



Published in final edited form as:

Org Biomol Chem. 2017 August 09; 15(31): 6640–6644. doi:10.1039/c7ob01707c.

Computationally Guided Discovery of a Reactive, Hydrophilic *trans*-5-Oxocene Dienophile for Bioorthogonal Labeling

William D. Lambert^a, Samuel L. Scinto^a, Olga Dmitrenko^a, Samantha J. Boyd^a, Ronald Magboo^e, Ryan A. Mehl^b, Jason W. Chin^c, Joseph M. Fox^a, and Stephen Wallace^{c,d}

^aBrown Laboratory, Department of Chemistry & Biochemistry, University of Delaware, Newark, Delaware 19716, USA

^bDepartment of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331, United States

^cMedical Research Council, Laboratory of Molecular Biology, Francis Crick Avenue, Cambridge Biomedical Campus, Cambridge CB2 0QH, UK

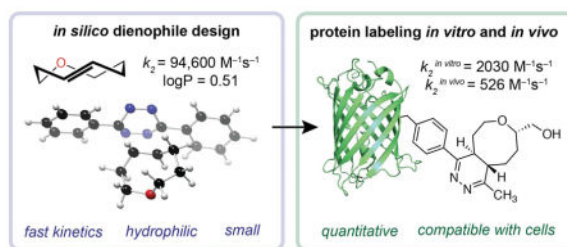
^dInstitute of Quantitative Biology, Biochemistry and Biotechnology, School of Biological Sciences, University of Edinburgh, UK

^eLotus Separations LLC, Newark DE 19711

Abstract

The use of organic chemistry principles and prediction techniques has enabled the development of new bioorthogonal reactions. As this “toolbox” expands to include new reaction manifolds and orthogonal reaction pairings, the continued development of existing reactions remains an important objective. This is particularly important in cellular imaging, where non-specific background fluorescence has been linked to the hydrophobicity of the bioorthogonal moiety. Here we report that *trans*-5-oxocene (oxoTCO) displays enhanced reactivity and hydrophilicity compared to *trans*-cyclooctene (TCO) in the tetrazine ligation reaction. Aided by *ab initio* calculations we show that the insertion of a single oxygen atom into the *trans*-cyclooctene (TCO) ring system is sufficient to impart aqueous solubility and also results in significant rate acceleration by increasing angle strain. We demonstrate the rapid and quantitative cycloaddition of oxoTCO using a water-soluble tetrazine derivative and a protein substrate containing a site-specific genetically encoded tetrazine moiety both *in vitro* and *in vivo*. We anticipate that oxoTCO will find use in studies where hydrophilicity and fast bioconjugation kinetics are paramount.

Graphical Abstract



Introduction

Biotechnology and biomedicine have been profoundly influenced by the development of new bioorthogonal reactions – abiotic transformations that occur selectively in a biological environment.^{1–9} Amongst these, the cycloaddition of alkenes/alkynes and *s*-tetrazines has become an important member of the bioorthogonal reaction “toolbox”.^{10–20} Since initial reports using *trans*-cyclooctene (TCO)²¹ and norbornene derivatives²², a complementary range of dienophiles has been developed – including cyclopropenes^{23,24}, cyclooctynes^{25,26} and simple α -olefins.^{27–29} However, *trans*-cyclooctene (TCO) still maintains the advantage of exceptional reaction kinetics in this process.^{3,10} For example, the cycloaddition of the equatorial diastereomer of 5-hydroxy-*trans*-cyclooctene and a 3,6-dipyridyl-*s*-tetrazine derivative occurs with a second-order rate constant of $22,600 \text{ M}^{-1}\text{s}^{-1}$ in H_2O at 25 C .³⁰ Faster reactivity can be realized by using the axial diastereomer of 5-hydroxy-*trans*-cyclooctene ($80,200 \text{ M}^{-1}\text{s}^{-1}$).^{11,30} However, the fastest bioorthogonal reactions described to date use the conformationally strained dienophiles *s*-TCO and *d*-TCO (Figure 1).^{30,31} These bicyclic molecules adopt a half-chair conformation that is 5.6–5.9 kcal/mol higher in energy than the crown conformation of monocyclic TCO. Cycloaddition of these compounds with tetrazines display second-order rate constants of up to $366,000 \text{ M}^{-1}\text{s}^{-1}$ for *d*-TCO and $3,300,000 \text{ M}^{-1}\text{s}^{-1}$ for *s*-TCO.

From a kinetic standpoint, *trans*-cyclooctene derivatives are excellent dienophiles for studies where high reactivity is essential such as in cellular imaging and nuclear medicine.^{32,33} However, the hydrophobicity of TCO and *s*-TCO has recently been linked to high levels of non-specific background fluorescence during imaging experiments, necessitating lengthy washout protocols (>2 h) to dissociate the excess reagent from the cell.^{34,35} While *d*-TCO displays reduced lipophilicity, the compound is relatively bulky compared to the parent TCO system. The development of new, low molecular weight dienophiles for the tetrazine ligation reaction that are fast and hydrophilic is therefore an important challenge.

