J|A|C|S
COMMUNICATIONS

Published on Web 09/18/2008

Tetrazine Ligation: Fast Bioconjugation Based on Inverse-Electron-Demand Diels−Alder Reactivity

Melissa L. Blackman, Maksim Royzen, and Joseph M. Fox*

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

Received July 11, 2008; E-mail: jmfox@udel.edu

Described herein is a bioorthogonal reaction that proceeds with unusually fast reaction rates without need for catalysis: the cycloaddition of *s*-tetrazine and *trans*-cyclooctene derivatives. The reactions tolerate a broad range of functionality and proceed in high yield in organic solvents, water, cell media, or cell lysate. The rate of the ligation between *trans*-cyclooctene and 3,6-di-(2-pyridyl)-*s*-tetrazine is very rapid (k_2 2000 M^{-1} s^{-1}). This fast reactivity enables protein modification at low concentration.

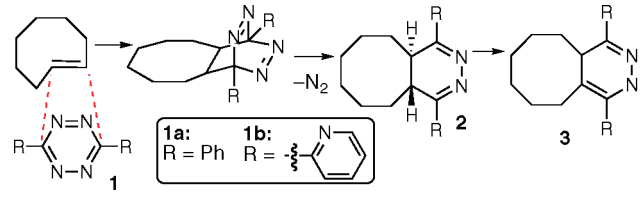
Bioorthogonal reactions, unnatural transformations that are unaffected by biological functionality, are broadly useful tools with applications that span synthesis, chemical biology, and materials science.[1] The utility of bioorthogonal reactivity has been augmented by recent developments in post-translational strategies for incorporating bioorthogonal functionality into proteins.[2] Bioorthogonal reactions must be exceptionally fast to be useful at the low concentrations relevant to many biological applications. Recently, Bertozzi and co-workers described strain-driven click reactions that take advantage of the intrinsic reactivity of cyclooctyne toward organic azides,[3,4] and work by Bertozzi[4a−d] and by Boons[4e] has shown that click reactions of cyclooctyne derivatives are fast (up to k_2 2.3 M^{-1} s^{-1}).[4] Importantly, this method avoids Cu-catalysts, which are cytotoxic, and the enhanced reactivity enables applications for dynamic *in vivo* imaging.[4] Recently, Lin and co-workers elegantly described bioconjugation based on a photoinducible 1,3-dipolar cycloaddition reaction that proceeds with fast rates (k_2 11 M^{-1} s^{-1}) with acrylamide.[5] Faster ligation chemistry will allow for the assembly of complex biomaterials under dilute conditions. Ultimately, fast bioconjugation reactions should facilitate the intracellular assembly of molecular structures that are too large to cross cell membranes.

Unlike normal-electron-demand Diels−Alder chemistry,[6] inverse-electron demand Diels−Alder reactions have not previously been applied to bioconjugation. Tetrazines are voracious dienes for inverse-electron-demand Diels−Alder reactions, and N_2 is produced as the only byproduct upon subsequent retro-[4 + 2] cycloaddition.[7] In 1990, Sauer described the kinetics of electron-deficient tetrazines (Scheme 1, structure **1**, where R = CO_2Me or CF_3) with a number of dienophiles and quantitatively demonstrated that their reactions with strained alkenes are exceptionally fast.[8] The most reactive dienophile is *trans*-cyclooctene, which is 7 orders of magnitude more reactive than *cis*-cyclooctene toward these tetrazines.[8] In protic solvents, the 4,5-dihydropyridizine **2** rapidly rearranges to isomeric **3**.

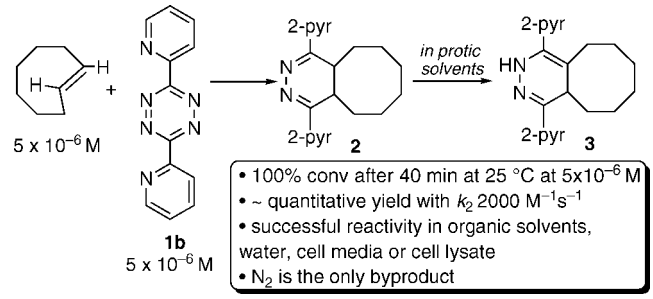
We surmised that such fast and selective reactivity could form the basis of a powerful bioorthogonal reaction. However, Sauer's conditions could not be directly applied to bioconjugation, as the tetrazines that he studied immediately react with water.[9] From a survey of substituted *s*-tetrazines, 3,6-diaryl-*s*-tetrazines were identified as suitable derivatives for bioorthogonal reactivity.[10]

