The Tetrazine Ligation: Fast Bioconjugation based on Inverse-electron-demand Diels-Alder Reactivity

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Abstract
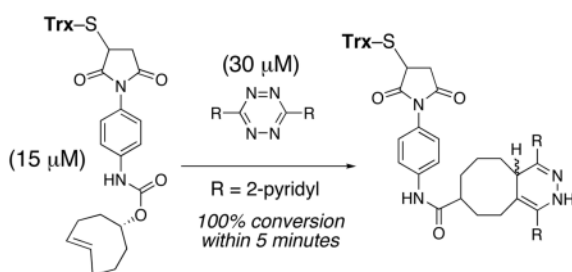
Described is a bioorthogonal reaction that proceeds with unusually fast reaction rates without need for catalysis: the cycloaddition of *s*-tetrazine and *trans*-cyclooctene derivatives. The reactions tolerate a broad range of functionality, and proceed in high yield in organic solvents, water, cell media or in 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.

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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 in order to be useful at the low concentrations relevant to many biological applications. Recently, Bertozzi and coworkers 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 coworkers 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

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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 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 1h 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 analogs of **1a** are known.[14] Unsymmetrical tetrazine **8** can be prepared in gram quantities from the reaction of hydrazine with commercially 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 (Fig

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 analog of **10** gave the analog of **11**, but reaction with **1b** did not give the analog 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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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.