In seminal work, Jendrella synthesized 4,6-dioxo-TCO **1** and showed it to be 20–1000 fold faster than *trans*-cyclooctene in cycloadditions with cyclopentadiene, 2,3-dimethylbutadiene, mesitronitroxide and diphenylketene.³⁶ More recently, Dudley, Alabugin and coworkers have shown (*in silico*) that 3-oxocyclooctynes display fast reactivity in cycloadditions with azides, and have attributed their fast reactivity partly to the hyperconjugative effect of the allylic oxygens.³⁷ Tomooka and Woerpel have synthesized *trans*-oxasilacycloalkenes, and have studied their reactivity in Diels-Alder and azide cycloadditions.^{38,39} Very recently, Lemke, Kele and coworkers reported the genetic incorporation of dioxo-TCO **2** and

demonstrated that the lower lipophilicity of this molecule resulted in improved washout times during imaging experiments. In Diels-Alder reactions with tetrazines, the reaction rate with **2** is similar to that with the parent TCO.^{36,40}

In the course of our synthetic studies on transannulations of *cis*- and *trans*-5-oxocenes, we queried whether such compounds would engage in rapid bioconjugation reactions.^{41,42} Here we report the computational design and synthesis of a *trans*-5-oxocene (“oxoTCO”, **3**) – a small, hydrophilic, and highly reactive dienophile for use in the bioorthogonal tetrazine ligation reaction. The reaction of **3** (2.2 : 1 d.r.) with a water-soluble 3,6-dipyridyl-*s*-tetrazine-mono-succinamic acid **10** occurs with a second order rate constant of 94,600 M⁻¹s⁻¹ in PBS at 25 °C (Figure 3), which is faster than either diastereomer of 5-hydroxy-*trans*-cyclooctene, and approaching the rate of bicyclic d-TCO. The oxoTCO heterocycle can be synthesized in seven high yielding steps from commercially available glycidol. Furthermore, oxoTCO **3** is small (MW 142) and hydrophilic with an experimental logP = 0.51. Finally, we describe the *in vitro* and *in vivo* kinetics of **3** on a recombinant protein substrate containing a site-specifically incorporated tetrazine-containing amino acid (*sf*GFP-150Tet-v.2.0).⁴³ We anticipate that oxoTCO **3** will find applications in cellular imaging studies where small hydrophilic probes with fast reaction kinetics, low background fluorescence and/or rapid data acquisition are required.

Results and discussion

Computation was used to assist the design of a reactive and soluble *trans*-oxocene dienophile. We reasoned that the short C–O bonds in the backbone of a *trans*-5-oxocene would augment the olefinic strain of the *trans*-cycloalkene, and thereby increase the reactivity in tetrazine ligation. As shown in Figure 2, ground state calculations were carried out for the parent *trans*-oxocenes **4** and **5** as well as *trans*-cyclooctene at the M06L/6-311+G(d,p) level. Indeed, the calculated C–C=C–C dihedral angle for **4** (134.6°) and **5** (134.4°) is significantly shorter than that for *trans*-cyclooctene (137.7°). M06L/6-311+G(d,p) and CAM-B3LYP/tzvp calculations were also carried out to compare the reactivity of **4** and **5** to *trans*-cyclooctene (Figure 2B). These calculations were carried out with diphenyl-*s*-tetrazine so that they could be benchmarked against previous calculations carried out in our labs.^{30,31} At the M06L/6-311+G(d,p) level, the barrier for the Diels-Alder reaction of *trans*-cyclooctene with 3,6-diphenyl-*s*-tetrazine is E[‡] 13.3 kcal/mol, E[‡](ZPE) 13.9 kcal/mol, H[‡] 12.9 kcal/mol. With *trans*-5-oxocene **4**, the barrier was significantly lower, with E[‡] –1.23 kcal/mol, E[‡](ZPE) –1.54 kcal/mol and H[‡] –1.44 kcal/mol relative to *trans*-cyclooctene. Interestingly, the isomeric *trans*-4-oxocene **5** is not predicted to be significantly more reactive than *trans*-cyclooctene. This computational result can be rationalized by considering the electron withdrawing nature of the allylic oxygen. Inverse electron demand Diels-Alder reactions are deactivated by electron withdrawing groups on the alkene, and the allylic oxygen of **5** is both inductively withdrawing and stereoelectronically positioned to deactivate the alkene through hyperconjugation. Thus, while the alkene of **5** is more strained than **4** (134.4° vs. 134.6° dihedral angle), the effect is attenuated by the electron withdrawing effect of the allylic oxygen.

