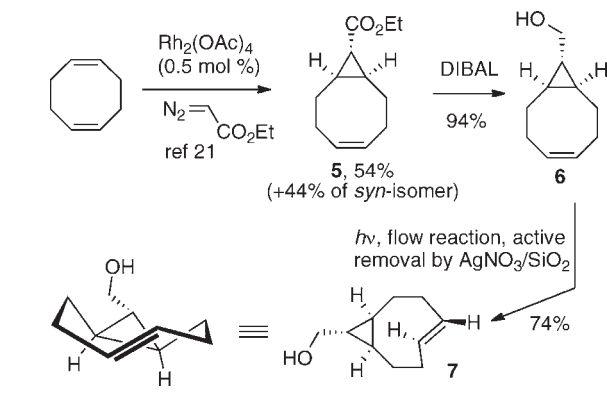
Figure 3. M06L/6-311+G(d,p)-optimized transition structures for the Diels–Alder reaction of *s*-tetrazine with the crown conformer of *trans*-cyclooctene (a), the *cis*-ring fused bicyclo[6.1.0]non-4-ene **3** (b), and the *trans*-ring fused bicyclo[6.1.0]non-4-ene **4** (c). The barrier (8.24 kcal/mol) for the reaction of **4** with *s*-tetrazine is 1.29 kcal/mol higher than the analogous reaction of **3**.

dienophiles for the tetrazine–*trans*-cyclooctene ligation have been derived from cyclooct-4-enol,⁷ a derivative of which was shown to adopt the crown conformation in a crystal structure.⁷ We recognized that the eight-membered ring of bicyclo[6.1.0]non-4-ene derivative **3** (Figure 3b), a *trans*-cyclooctene annealed to cyclopropane with a *cis* ring fusion, would be forced to adopt a strained conformation similar to that of **1b** (Figure 2).¹⁶ Computation was used to predict whether the added strain in **3** would manifest in faster reactions with tetrazines.

Transition state calculations in the gas phase for the inverse electron demand Diels–Alder reaction between *s*-tetrazine and *trans*-cyclooctene derivatives were studied by us at the M06L/6(311)+G(d,p) level.^{17,18} The reaction between *trans*-cyclooctene in the crown conformation (**1a**) and *s*-tetrazine proceeded with a barrier of $\Delta G^\ddagger = 8.92$ kcal/mol (Figure 3a). By comparison, the reaction of *s*-tetrazine and *cis*-fused bicyclo[6.1.0]non-4-ene **3** proceeded with a significantly lower barrier of $\Delta G^\ddagger = 6.95$ kcal/mol (Figure 3b). These barriers are consistent with those that have been calculated for other Diels–Alder reactions that proceed with fast rate constants.¹⁹ These calculations predict that the reaction of *s*-tetrazine with **3** would be 29 times faster than the reaction with **1a**.²⁰

We also computed the barrier for the reaction between *s*-tetrazine and *trans*-fused bicyclo[6.1.0]non-4-ene **4**. Because **4** bears a

Scheme 1. Synthesis of a Highly Reactive Dienophile



trans-ring fusion, the eight-membered ring adopts a crown conformation (similar to **1a**) in its minimized conformation (Figure 3c). The barrier for the reaction between **4** and *s*-tetrazine, $\Delta G^\ddagger = 8.24$ kcal/mol, is similar to that for **1a** and significantly higher than that for **3**. Compounds **3** and **4** are diastereomers and the cyclopropyl moiety is distant from the tetrazine in each transition state. We therefore conclude that the low barrier computed for the reaction of **3** with *s*-tetrazine is attributable to the higher strain of the eight-membered ring.

Based on these calculations, we sought to prepare compound **7** (Scheme 1). Thus, a Rh-catalyzed reaction of ethyl diazoacetate in neat ^{21c} 1,5-cyclooctadiene gave **5** in 54% yield (along with 44% of the separable *syn*-isomer).²¹ DIBAL reduction of **5** gave the known alcohol **6**.^{21a,22} The flow-chemistry method developed in our laboratories was used to photoisomerize **6** to *trans*-isomer **7** in 74% yield.⁷

During the completion of our studies, van Delft et al. elegantly demonstrated that cyclooctyne-based bioconjugations can be accelerated through fusion of a cyclopropane ring.^{21a} This group reported the synthesis of **6** and readily converted it to the corresponding cyclooctyne derivative for bioorthogonal labeling and cell imaging using **3** + **2** cycloaddition strategies.^{21a} The rates of these conjugations were as high as $1.66 \text{ M}^{-1} \text{ s}^{-1}$ for nitrone cycloadditions.

