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

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Abstract

Computation was used to design a *trans*-cyclooctene derivative that displays enhanced reactivity in the tetrazine-*trans*-cyclooctene ligation. The optimized derivative is a (*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.

Bioorthogonal reactions— 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 application,² 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 version of this transformation based upon the 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 (Fig 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 synthesized,⁶ 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 have been 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

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Supporting Information Available: Full experimental details, ¹H and ¹³C-NMR spectra, kinetic and computational details are provided. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Pipkorn and Braun¹³ described ligations between tetrazines and less reactive strained alkenes. However, 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**, Fig 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**, Fig 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 significant increase in strain energy associated with non-crown conformations of *trans*-cyclooctene could be used to accelerate the reactivity toward tetrazines. Previously, dienophiles for the tetrazine-*trans*-cyclooctene ligation have been derived from cyclooct-4-enol⁷ — 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 compound **3** would manifest in faster kinetics in 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

To provide further evidence that the low barrier for **3** was a manifestation of additional strain to the eight-membered ring, 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 *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**. As compounds **3** and **4** are diastereomers and the cyclopropyl moiety is distant from the tetrazine in each transition state, the primary difference between the two compounds is the eight-membered ring conformation. 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**— an analog of **3** with a functionalizable group (Scheme 1). Thus, 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 labs was used to photoisomerize **6**: active removal on AgNO₃-impregnated silica gel provided the *trans*-isomer **7** in 74% yield.⁷

During the completion of our studies, van Delft and coworkers elegantly demonstrated that cyclooctyne-based bioconjugations can be accelerated through fusion of a cyclopropane

ring.^{21a} This group reported the synthesis of compound **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 nitron cycloadditions.

Compound **7** combines with 3,6-di(2-pyridyl)-*s*-tetrazine 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 compound **7** with 3,6-di(2-pyridyl)-*s*-tetrazine 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 di(2-pyridyl)-*s*-tetrazine (**2**, 6.5 mM) in CD_3OD gave an 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

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**), compound **10** reacted more slowly: compounds **9** : **11** were formed in a 1.0 : 0.72 ratio. Based on these relative rates, the functionalizable alcohol **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**, additional rate constants were measured to be $27.2 (\pm 1.5) \text{ M}^{-1}\text{s}^{-1}$ at 35 °C, and $36.4 (\pm 1.3) \text{ M}^{-1}\text{s}^{-1}$ at 45 °C, and Eyring analysis showed $\Delta H^\ddagger$ to be $5.41 (\pm 0.7) \text{ kcal/mol}$, $\Delta S^\ddagger$ to be $-33.6 (\pm 2.3) \text{ e.u.}$, and $\Delta G^\ddagger$ to be $15.4 (\pm 0.9) \text{ kcal/mol}$. Based on the relative rate data at 25 °C in MeOH, $\Delta G^\ddagger$ for the reaction of **7** and **12** was calculated to be $12.4 (\pm 0.9) \text{ kcal/mol}$ at 25 °C in MeOH. Computation at the M06L/6-311+G(d,p) level of theory was also used to study the Diels-Alder reactions of **12**. For gas phase Diels-Alder reactions with **12** 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}

In addition to excellent reactivity, **7** also displays excellent stability. Compound **7** shows no degradation in water or in human serum after 24 hours. Compound **7** (30 mM) also shows no decomposition upon exposure to 30 mM *n*-butylamine in solvent for 24 hours, or to 5 mM ethanethiol in MeOH for 12 hours.²⁸ Compound **7** could be converted into derivatives readily. 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 μM) could be derivatized with an excess (120 μM) of maleimide **13** to give adduct **14**. Subsequent reaction with 3,6-di(2-pyridyl)-*s*-tetrazine (**2a**, 120 μM) 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 optimized derivative is a (*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. The strained *trans*-cyclooctene derivative not only displays enhanced reactivity, but it can be easily derivatized, and bioconjugation to the protein thioredoxin has been demonstrated. The strained *trans*-cyclooctene displays good stability in water, serum, and in the presence of amine and thiol nucleophiles. Toward 3,6-dipyridyl-*s*-tetrazine in MeOH at 25 °C, the strained derivative reacts with a 2nd order rate constant of 22,000 M⁻¹s⁻¹, which 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, the strained derivative reacts with a rate constant of 3,100 M⁻¹s⁻¹, which is 160 times faster than the parent *trans*-cyclooctene.

Supplementary Material

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Acknowledgments

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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 *trans*-cyclooctene in the crown conformation (**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 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, Metodiewa D. Free Radical Biology & Medicine. 1999; 27:322. [PubMed: 10468205]
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30. Trx (15 μ M) was reduced with tri(3-hydroxypropyl)phosphine (THP, 1 mM), and combined with **12** (100 μ M) in acetate buffer (pH 6). As THP also reduces maleimide functionalities, an excess of **12** was required

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

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**.

Figure 4. Relative rates of reactions with (a) 3,6-diaryltetrazines (**2a**) in CD₃OD at 25 °C. NMR spectra (400 MHz, CD₃OD) of competition experiments are shown in the insets.

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**.

Scheme 1.
Synthesis of a highly reactive dienophile

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