Based on these computational predictions, we synthesized the alcohol-functionalized *trans*-5-oxocene **3** in 7 steps from commercially available glycidol **6** (Scheme 1). The synthesis began with TBS-protection and the addition of allyl magnesium chloride to provide alcohol **7**. Our attempts to access **8** directly from **7** via Williamson etherification or Mitsunobu chemistry were unsuccessful. Fortunately, we found that the treatment of the MOM ether of **7** with Lewis acidic stannic chloride generated a putative oxocarbenium ion that could be quenched via Sakurai allylation to afford butenyl ether **8** in 78% yield. Ring-closing metathesis of **8** using the Grubbs first-generation catalyst proceeded efficiently to afford *cis*-oxocene **9** in 84% yield. Finally, desilylation and photoisomerization using our closed-loop flow reactor⁴⁴ afforded a 2.2:1 diastereoisomeric mixture of *trans*-oxocenes in 70% yield (37% overall yield over 7 steps). Separation of the diastereomers using preparative thin layer or silica gel chromatography was unsuccessful. An analytical sample of the major diastereomer of **3** was obtained by preparative supercritical fluid chromatography, however, given the difficulty of separation we continued the majority of further studies on oxoTCO using a 2.2:1 mixture of diastereomers. The logP of **3** was experimentally determined to be 0.51 whereas equatorial 5-hydroxy-*trans*-cyclooctene and d-TCO were both determined to be more hydrophobic with logP = 1.11 and 0.94, respectively.³⁰

The stability of oxoTCO **3** was studied under several conditions by ¹H NMR spectroscopy. Over a 14 day period at room temperature, a 2.2:1 diastereoisomeric mixture of oxoTCO **3** (33 mM) showed no degradation in CD₃OD. In D₂O-PBS (pD = 7.4), the major, equatorial diastereomer of **3** showed less than 10% degradation after 1 week. The more reactive minor diastereomer degraded more rapidly in PBS, and decomposed with a half-life of 36 hours, with complete degradation after 9 days. oxoTCO **3** (25 mM) in the presence of mercaptoethanol (25 mM) showed only 8% isomerization in CD₃OD over a 22 hour period while 92% was isomerized in phosphate buffered D₂O (pD = 7.4) over the same period of time. Under similar conditions, oxoTCO stability to thiols in methanol is improved relative to d-TCO (92% isomerization after 14h) and s-TCO (100% isomerization after 4h).³⁰ In D₂O (pD = 7.4) containing 25 mM mercaptoethanol, the major diastereomer isomerized with a half-life of 2.2 hours, and the minor diastereomer isomerized with a half life of 1.6 hours. Overall, the stability of the oxoTCO diastereomers is similar to that of dTCO.³⁰

We next measured the rate constant for the inverse electron-demand Diels-Alder (IEDDA) cycloaddition of oxoTCO and tetrazine **10** under pseudo-first order conditions (Figure 3). PBS was chosen as a solvent for two reasons: aqueous solvent considerably accelerates the IEDDA reaction by the hydrophobic effect and initial kinetic studies indicated tetrazine **10**, though more water soluble than 3,6-dipyridyl-*s*-tetrazine, was aggregating in unbuffered H₂O, thus giving inconsistent first-order rates. Using a stopped-flow spectrophotometer and by following the exponential decay in tetrazine absorbance at 325 nm the second-order rate constant (k_2) was determined to be $94,600 \pm 5,700 \text{ M}^{-1}\text{s}^{-1}$ in PBS at 25 °C for the 2.2 : 1 diastereomeric mixture of **3**. This is faster than the reaction of a similar tetrazine with both diastereomers of 5-hydroxy-*trans*-cyclooctene (equatorial isomer $22,600 \text{ M}^{-1}\text{s}^{-1}$; axial isomer $80,200 \text{ M}^{-1}\text{s}^{-1}$), and is approximately 1/4 as fast as a bicyclic d-TCO under comparable conditions ($366,000 \text{ M}^{-1}\text{s}^{-1}$).³⁰ The diastereomerically pure equatorial isomer

of **3** was obtained by SFC, and found to react with **10** with a rate constant of $44,100 \pm 2,600 \text{ M}^{-1}\text{s}^{-1}$ in PBS at 25 °C. While we were unable to obtain a diastereomerically pure sample of the axial diastereomer, the rate constant can be calculated to be $310,000 \text{ M}^{-1}\text{s}^{-1}$ based on the rates observed for the diastereomer mixture and the pure equatorial isomer. The 7-fold rate acceleration for the axial isomer is consistent with prior reports for 5-hydroxy-*trans*-cyclooctene.^{11,30}

We also studied the *in vitro* cycloaddition of oxoTCO and a green fluorescent protein encoded with an unnatural tetrazine-containing amino acid **11** (*sfGFP*-150Tet-v.2.0) via the procedure of Mehl and coworkers.⁴³ Thus, 4-(6-methyl-*s*-tetrazin-3-yl)phenylalanine was site-specifically introduced into a C-terminally hexahistidine-tagged GFP (*sfGFP*-150TAG-His₆) via orthogonal translation using the evolved aminoacyl-tRNA synthetase *MjRS*/tRNA_{CUA} pair. Co-expression of these components in *E. coli* resulted in the amino acid-dependent synthesis of full-length recombinant GFP **11** which was purified by Ni-NTA chromatography and confirmed by ESI-MS. The tetrazine moiety of this protein quenches the fluorescence of the GFP chromophore, whereas the dihydropyridazine product of the TCO ligation does not. It is therefore possible to determine the kinetics of the reaction by monitoring the increase in GFP fluorescence (Figure 4A). Accordingly, the second order rate constant of the reaction between oxoTCO and *sfGFP*150Tet-v.2.0 was determined to be $2,030 \pm 180 \text{ M}^{-1}\text{s}^{-1}$ in phosphate buffer at room temperature (Figure 4B). The reaction was quantitative under these conditions as determined by ESI-MS (Figure 4C). The slower rate relative to that observed with **10** is due to the less reactive nature of the tetrazine **11** and in line with rate decreases observed with other TCOs.³⁰