As a model study, it was shown that **1b** and *trans*-cyclooctene combine to give **3** in quantitative yield after epimerization (Scheme 2). In separate experiments, EtSH (100 mM) and $BuNH_2$ (100 mM) were introduced to *trans*-cyclooctene (120 mM) before combination

Scheme 1. Diels−Alder Reactions of Tetrazines with *trans*-Cyclooctene



Scheme 2. Fast Reactivity at Low Micromolar Concentrations



with **1b** (100 mM), but these had no effect on the efficiency of the Diels−Alder reaction. As more stringent tests of tolerance of biological functionality, it was shown that the reaction of **1b** and *trans*-cyclooctene can be carried out in cell media (DMEM +5% FBS) or in an aqueous solution containing 10% untreated rabbit reticulocyte lysate. The reactions were carried out at rt for 1 h with 50 μM **1b** and 500 μM *trans*-cyclooctene and monitored by ESI-MS. The yields in cell lysate and media were estimated to be >80% (vs internal MS standards).

The second-order rate constant for **1b** + *trans*-cyclooctene at 25 °C is k_2 2000 (± 400) M^{-1} s^{-1} in 9:1 methanol/water. As anticipated,[10] there is a hydrophobic effect for the Diels−Alder reaction: slower rates were observed for reactions in pure methanol [k_2 1140 (± 40) M^{-1} s^{-1}] and in THF [k_2 400 (± 20) M^{-1} s^{-1}]. The reaction to form **2** is complete within 40 min at 25 °C at 5 μM in THF without using an excess of either **1b** or *trans*-cyclooctene. The half-life is 7 s when **1b** (20 μM) is reacted with excess *trans*-cyclooctene (200 μM) in 9:1 methanol/water at 25 °C. The reaction between **1b** and *trans*-cyclooctene is much faster than background reactivity toward water or exogenous nucleophiles.[9,11] Further, **1a** also displays fast reactivity toward *trans*-cyclooctene [k_2 3.1 (± 0.1) M^{-1} s^{-1} in THF] and does not display background reactivity toward BuSH or $BuNH_2$ after 8 h at rt.[11,12]

The practicality of the tetrazine ligation is augmented by facile access to starting materials. *trans*-Cyclooctene derivative **5** is readily accessible from *cis*-cyclooctene **4** by a photochemical protocol that we described recently (Scheme 3a).[13] Functionalized analogues of **1a** are known.[14] Unsymmetrical tetrazine **8** can be prepared in gram quantities from the reaction of hydrazine with commercially

10.1021/ja8053805 CCC: $40.75

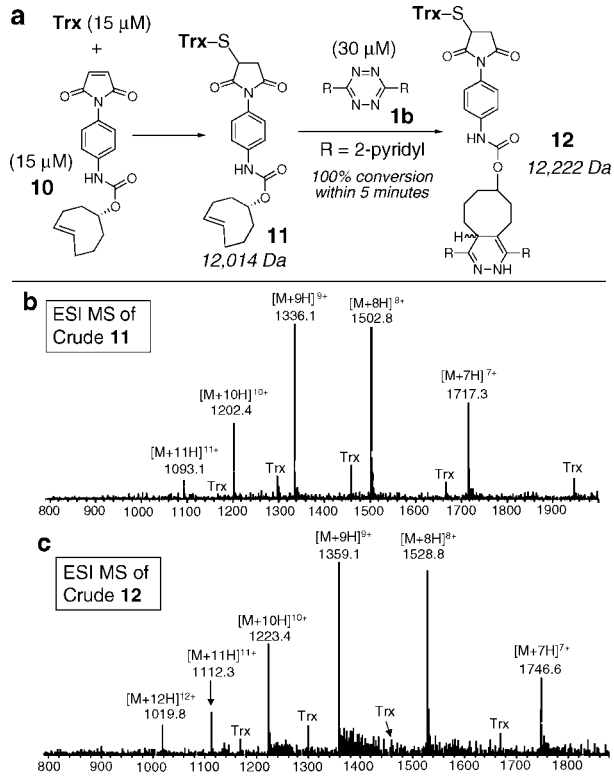
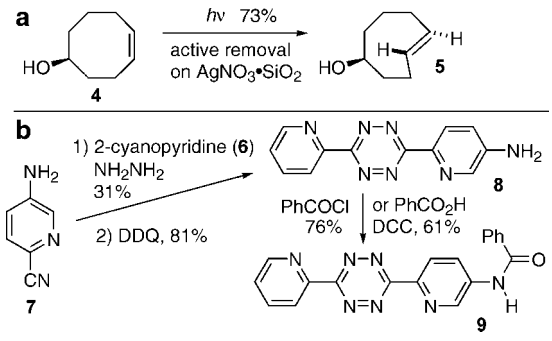


Figure 1. (a) Rapid reactivity to form **12** was monitored by ESI-MS and HPLC. (b,c) Crude ESI-MS data for **11** and **12** in experiments that began with 15 μM Trx.

