JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

COMMUNICATION

pubs.acs.org/JACS

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

Michael T. Taylor, Melissa L. Blackman, Olga Dmitrenko, and Joseph M. Fox*

Brown Laboratories, Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716, United States

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.

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 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 $k_2 = 2000\ M^{-1}\ s^{-1}$.[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′ = DOTA[10] or cyclic RGD peptide[11]) have been used by Robillard[10] and our group[11] 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

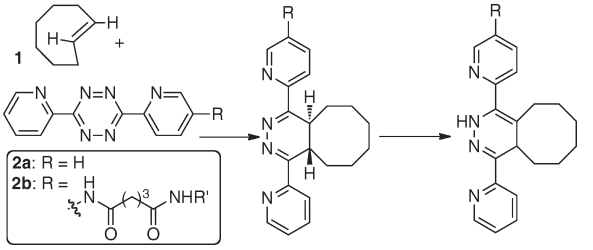


Figure 1. Tetrazine–*trans*-cyclooctene ligation.

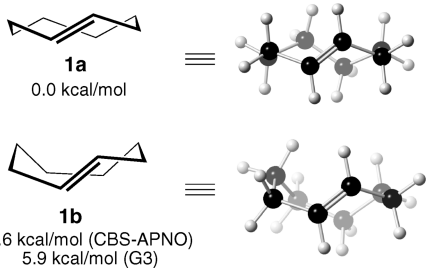


Figure 2. Calculated relative energies (0 K) for two conformations of *trans*-cyclooctene at the CBS-APNO and G3 levels of theory.

ligations, the groups of Hilderbrand and Weissleder[12] and Pipkorn and Braun[13] 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*-cyclooctene[15] (**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,

Received: February 28, 2011
Published: May 20, 2011

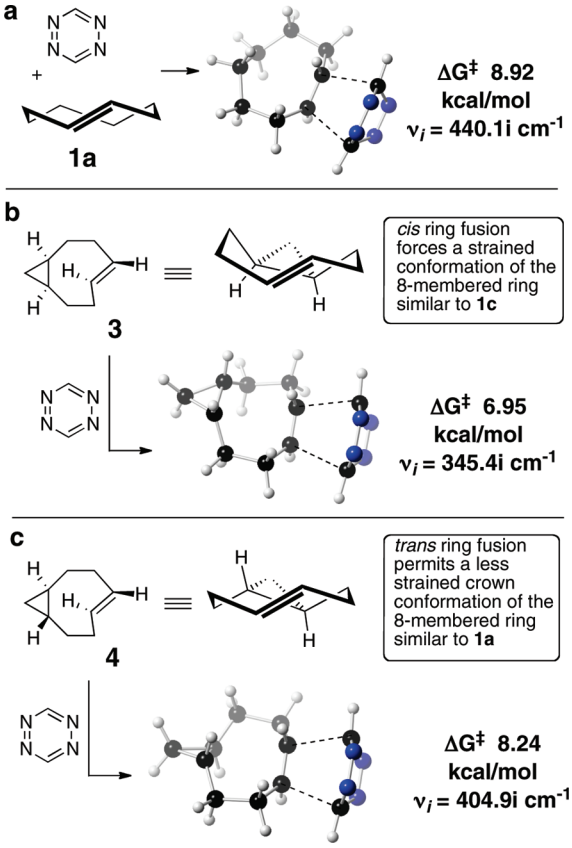


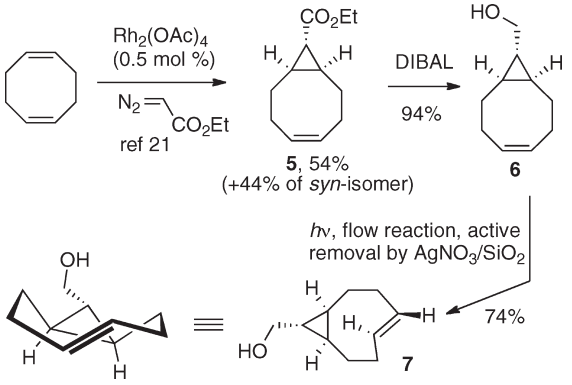
Figure 3. M06L/6-311+G(d,p)-optimized transition structures for the Diels–Alder reaction of *s*-tetrazine with the crown conformer of *trans*-cyclooctene (a), the *cis*-ring fused bicyclo[6.1.0]non-4-ene **3** (b), and the *trans*-ring fused bicyclo[6.1.0]non-4-ene **4** (c). The barrier (8.24 kcal/mol)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