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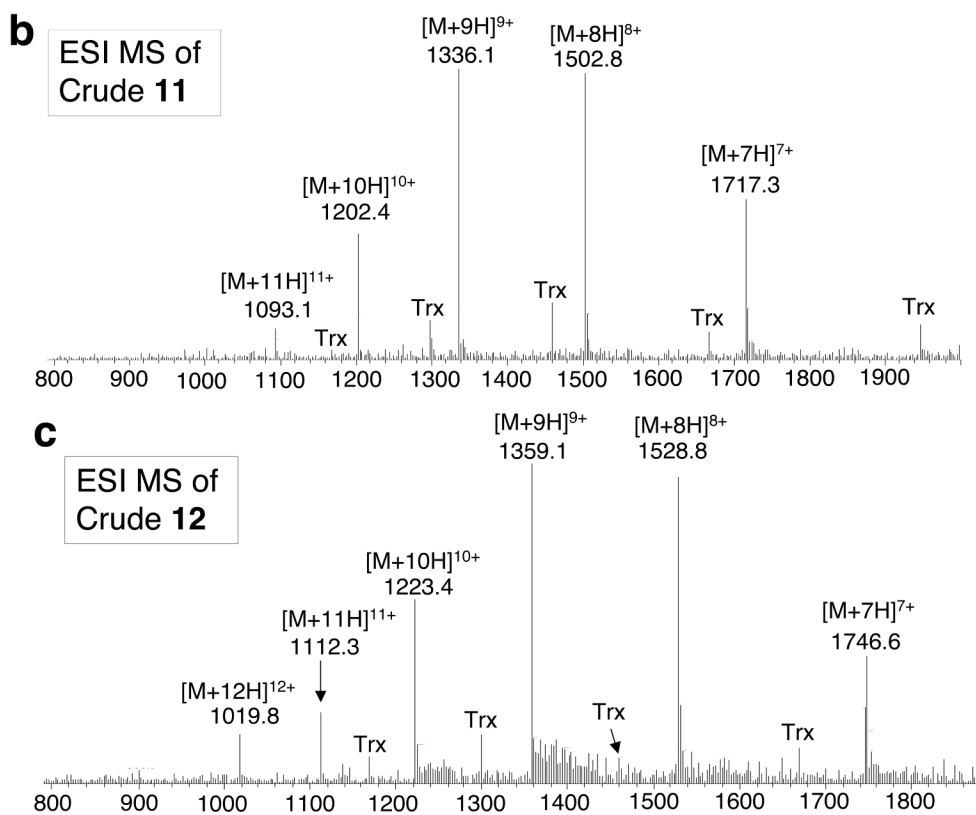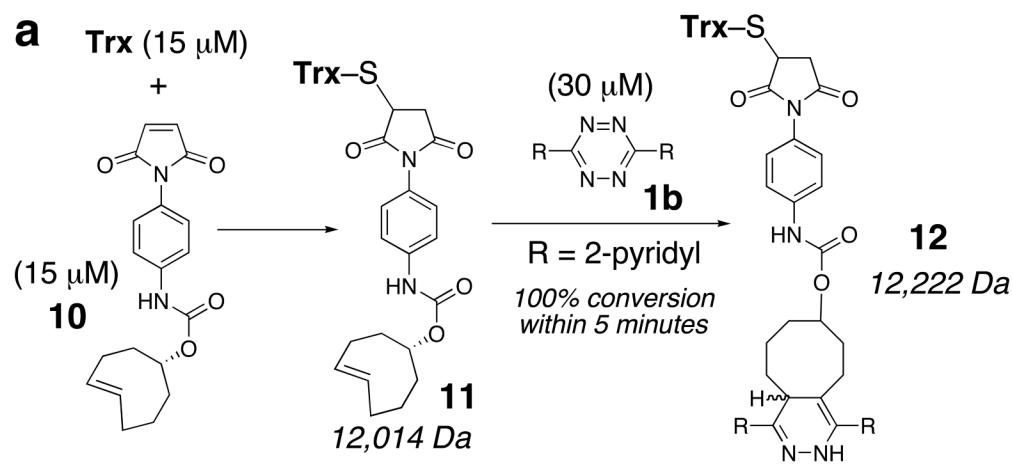16. Kallis G-B, Holmgren A. J Biol Chem 1980;255:10261. [PubMed: 7000775]
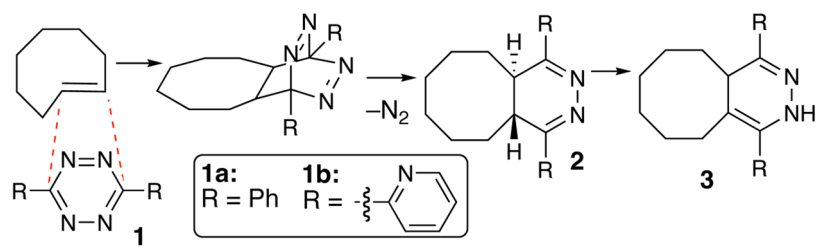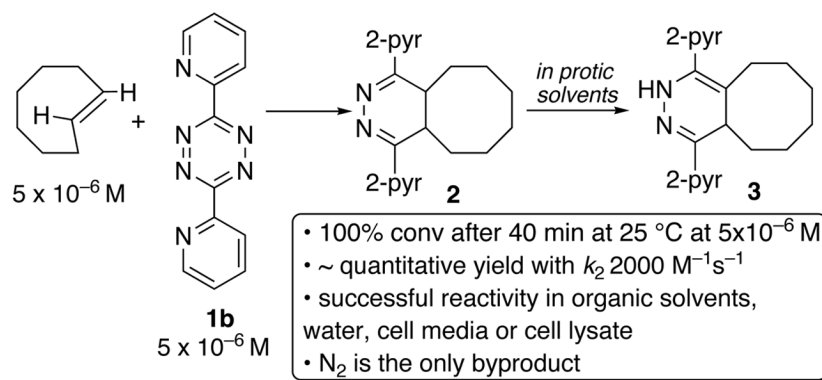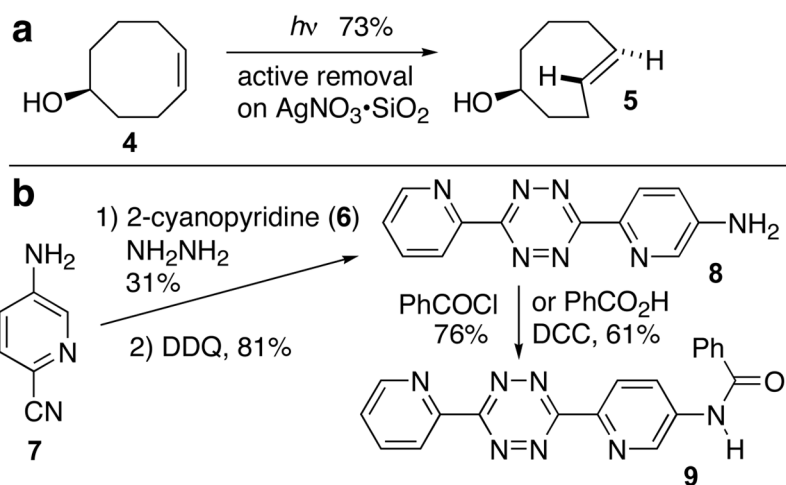
Figure 1.
(a) Rapid reactivity to form **12** was monitored by ESI-MS and HPLC. (b,c) Crude ESI-MS data for **11** and **12** in experiments that began with 15 μM Trx.

Scheme 1.
Diels-Alder reactions of tetrazines with *trans*-cyclooctene

Scheme 2.
Fast reactivity at low micromolar concentrations

Scheme 3.
Synthesis of *trans*-cyclooctene and tetrazine derivatives