Finally, the small size and good hydrophilicity of oxoTCO make it an excellent candidate for labeling *in vivo*. The cycloaddition was monitored in a suspension (PBS) of *E. coli* overexpressing *sfGFP*150Tet-v.2.0 by measuring the increase in whole-cell fluorescence upon addition of **3**. At room temperature oxoTCO displays a second-order rate constant of $526 \pm 11 \text{ M}^{-1}\text{s}^{-1}$, which is approximately ¼ as fast as the *in vitro* ligation. Quantitative determination of the biorthogonal reaction was verified by ESI-MS. Cells were washed before lysis and the protein was purified via nickel affinity chromatography. The resulting protein mass was as expected for the cycloaddition product. This, alongside the whole-cell fluorescence experiment, provides evidence to suggest that oxoTCO crosses the bacterial cell membrane.

Conclusions

In summary, computation was used to design a hydrophilic 5-oxo-*trans*-cyclooctene derivative with high reactivity attributed to increased angle strain. A short synthesis was developed involving Sakurai allylation, olefin metathesis and flow-enabled photoisomerization as key steps. This heterocyclic *trans*-cyclooctene displays improved hydrophilicity, with an experimental logP value of 0.51. Kinetic analysis revealed that oxoTCO displays faster reactivity than mono-substituted TCO dienophiles, and is less bulky than bicyclic *trans*-cyclooctenes we have described previously. Quantitative labeling of GFP containing a genetically encoded tetrazine amino acid was studied in solution and in whole bacteria cells with complete labeling within minutes at room temperature. The high

reactivity and lower hydrophobicity of oxoTCO-based probes should prove useful for *in vivo* applications, and in this context is the focus of active study in our labs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH R01DC014461, R01EB014354, NSF DMR-1506613 and by Medical Research Council, UK (grants MC_U105181009 and MC_UP_A024_1008). Spectra were obtained with instrumentation supported by NIH grants P20GM104316, P30GM110758, S10RR026962, S10OD016267 and NSF grants CHE-0840401, CHE-1229234. The authors declare no competing financial interest. SLS is grateful to training at the genetic code expansion workshop at Oregon State University funded through NSF MCB-1518265.

Notes and references

1. Sletten EM, Bertozzi CR. *Angew Chem Int Ed*. 2009; 48:6974–6998.
2. Patterson DM, Nazarova LA, Prescher JA. *ACS Chem Biol*. 2014; 9:592–605. [PubMed: 24437719]
3. Lang K, Chin JW. *ACS Chem Biol*. 2014; 9:16–20. [PubMed: 24432752]
4. Kay CSM, Finn MG. *Chem Biol*. 2014; 21:1075–1101. [PubMed: 25237856]
5. Ramil CP, Lin Q. *Chem Commun*. 2013; 49:11007–11022.
6. Rossin R, Robillard MS. *Curr Opin Chem Biol*. 2014; 21:161–169. [PubMed: 25159021]
7. Meyer J-P, Adumeau P, Lewis JS, Zeglis BM. *Bioconj Chem*. 2016; doi: 10.1021/acs.bioconjchem.6b00561
8. Vrabel, M., Carell, T. *Cycloadditions in Bioorthogonal Chemistry*. Springer International Publishing; Switzerland: 2016.
9. Niki I, Lemke EA. *Curr Opin Chem Biol*. 2015; 28:164–173. [PubMed: 26302384]
10. Selvaraj R, Fox JM. *Curr Opin Chem Biol*. 2013; 17:753–760. [PubMed: 23978373]
11. Rossin R, van den Bosch SM, ten Hoeve W, Carvelli M, Versteegen RM, Lub J, Robillard MS. *Bioconj Chem*. 2013; 24:1210–1217.
12. Lang K, Chin JW. *Chem Rev*. 2014; 114:4964–4806.
13. Wang K, Sachdeva A, Cox DJ, Wilf NM, Wallace S, Mehl RA, Chin JW. *Nat Chem*. 2014; 6:393–403. [PubMed: 24755590]
14. Peng T, Hang HC. *J Am Chem Soc*. 2016; 138:14426–14433.
15. Tsai Y, Essig S, James JR, Lang K, Chin JW. *Nat Chem*. 2015; 7:554–561. [PubMed: 26100803]
16. Billaud EMF, Shahbazali E, Ahamed M, Cleeren F, Noël T, Koole M, Verbruggen A, Hessel V, Bormans G. *Chem Sci*. 2016; doi: 10.1039/c6sc02933g
17. Liu S, Zhang H, Remy RA, Deng F, Mackay ME, Fox JM, Jia X. *Adv Mater*. 2015; 27:2783–2790. [PubMed: 25824805]
18. Devaraj NK, Weissleder R. *Acc Chem Res*. 2011; 44:816–827. [PubMed: 21627112]
19. Wu H, Devaraj NK. *Top Curr Chem*. 2015; 374:3.
20. Knall A-C, Slugovc C. *Chem Soc Rev*. 2013; 42:5131. [PubMed: 23563107]
21. Blackman ML, Royzen M, Fox JM. *J Am Chem Soc*. 2008; 130:13518–13519. [PubMed: 18798613]
22. Devaraj NK, Weissleder R, Hilderbrand SA. *Bioconj Chem*. 2008; 19:2297–2299.
23. Yang J, Seckute J, Cole CM, Devaraj NK. *Angew Chem Int Ed*. 2012; 51:7476–7479.
24. Patterson DM, Nazarova LA, Xie B, Kamber DN, Prescher JA. *J Am Chem Soc*. 2012; 134:18638–18643. [PubMed: 23072583]
25. Chen W, Wang D, Dai C, Hamelberg D, Wang B. *Chem Comm*. 2012; 48:1736–1738. [PubMed: 22159330]