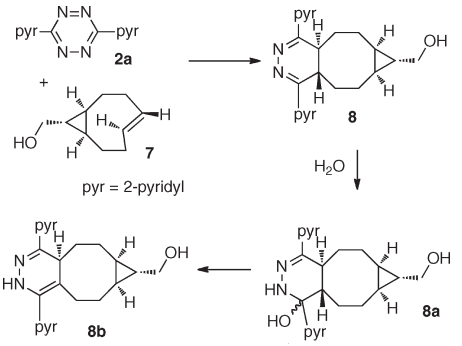
During the completion of our studies, van Delft et al. elegantly demonstrated that cyclooctyne-based bioconjugations can be accelerated through fusion of a cyclopropane ring.[21a] This group reported the synthesis of **6** and readily converted it to the corresponding cyclooctyne derivative for bioorthogonal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

Compound **7** combines with **2a** to give product **8** in >95% yield by ^{1}H 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 aminal 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 ^{1}H 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 CD_3OD 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 M^{-1} s^{-1} (± 40) in MeOH, these NMR experiments show the rate of reaction between **7** and **2a** to be k_2 = 22 000 M^{-1} 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]

dx.doi.org/10.1021/ja201844c | J. Am. Chem. Soc. 2011, 133, 9646–9649

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



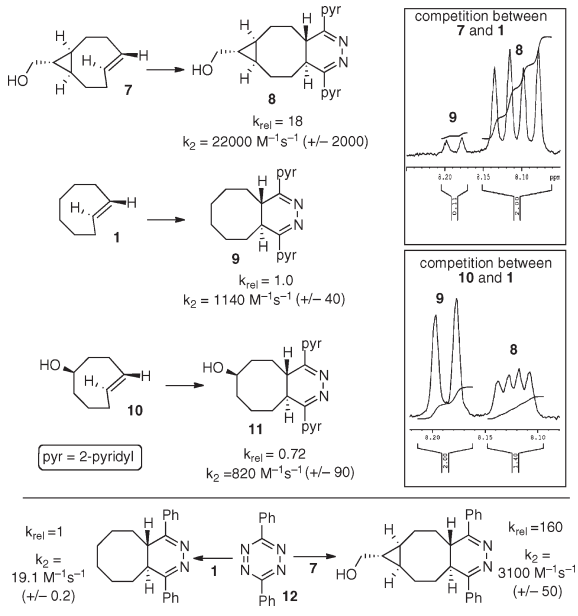


Figure 4. Relative rates of reactions with 3,6-diaryltetrazines (**2a**) in CD_3OD at 25 °C. NMR spectra (400 MHz, CD_3OD) of competition experiments are shown in the insets.

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 M^{-1} 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 (± 0.2) $M^{-1}s^{-1}$, whereas **7** reacted with a rate of 3100 (± 50) $M^{-1}s^{-1}$. For the reaction of **1** and **12**, Eyring analysis showed $\Delta H^{\ddagger}$ to be 5.41 (± 0.7) kcal/mol, $\Delta S^{\ddagger}$ to be −33.6 (± 2.3) e.u., and $\Delta G^{\ddagger}$ to be 15.4 (± 0.9) 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 (± 0.9) 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]