Compound **7** combines with **2a** to give product **8** in >95% yield by ¹H NMR analysis (Scheme 2). As expected,^{6,14,23} the initially formed 4,5-dihydropyrazine derivative **8** slowly isomerizes in the presence of water to the 1,4-dihydropyrazine derivative **8b** via the amination intermediates **8a**.²⁴

The rate of the reaction between **7** and **2a** was studied. As the reaction was too rapid for reliable rate determination by UV–vis kinetics, we determined the relative rate by ¹H NMR through a competition experiment with *trans*-cyclooctene at 25 °C. NMR analysis was conducted immediately upon mixing, and product mixtures were analyzed for the formation of conjugated 4,5-dihydropyrazine products **8** and **9** (Figure 4). Thus, competition of **7** (10 equiv) and **1** (10 equiv) with **2a** (6.5 mM) in CD₃OD gave a 19:1 ratio of **8**:**9**. As the rate of the reaction between **1** and **2a** had been previously measured to be $k_2 = 1140 \text{ M}^{-1} \text{ s}^{-1}$ (± 40) in MeOH, these NMR experiments show the rate of reaction between **7** and **2a** to be $k_2 = 22\,000 \text{ M}^{-1} \text{ s}^{-1}$ (± 2000). As inverse-demand Diels–Alder reactions of tetrazines show significant accelerations due to the hydrophobic effect,^{6,25} it is possible that rates may be even faster in aqueous solvents.²⁶

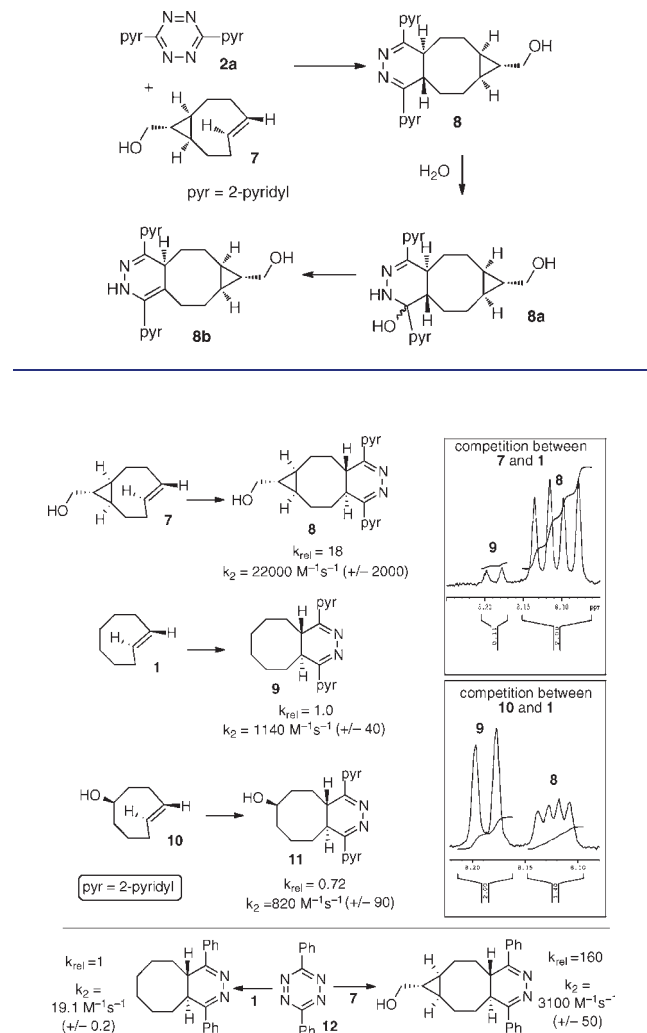
Scheme 2. Reaction of *trans*-Cyclooctene 7 with 2a

Figure 4. Relative rates of reactions with 3,6-diaryltetrazines (**2a**) in CD_3OD at 25°C . NMR spectra (400 MHz, CD_3OD) of competition experiments are shown in the insets.

In prior studies on the tetrazine–*trans*-cyclooctene ligation, functionalized derivatives of 4*E*-cyclooct-4-enol (**10**) have been utilized.^{6,10,13,14} In a competition experiment against *trans*-cyclooctene (**1**), **9** and **11** were formed in a 1.0:0.72 ratio. Based on these relative rates, **10** reacts in methanol with a rate of $820 \text{ M}^{-1} \text{ s}^{-1}$ (± 90) and a relative rate that is 27 times slower than **7**.^{27a}

The reaction rates of 3,6-diphenyl-*s*-tetrazine (**12**) and cyclooctenes **1** and **7** were directly measured by UV–vis spectroscopy. In MeOH at 25°C , a large rate difference was observed, as **12** reacted with **7** 160 times faster than did **1**. Thus, **1** reacted with a rate of $19.1 (\pm 0.2) \text{ M}^{-1} \text{ s}^{-1}$, whereas **7** reacted with a rate of $3100 (\pm 50) \text{ M}^{-1} \text{ s}^{-1}$. For the reaction of **1** and **12**, Eyring analysis showed ΔH^\ddagger to be $5.41 (\pm 0.7) \text{ kcal/mol}$, ΔS^\ddagger to be $-33.6 (\pm 2.3) \text{ e.u.}$, and ΔG^\ddagger to be $15.4 (\pm 0.9) \text{ kcal/mol}$. Based on the relative rate data at 25°C , in MeOH, ΔG^\ddagger for the reaction of **7** and **12** was calculated to be $12.4 (\pm 0.9) \text{ kcal/mol}$ at 25°C in MeOH. In gas phase computations at the M06L/6-311+G(d,p) level of theory at 25°C the experimental $\Delta\Delta G^\ddagger$ (3.0 kcal/mol) for **7** vs **1** correlated closely with the calculated $\Delta\Delta G^\ddagger$ (3.34 kcal/mol) for **3** vs **1a**.^{27b}