26. Lang K, Davis L, Wallace S, Mahesh M, Cox DJ, Blackman ML, Fox JM, Chin JW. *J Am Chem Soc.* 2012; 134:10317–10320. [PubMed: 22694658]
27. Niederwieser A, Späte A, Nguyen LD, Jüngst C, Reutter W, Wittmann V. *Angew Chem Int Ed.* 2013; 52:4265–4268.
28. Lee Y, Kurra Y, Yang Y, Torres–Kolbus J, Deiters A, Liu WR. *Chem Comm.* 2014; 50:13085–13088. [PubMed: 25224663]
29. Oliveira BL, Guo Z, Boutureira O, Guerreiro A, Jiménez-Osés G, Bernardes GJL. *Angew Chem.* 2016; 128:14903–14907.
30. Darko A, Wallace S, Dmitrenko O, Machovina MM, Mehl R, Chin JW, Fox JM. *Chem Sci.* 2014; 5:3770–3776. [PubMed: 26113970]
31. Taylor MT, Blackman ML, Dmitrenko O, Fox JM. *J Am Chem Soc.* 2011; 133:9646–9649. [PubMed: 21599005]
32. Murrey HE, Judkins JC, am Ende CW, Ballard TE, Fang Y, Riccardi K, Di L, Guilmette ER, Schwartz JW, Fox JM, Johnson DS. *J Am Chem Soc.* 2015; 137:11461–11475. [PubMed: 26270632]
33. Selvaraj R, Giglio B, Liu S, Wang H, Wang M, Yuan H, Chintala SR, Yap L, Conti PS, Fox JM, Li Z. *Bioconj Chem.* 2015; 26:435–442.
34. Uttamapinant C, Howe JD, Lang K, Beranek V, Davis L, Mahesh M, Barry NP, Chin JW. *J Am Chem Soc.* 2015; 137:4602–4605. [PubMed: 25831022]
35. Kozma E, Niki I, Varga BR, Aramburu IV, Kang JH, Fackler OT, Lemke EA, Kele P. *ChemBioChem.* 2016; 17doi: 10.1002/cbic.201600284
36. Jendralla H. *Tetrahedron.* 1983; 39:1359–1363.
37. Gold B, Dudley GB, Alabugin IV. *J Am Chem Soc.* 2013; 135:1558–1569. [PubMed: 23272641]
38. Tomooka K, Miyasaka S, Motomura S, Igawa K. *Chem Eur J.* 2014; 20:798–7602.
39. Santucci J III, Sanzone JR, Woerpel KA. *Eur J Org Chem.* 2016; 17:2933–2943.
40. Knorr G, Kozma E, Herner A, Lemke E, Kele P. *Chem Eur J.* 2016; 22:8972–8979. [PubMed: 27218228]
41. Royzen M, Taylor MT, DeAngelis A, Fox JM. *Chem Sci.* 2011; 2:2162–2165. [PubMed: 23125911]
42. Taylor MT, Fox JM. *Tet Lett.* 2015; 56:3560–3563.
43. Blizzard RJ, Backus DR, Brown W, Bazewicz CG, Li Y, Mehl RA. *J Am Chem Soc.* 2015; 137:10044–10047. [PubMed: 26237426]
44. Royzen M, Yap GPA, Fox JM. *J Am Chem Soc.* 2008; 130:3760–3761. [PubMed: 18321114]