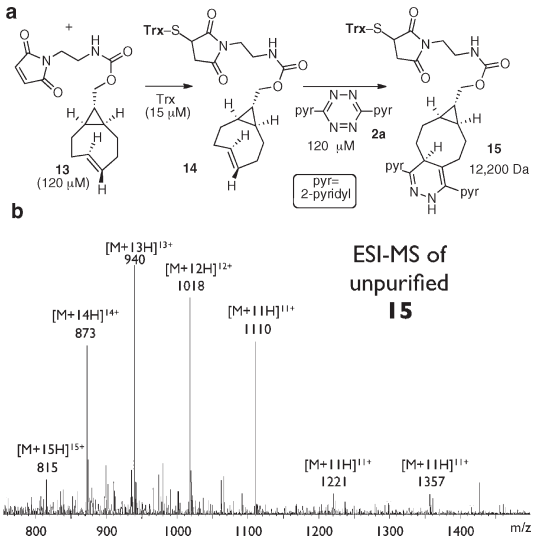


Figure 5. (a) Preparation of a thioredoxin–*trans*-cyclooctene conjugate **14**, and ligation with **2a** to give adduct **15**. (b) Analysis of **15** within 5 min of combination of **14** and **2a**.

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 h[28]. 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 form[30] 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 **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 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

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.

■ AUTHOR INFORMATION

Corresponding Author
jmfox@udel.edu

■ ACKNOWLEDGMENT

We thank NIH P20 RR017716 (NCCR COBRE) and NSF CRIF: MU, CHE 0840401 for NMR instrumentation. We thank Colin Thorpe for insightful discussions and a gift of Trx.

REFERENCES

(1) (a) Sletten, E. M.; Bertozzi, C. R. *Angew. Chem., Int. Ed.* **2009**, *48*, 6974. (b) Lim, R. K. V.; Lin, Q. *Chem. Commun.* **2010**, *46*, 1589. (c) Kalia, J.; Raines, R. T. *Curr. Org. Chem.* **2010**, *14*, 138.

(2) Click chemistry reviews: (a) Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2001**, *40*, 2004. (b) Moses, J. E.; Moorhouse, A. D. *Chem. Soc. Rev.* **2007**, *36*, 1249. (c) Best, M. D. *Biochemistry* **2009**, *48*, 6571. (d) Iha, R. K.; Wooley, K. L.; Nystrm, A. M.; Burke, D. J.; Kade, M. J.; Hawker, C. J. *Chem. Rev.* **2009**, *109*, 5620. (e) Mendal, M.; Tornøe, C. W. *Chem. Rev.* **2008**, *108*, 2952.

(3) Reviews of Cu-free bioconjugation chemistry: (a) Jewett, J. C.; Bertozzi, C. R. *Chem. Soc. Rev.* **2010**, *39*, 1272. (b) Becer, C. R.; Hoogenboom, R.; Schubert, U. S. *Angew. Chem., Int. Ed.* **2009**, *48*, 4900. (c) Baskin, J. M.; Bertozzi, C. R. *Aldrichimica Acta* **2010**, *43*, 15. (d) Lutz, J.-F. *Angew. Chem., Int. Ed.* **2008**, *47*, 2182. Selected examples: (e) Lim, R. K. V.; Lin, Q. *Chem. Commun.* **2010**, *46*, 7993. (f) Wang, Y.; Song, W.; Hu, W.; Lin, Q. *Angew. Chem., Int. Ed.* **2009**, *48*, 5330. (g) Ning, X.; Temming, R. P.; Dommerholt, J.; Guo, J.; Ania, D. B.; Debets, M. F.; Wolfert, M. A.; Boons, G. J.; van Delft, F. L. *Angew. Chem., Int. Ed.* **2010**, *49*, 3065. For the recent development of a Cu-catalyst that functions for cell-surface labeling in alkyne/azide cycloadditions:(h) Hong, V.; Steinmetz, N. F.; Manchester, M.; Finn, M. G. *Bioconjugate Chem.* **2010**, *21*, 1912.