Scheme 3. Synthesis of *trans*-Cyclooctene and Tetrazine Derivatives



available 5-amino-2-cyanopyridine (**7**) and 2-cyanopyridine (**6**) (Scheme 3b). The amino group of tetrazine **8** provides a handle for functionalization via acyl transfer (e.g., **9**).[15]

To illustrate compatibility of the tetrazine ligation with proteins, we functionalized thioredoxin (Trx) with *trans*-cyclooctene derivative **10**. Trx is a 11.7 kDa protein that contains a single disulfide. Upon reduction, the solvent exposed cysteine can be selectively functionalized by maleimides.[16] Thus, Trx (15 μM) was reduced with tri(3-hydroxypropyl)phosphine (THP, 1 mM) and combined with **10** (15 μM) in acetate buffer (pH 6). ESI mass spectral analysis (Figure 1b) indicated that most of the Trx had been consumed and that the conjugate **11** had formed. Subsequent combination of **11** with **1b** (30 μM) indicated that the formation of **12** was complete within 5 min. A control experiment with the *cis*-cyclooctene analogue of **10** gave the analogue of **11**, but reaction with **1b** did not give the analogue of **12** even after 24 h.

HPLC was also used to monitor the bioconjugation reactions that gave **11** and **12** and to demonstrate that the tetrazine ligation to form **12** was fast and high yielding (see Supporting Information).

In summary, a new method for bioconjugation based on inverse-electron demand Diels−Alder chemistry has been described. The reaction proceeds with very fast rates and tolerates a broad range of biological functionality.

Acknowledgment. This work was supported by NIH Grant GM068640-01. We thank Colin Thorpe for donating Trx and Danny Ramadan for HPLC assistance. We thank Ryan Mehl for insight into the aqueous stability of **1b**. We thank Colin Thorpe and John Koh for insightful discussions.

Supporting Information Available: Experiments in which HPLC was used to monitor bioconjugation reactions are described. Full experimental details and ^{1}H,^{13}C NMR spectra are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2001**, *40*, 2004. (b) Kohn, M.; Breinbauer, R. *Angew. Chem., Int. Ed.* **2004**, *43*, 3106. (c) Chen, I.; Ting, A. Y. *Curr. Opin. Biotechnol.* **2005**, *16*, 35. (d) Antos, J. M.; Francis, M. B. *Curr. Opin. Chem. Biol.* **2006**, *10*, 253. (e) Hahn, M. E.; Muir, T. W. *Trends Biochem. Sci.* **2005**, *30*, 26. (f) Soellner, M. B.; Nilsson, B. L.; Raines, R. T. *J. Am. Chem. Soc.* **2006**, *128*, 8820. (g) Lin, F. L.; Hoyt, H. M.; van Halbeek, H.; Bergman, R. G.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2005**, *127*, 2686. (h) Kolakowski, R. V.; Shangguan, N.; Sauers, R. R.; Williams, L. J. *J. Am. Chem. Soc.* **2006**, *128*, 5695.

(2) Recent examples: Duckworth, B. P.; Xu, J. H.; Taton, T. A.; Guo, A.; Distefano, M. D. *Bioconjugate Chem.* **2006**, *17*, 967. (b) Gauchet, C.; Labadie, G. R.; Poulter, C. D. *J. Am. Chem. Soc.* **2006**, *128*, 9274. (c) Esser-Kahn, A. P.; Francis, M. B. *Angew. Chem., Int. Ed.* **2008**, *47*, 3751.

(3) (a) Wittig, G.; Krebs, A. *Chem. Ber.* **1961**, *94*, 3260.