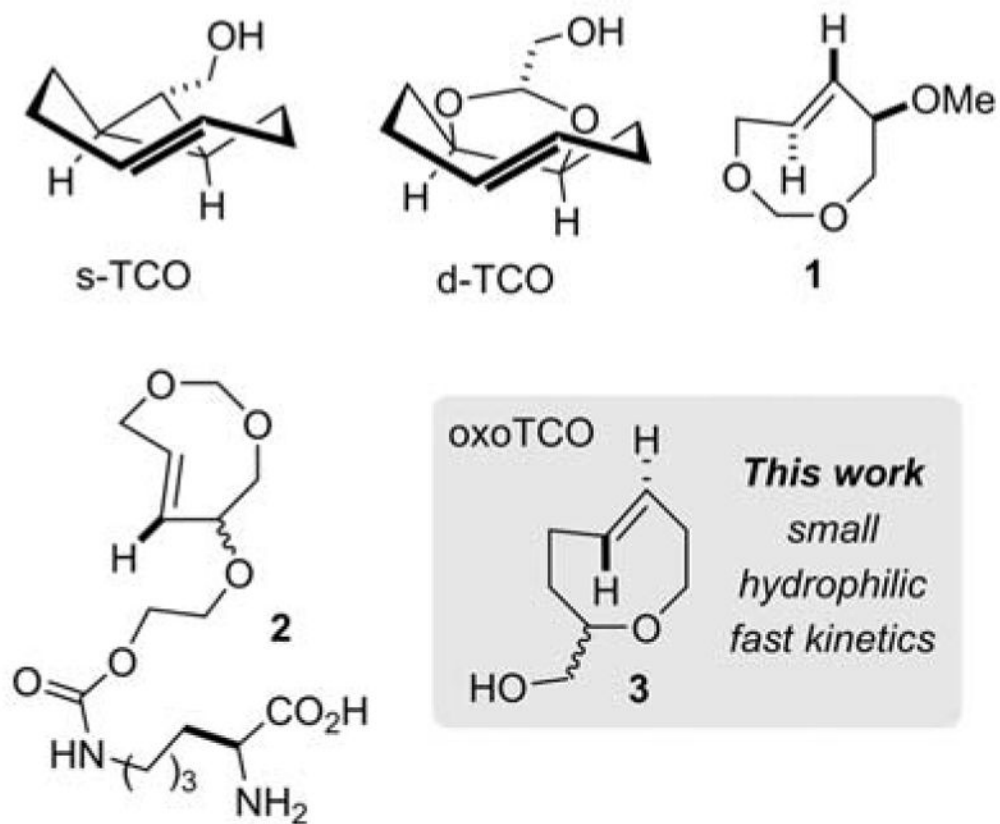
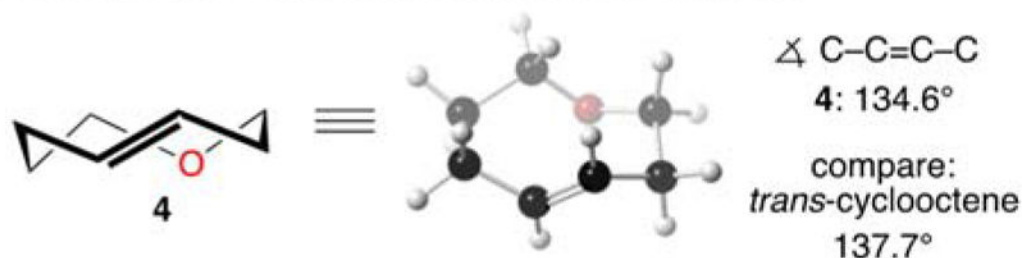


Figure 1.
Conformationally strained and heterocyclic *trans*-cyclooctenes

A Ground state calculations (M06L/6-311+G(d,p))



B Transition state calculations (M06L/6-311+G(d,p))