(4) (a) Agard, N. J.; Prescher, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2004**, *126*, 15046. (b) Jewett, J. C.; Sletten, E. M.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2010**, *132*, 3688. (c) Chang, P. V.; Prescher, J. A.; Sletten, E. M.; Baskin, J. M.; Miller, I. A.; Agard, N. J.; Lo, A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 1821. (d) Codelli, J. A.; Baskin, J. M.; Agard, N. J.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2008**, *130*, 11486.

(5) (a) Poloukhtine, A. A.; Mbua, N. E.; Wolfert, M. A.; Boons, G.-J.; Popik, V. V. *J. Am. Chem. Soc.* **2009**, *131*, 15769. (b) Ning, X.; Guo, J.; Wolfert, M. A.; Boons, G.-J. *Angew. Chem., Int. Ed.* **2008**, *47*, 2253. (c) McKay, C. S.; Moran, J.; Pezacki, J. P. *Chem. Commun.* **2010**, *46*, 931.

(6) Blackman, M. L.; Royzen, M.; Fox, J. M. *J. Am. Chem. Soc.* **2008**, *130*, 13518.

(7) Royzen, M.; Yap, G. P. A.; Fox, J. M. *J. Am. Chem. Soc.* **2008**, *130*, 3760.

(8) (a) Thalhammer, F.; Wallfahrer, U.; Sauer, J. *Tetrahedron Lett.* **1990**, *31*, 6851. (b) Boger, D. L. *Chem. Rev.* **1986**, *86*, 781.

(9) Acetamido-substituted dipyridyltetrazines (**1b**) are stable for prolonged periods (>24 h) in water and toward amines and thiols in MeOH. See ref 11.

(10) Rossin, R.; Verkerk, P. R.; Bosch, S. M. v. d.; Vulders, R. C. M.; Verel, I.; Lub, J.; Robillard, M. S. *Angew. Chem., Int. Ed.* **2010**, *49*, 3375.

(11) (a) Selvaraj, R.; Liu, S.; Hassink, M.; Huang, C.-w.; Yap, L.-p.; Park, R.; Fox, J. M.; Li, Z.; Conti, P. S. *Bioorg. Med. Chem. Lett.* **2011**, doi: 10.1016/j.bmcl.2011.04.116. (b) Li, Z.; Cai, H.; Hassink, M.; Blackman, M. L.; Brown, R. C. D.; Conti, P. S.; Fox, J. M. *Chem. Commun.* **2010**, *46*, 8043.

(12) Devaraj, N. K.; Weissleder, R.; Hilderbrand, S. A. *Bioconjugate Chem.* **2008**, *19*, 2297.

(13) Pipkorn, R.; Waldeck, W.; Didinger, B.; Koch, M.; Mueller, G.; Wiessler, M.; Braun, K. *J. Peptide Sci.* **2009**, *15*, 235.

(14) (a) Devaraj, N. K.; Upadhyay, R.; Haun, J. B.; Hilderbrand, S. A.; Weissleder, R. *Angew. Chem., Int. Ed.* **2009**, *48*, 7013. (b) Devaraj, N. K.; Hilderbrand, S.; Upadhyay, R.; Mazitschek, R.; Weissleder, R. *Angew. Chem., Int. Ed.* **2010**, *19*, 2869. For a subsequent study on the same F-18 probe described in ref 11b:(c) Keliher, E. J.; Reiner, T.; Turetsky, A.; Hilderbrand, S. A.; Weissleder, R. *ChemMedChem* **2011**, *6*, 424.

(15) (a) Bach, R. D. *J. Am. Chem. Soc.* **2009**, *131*, 5233. (b) Allinger, N. L.; Sprague, J. T. *J. Am. Chem. Soc.* **1972**, *94*, 5734. (c) Leong, M. K.; Mastryukov, V. S.; Boggs, J. E. *J. Mol. Struct.* **1998**, *445*, 149. (d) Barrows, S. E.; Eberlein, T. H. *J. Chem. Educ.* **2005**, *82*, 1334.

(16) For prior synthetic studies on *cis*- and *trans*-fused derivatives of (*E*)-bicyclo[6.1.0]non-4-ene: Deyru, J. A.; Betkouski, M. F. *J. Org. Chem.* **1975**, *40*, 284.

