

Communication

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1,2,4-Triazines Are Versatile Bioorthogonal Reagents

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Supporting Information Placeholder

ABSTRACT: A new class of bioorthogonal reagents—1,2,4-triazines—is described. These scaffolds are stable in biological media and capable of robust reactivity with *trans*-cyclooctene (TCO). The enhanced stability of the triazine scaffold enabled its direct use in recombinant protein production. The triazine-TCO reaction can also be used in tandem with other bioorthogonal cycloaddition reactions. These features fill current voids in the bioorthogonal toolkit.

The bioorthogonal chemical reporter strategy has emerged as a robust method for visualizing biomolecule structures and functions.¹ This strategy involves the attachment of bioinert functional groups (i.e., chemical reporters) to biomolecules of interest; the reporters are detected in a second step via selective (i.e., bioorthogonal) reactions with complementary probes.^{2,3} Numerous chemical reporters and bioorthogonal reactions have been reported in recent years, but significant limitations remain.¹⁴ Many of the reagents are too bulky for general use or prone to hydrolysis in cellular environments. Moreover, several popular bioorthogonal reagents cross-react with one another and cannot be used concurrently to visualize collections of biomolecules.⁴

To address these issues and expand the scope of the chemical reporter strategy, new bioorthogonal reactions—and combinations of reactions—are being pursued. In recent years, we and others have developed compatible chemistries based on cyclopropenes and other strained alkenes.⁵⁻⁸ These motifs are stable in physiological environments and have been used to target numerous biomolecules in live cells.⁹⁻¹² In nearly all cases, the strained alkenes were detected via inverse-electron-demand Diels-Alder (IED-DA) reactions with 1,2,4,5-tetrazines. A handful of tetrazine ligations can also be used simultaneously with azide-alkyne cycloadditions,^{5,11} setting the stage for multi-component bioorthogonal imaging *in vivo*.^{8,11,13}

While much attention has been paid to strained alkenes for bioorthogonal reaction development, less attention has been given to the other half of the IED-DA reaction: the electron-deficient dienes. To date, tetrazines have dominated the IED-DA landscape.¹⁴ These moieties react robustly with *trans*-cyclooctene (TCO) and other strained dienophiles in a variety of settings.¹⁵ Unfortunately, the most rapid-reacting tetrazines also tend to be the least stable in cells and *in vivo*.¹⁶ Tetrazines are prone to hydrolysis and side reactions with endogenous thiols, limiting their applications in the most stringent environments (e.g., inside cells).¹⁷⁻¹⁹ More stable tetrazines are being pursued, but these reagents remain large in size.²⁰

To develop improved bioorthogonal IED-DA reactions, we were drawn to triazine scaffolds. 1,2,4-Triazines have been identified in microbial natural products and pigments, suggesting that they are stable in physiological environments.²¹⁻²³ These motifs also react efficiently with electron-rich alkenes in IED-DA reactions.²⁴⁻²⁹ Boger further showed that 1,2,3-triazines react with electron-rich dienophiles.³⁰ To compare the intrinsic DA reactivities of 1,2,3- and 1,2,4-triazines with that of 1,2,4,5-tetrazine, we evaluated the activation free energies for their reactions with ethylene by density functional theory (DFT) calculations (Figure 1A, Table S1).³¹⁻³³ The computational analysis suggested that 1,2,4-triazine is much more reactive than 1,2,3-triazine (activation free energy: 29.3 versus 41.0 kcal/mol), but less reactive than 1,2,4,5-tetrazine (29.3 versus 21.9 kcal/mol). This is consistent with the inverse-electron-demand nature of the cycloaddition: the LUMO+1 (the π^* orbital that interacts with the dienophile HOMO in the DA reaction) of 1,2,4-triazine is increased by 0.49 eV as compared to 1,2,4,5-tetrazine (2.18 versus 1.69 eV, Figures 1A and S1). While highly reactive, tetrazines are prone to decomposition by biological nucleophiles.¹⁸⁻²⁰ Seitz and co-workers found that thiols rapidly decompose tetrazines via 1,4-addition and subsequent release of nitrogen.³⁴ DFT calculations revealed that formation of the 1,4-adduct of 3-phenyl-1,2,4,5-tetrazine and methanethiol is endergonic by 23.4