(4) (a) Agard, N. J.; Prescher, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2004**, *126*, 15046. (b) Baskin, J. M.; Prescher, J. A.; Laughlin, S. T.; Agard, N. J.; Chang, P. V.; Miller, I. A.; Lo, A.; Codelli, J. A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 16793. (c) Codelli, J. A.; Baskin, J. M.; Agard, N. J.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2008**, *130*, 11486. (d) Sletten, E. M.; Bertozzi, C. R. *Org. Lett.* **2008**, *10*, 3097. (e) Ning, X. H.; Guo, J.; Wolfert, M. A.; Boons, G.-J. *Angew. Chem., Int. Ed.* **2008**, *47*, 2253.

(5) Song, W.; Wang, Y.; Qu, J.; Madden, M. M.; Lin, Q. *Angew. Chem., Int. Ed.* **2008**, *47*, 2832. (b) Song, W.; Wang, Y.; Qu, J.; Lin, Q. *J. Am. Chem. Soc.* **2008**, *130*, 9654.

(6) (a) Dantas de Araujo, A.; Palomo, J. M.; Cramer, J.; Seitz, O.; Alexandrov, K.; Waldmann, H. *Angew. Chem., Int. Ed.* **2006**, *45*, 296. (b) Latham-Timmons, H. A.; Wolter, A.; Roach, J. S. *Nucleosides, Nucleotides Nucleic Acids* **2003**, *22*, 1495. (c) Yousaf, M. N.; Houseman, B. T.; Mrksich, M. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 5992. (d) Yousaf, M. N.; Mrksich, M. *J. Am. Chem. Soc.* **1999**, *121*, 4286. (e) Seelig, B.; Jaschke, A. *Tetrahedron Lett.* **1997**, *38*, 7729.

(7) Boger, D. L. *Chem. Rev.* **1986**, *86*, 781.

(8) Thalhammer, F.; Wallfahrer, U.; Sauer, J. *Tetrahedron Lett.* **1990**, *31*, 6851.

(9) Hunter, D.; Nielson, D. G.; Tweakley, T., Jr. *J. Chem. Soc., Perkin Trans. 1* **1985**, 2709, and references therein.

(10) For Diels−Alder reactions of styrene and **1b** in water: Wijnen, J. W.; Zavarise, S.; Engberts, J. B. F. N.; Charton, M. *J. Org. Chem.* **1996**, *61*, 2001.

(11) No reaction was observed by ^{1}H NMR after 8 h when **1a** (2×10^{-2} M) was combined with $BuNH_2$ (2×10^{-2} M) or EtSH (2×10^{-2} M) in CD_3OD. When a CD_3OD solution of **1b** (2×10^{-2} M) was combined with $BuNH_2$ (2×10^{-2} M), 50% of **1b** had reacted after 4 h. In a similar experiment with EtSH (2×10^{-2} M), 50% of **1b** had reacted after 10 min. When **1b** (2×10^{-2} M) was allowed to stir in pure water, 20% decomposition of **1b** was noted after 2 h.

(12) The product from *trans*-cyclooctene and **1a** aromatizes upon workup. See: Padwa, A.; Rodriguez, A.; Tohidi, M.; Fukunaga, T. *J. Am. Chem. Soc.* **1983**, *105*, 933.

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

(14) Soloducho, J.; Doskocz, J.; Cabaj, J.; Roszak, S. *Tetrahedron* **2003**, *59*, 4761.

(15) Acyl transfer to **8** was most effective when aromatic acylating agents were employed. When aliphatic acylating agents were used, undesired reactivity (presumably via ketene formation) competes with the rate of acylation. It is likely that the rate of acyl transfer to **8** is slow because it is a very electron-deficient aniline. Sugars and peptides can be appended to aromatic acylating agents via terephthaloyl linkers, e.g.: (a) Angelastro, M. R.; Baugh, L. E.; Bey, P.; Burkhart, J. P.; Chen, T.-M.; Durham, S. L.; Hare, C. M.; Huber, E. W.; Janusz, M. J.; Koehl, J. R.; Marquart, A. L.; Mehdi, S.; Peet, N. P. *J. Med. Chem.* **1994**, *37*, 4538. (b) Kanie, O.; Grotenbreg, G.; Wong, C.-H. *Angew. Chem., Int. Ed.* **2000**, *39*, 4545. (c) Czifrák, K.; Hadady, Z.; Docsa, T.; Gergely, P.; Schmidt, J.; Wessjohann, L.; Somsák, L. *Carbohydr. Res.* **2006**, *341*, 947.

(16) Kallis, G.−B.; Holmgren, A. *J. Biol. Chem.* **1980**, *255*, 10261.

JA8053805