homoallylic ether: lower barrier
relative to *trans*-cyclooctene

allylic ether: similar barrier
to *trans*-cyclooctene

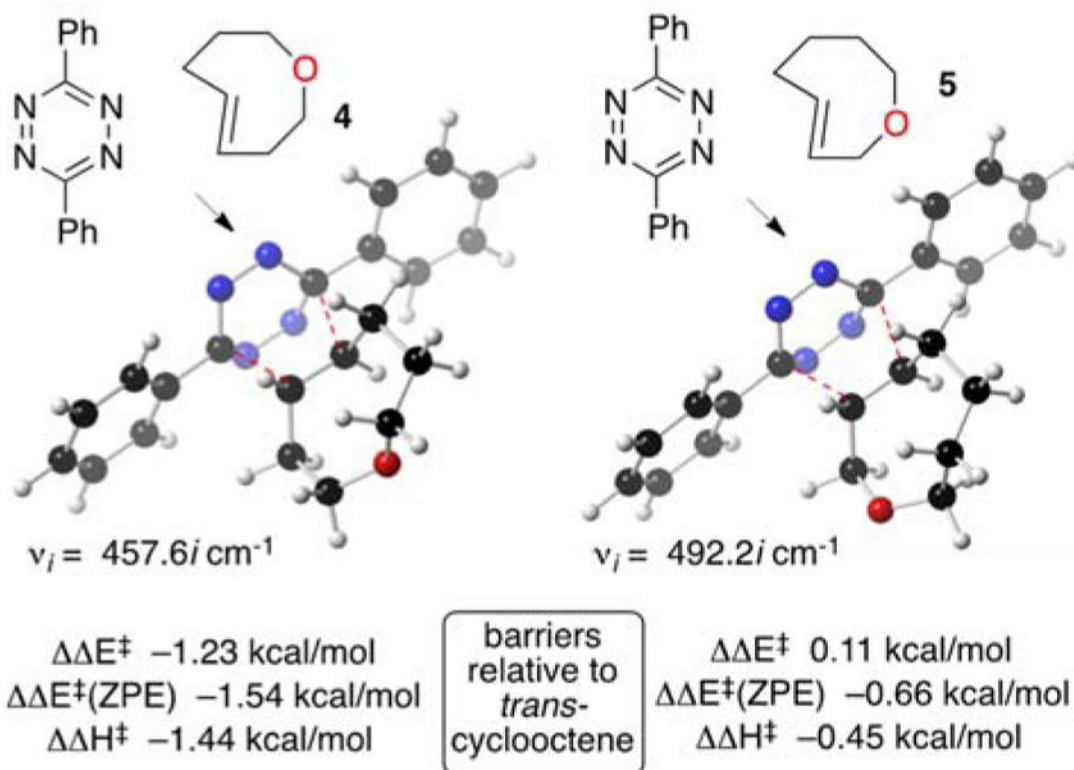


Figure 2.

DFT transition state calculations predict that *trans*-5-oxocene **4**, but not *trans*-4-oxocene **5**, would be more reactive than *trans*-cyclooctene. While both **4** and **5** are more strained than *trans*-cyclooctene, that the reactivity of **5** is attenuated by the electron withdrawing allylic oxygen.

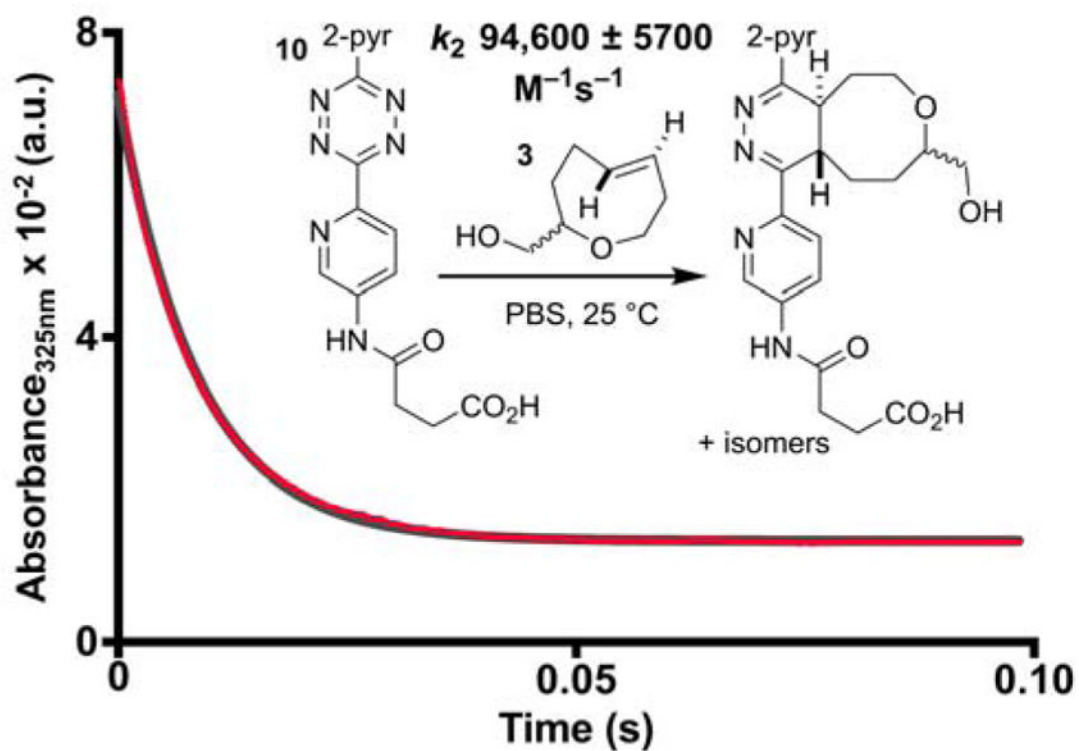


Figure 3.

The kinetics of the cycloaddition of oxoTCO **3** with water-soluble 3,6-dipyridyl-*s*-tetrazine-mono-succinamic acid **10** in PBS buffer (pH 7.4). Second order rate constants (k_2) were determined with a stopped-flow spectrophotometer under pseudo-first order conditions using ca. 10–30 equivalents of oxoTCO **3** (2.2 : 1 d.r.) by monitoring the decrease in tetrazine absorbance at 325 nm.