(17) For prior calculations on Diels–Alder reactions of tetrazines and alkynes: (a) Cioslowski, J.; Sauer, J.; Hetzenegger, J.; Karcber, T.; Hierstetter, T. *J. Am. Chem. Soc.* **1993**, *115*, 1353. For prior calculations on reactions between *trans*-cyclooctene and azides, see ref 15a and(b) Schoenebeck, F.; Ess, D. H.; Jones, G. O.; Houk, K. N. *J. Am. Chem. Soc.* **2009**, *131*, 8121. (c) Ess, D. H.; Houk, K. N. *J. Am. Chem. Soc.* **2008**, *130*, 10187.

(18) A barrier of $\Delta G^{\ddagger}$ = 7.12 kcal/mol for the reaction between **1a** and *s*-tetrazine was also calculated at the MP4SDTQ/6-31G(d)//MP2/6-311+G(d,p) level of theory. This calculation is in good agreement with the barrier of 8.92 kcal/mol calculated using the M06L/6-311+G-(d,p) method (see Figure 3 and the Supporting Information, Table 3S). Calculations at the B3LYP/6-311+G(d,p) level overestimated the barrier for the reaction between **1a** and *s*-tetrazine relative to MP4SDTQ/6-31G(d)//MP2/6-311+G(d,p).

(19) Jones, G. O.; Guner, V. A.; Houk, K. N. *J. Phys. Chem. A* **2006**, *110*, 1216.

(20) k_{rel} for **3**/**1a** was determined according to the equation $\Delta\Delta G^{\ddagger} = -RT \ln(k1/k2)$.

(21) (a) Dommerholt, J.; Schmidt, S.; Temming, R.; Hendriks, L. J. A.; Rutjes, F. P. J. T.; van Hest, J. C. M.; Lefeber, D. J.; Friedl, F.; van Delft, F. L. *Angew. Chem., Int. Ed.* **2010**, *49*, 9422. (b) Ast, W.; Rheinwald, G.; Kerber, R. *Makromol. Chem.* **1976**, *177*, 1349. (c) Anciaux, A. J.; Hubert, A. J.; Noels, A. F.; Petiniot, N.; Teyssié, P. *J. Org. Chem.* **1980**, *45*, 695.

(22) Dehmlow, E. V.; Plueckebaum, O. *J. Chem. Res., Miniprint* **2001**, *4*, 451.

(23) Aminal formation has been observed in studies on 3,4-diaza-norcaradienes with alcohols and water. Steigel, A.; Sauer, J.; Kleier, D. A.; Binsch, G. *J. Am. Chem. Soc.* **1972**, *94*, 2770.

(24) In CD_3CN with 10 equiv of D_2O, the conversion of **8** to **8b** was 50% complete after 60 h. In 10% (202 equiv) D_2O/CD_3CN, the conversion to **8b** was slower (12% after 84 h) and hemiaminals **8a** could be observed by ^{1}H NMR as **8** converted to **8b** over the course of 206 h. In CD_3OD, only 10% of **8** remained after 12 h, with 90% aminal formation from CD_3OD. In CD_3OD, the formation of **8b** was not observed until after 96 h, at which point 7% of **8b** was observed. See the Supporting Information for full experimental details.

(25) Wijnen, J. W.; Zavarise, S.; Engberts, J. B. F. N.; Charton, M. *J. Org. Chem.* **1996**, *61*, 2001.

(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 ^{1}H 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 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 Biol. Med.* **1999**, *27*, 322.

(29) (a) Ning, X.; Guo, J.; Wolfert, M. A.; Boons, G. *Angew. Chem., Int. Ed.* **2008**, *47*, 2253. (b) Krishnamurthy, V. R.; Wilson, J. T.; Cui, W.; Song, X.; Yi, L.; Cummings, R. D.; Chaikof, E. L. *Langmuir* **2010**, *26*, 7675.

(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.