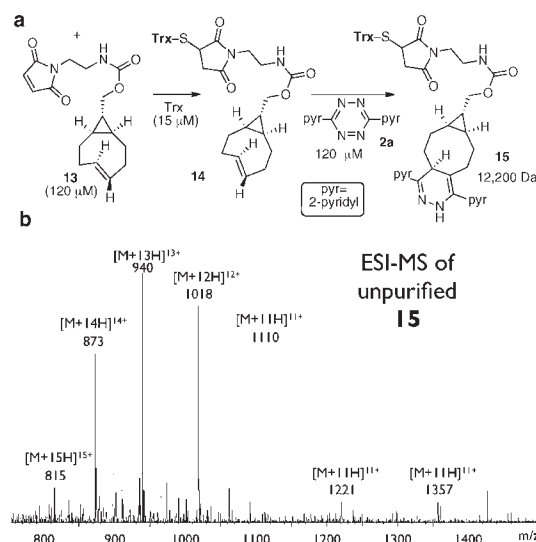


Figure 5. (a) Preparation of a thioredoxin–*trans*-cyclooctene conjugate **14**, and ligation with **2a** to give adduct **15**. (b) Analysis of **15** within 5 min of combination of **14** and **2a**.

In addition to excellent reactivity, **7** also displays excellent stability; it shows no degradation in water or human serum after 24 h. Compound **7** (30 mM) also shows no decomposition upon exposure to 30 mM *n*-butylamine in CD_3OD solvent for 24 h or to 5 mM ethanethiol in CD_3OD for 12 h²⁸. Treatment with 4-nitrophenylchloroformate^{21a,29} gave a carbonate which combined with *N*-(2-aminoethyl)maleimide to give **13** (Figure 5a). As shown in Figure 5a, the reduced form³⁰ of the 11.7 kDa protein thioredoxin (Trx, $15 \mu\text{M}$) could be derivatized with an excess ($120 \mu\text{M}$) of maleimide **13** to give adduct **14**. Subsequent reaction with **2a** ($120 \mu\text{M}$) was rapid, and the crude ESI-MS indicated that the formation of adduct **15** was completed as quickly as we were able to take a measurement (<5 min) (Figure 5b). By contrast, Trx derivatized by a *cis*-cyclooctene does not react with **2a**.⁶

In summary, computation was used to design a *trans*-cyclooctene derivative with enhanced reactivity in the tetrazine–*trans*-cyclooctene ligation. The strained *trans*-cyclooctene derivative not only displays enhanced reactivity but also can be easily derivatized, and bioconjugation to the protein thioredoxin has been demonstrated.

■ ASSOCIATED CONTENT

S Supporting Information. NMR spectra, experimental, kinetic and computational details are provided. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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- (26) We attempted to measure the rate of the reaction between **7** and **2a** in 9:1 MeOH/water, but the rate was too fast to measure accurately by UV–vis spectroscopy. Compound **12** is too poorly soluble in aqueous solvent mixtures for direct rate measurements.
- (27) (a) In a direct competition between **7** and **10** for **2a**, the amount of **11** was below the reliable ¹H NMR detection limit (<5%). (b) The barriers for the gas phase reaction between **1a** and **12** were $\Delta H^\ddagger = 12.92$ kcal/mol and $\Delta G^\ddagger = 16.09$ kcal/mol at 25 °C. The calculated barriers for the reaction of **12** and **3** were significantly lower: $\Delta H^\ddagger = 9.59$ kcal/mol and $\Delta G^\ddagger = 12.74$ kcal/mol.
- (28) At a high concentration of ethanethiol (30 mM) in MeOH, we observed isomerization of **7** (30 mM) to **6**: 0% isomerization after 2 h, 25% after 2.5 h, and 58% after 3.5 h. Because the transformation of **6** to **7** has a long induction time and does not follow second-order behavior, we suspect that the conversion of **6** to **7** may be a radical catalyzed at high thiol concentrations. For a review of radical processes that involve low concentrations of thiol-based radicals, see: Winterbourn, C.; Metodiowa, D. *Free Radical Biol. Med.* **1999**, *27*, 322.
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- (30) Trx (15 μM) was reduced with tri(3-hydroxypropyl)phosphine (THP, 1 mM) and combined with **12** (100 μM) in acetate buffer (pH 6). As THP also reduces maleimide functionalities, an excess of **12** was required.