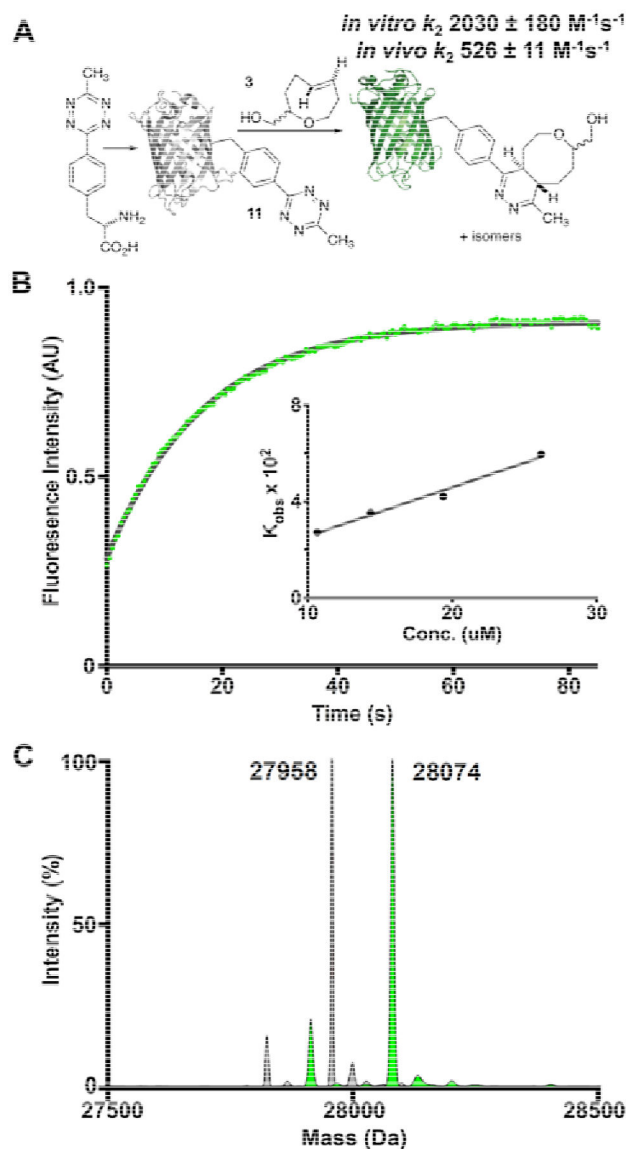
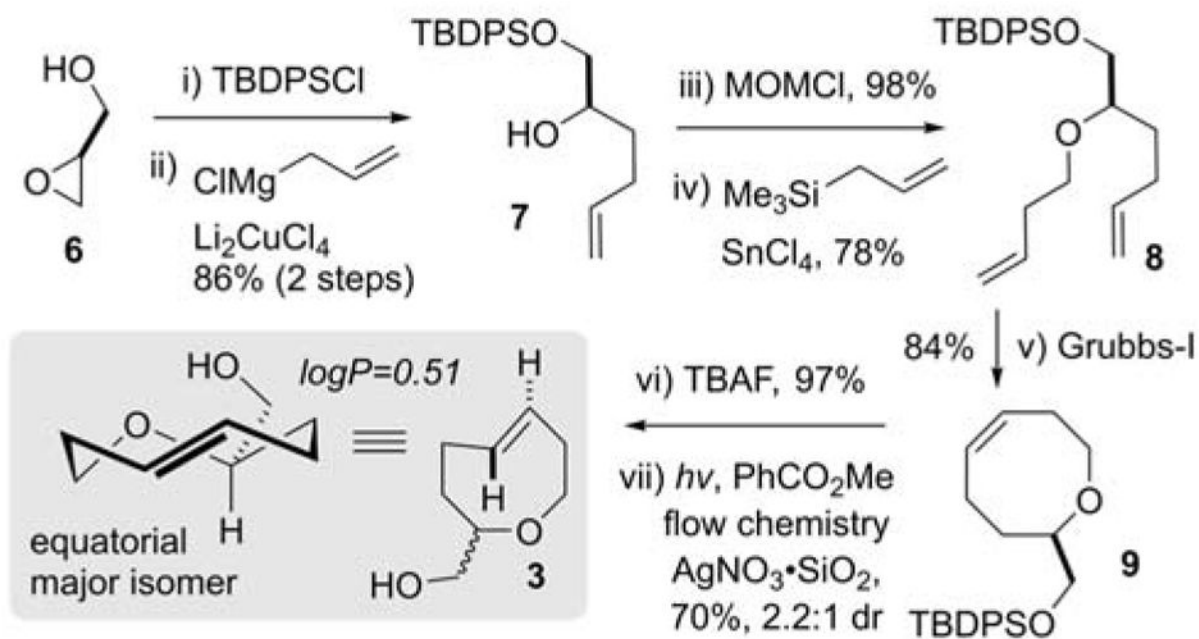


Figure 4.

(A) The oxoTCO-tetrazine cycloaddition using a recombinant protein substrate containing a site-specifically incorporated tetrazine. Experiments were carried out both *in vitro* (PBS) and *in vivo* (*E. coli*) using a 2.2 : 1 eq/ax mixture of oxoTCO diastereomers. (B) Second order rate constants (k_2) were determined under pseudo-first order conditions using ca. 100–260 equivalents of oxoTCO **3** by monitoring the increase in GFP fluorescence. (*in vitro* study shown here) (C) Quantitative determination of the cycloadduct was confirmed by ESI-MS.



Scheme 1.
Synthesis of oxoTCO 3