kcal/mol in water, and that the overall barrier for N₂ release is 28.6 kcal/mol (Figure 1B). However, the corresponding adduct and transition state of 6-phenyl-1,2,4-triazine are significantly higher in energy. This implies that 6-aryl-1,2,4-triazine is quite inert to thiol relative to monoaryl tetrazine, although both are very similar in size. Thus, while 1,2,4-triazine is less reactive in the IED-DA reaction, considering the extremely fast rates of the tetrazine-TCO cycloaddition ($k_2 = 10^2$ – 10^4 M⁻¹ s⁻¹),¹⁷ we hypothesized that triazines would be good candidates for bioorthogonal reaction development based on their size and stability.

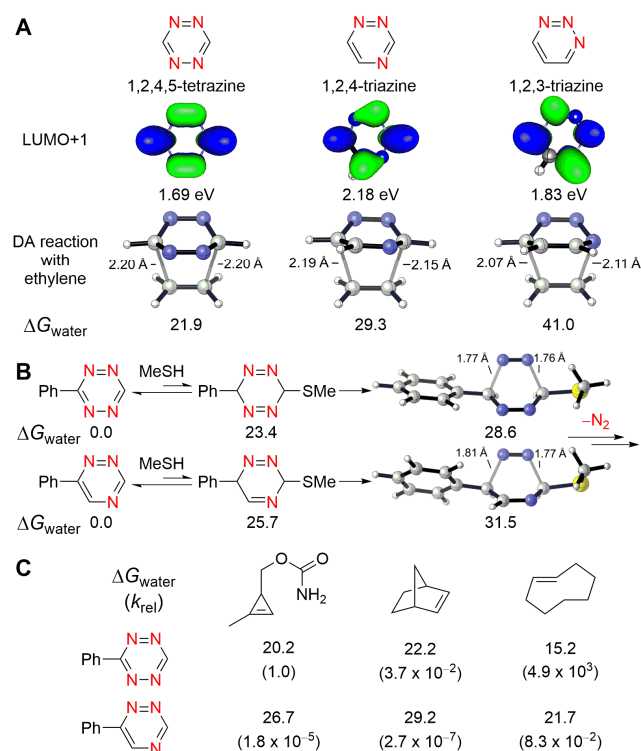


Figure 1. (A) Diels-Alder reactions of 1,2,4,5-tetrazine, 1,2,4-triazine, and 1,2,3-triazine with ethylene. LUMO+1 energies were computed with HF/6-311+G(d,p)//M06-2X/6-31G(d), and activation free energies (in kcal/mol) in water were computed with CPCM(water)-M06-2X/6-311+G(d,p)//M06-2X/6-31G(d). (B) Energetics of 1,4-adduct formation and subsequent N₂ release transition state for methanethiol and 3-phenyl-1,2,4,5-tetrazine or 6-phenyl-1,2,4-triazine. (C) DFT-computed activation free energies and predicted relative rate constants for tetrazine or triazine cycloaddition with 3-carbamoyloxymethyl-1-methylcyclopropene, norbornene, or *trans*-cyclooctene, in water at 25 °C.

We also predicted relative rate constants for the DA reactions of 3-phenyl-1,2,4,5-tetrazine or 6-phenyl-1,2,4-triazine with 3-carbamoyloxymethyl-1-methylcyclopropene, norbornene, and *trans*-cyclooctene (Figures 1C and S2). These data suggest that 6-aryl-1,2,4-triazines react efficiently with TCO, yet remain inert to other bioorthogonal scaffolds, including cyclopropene and norbornene. This unique reactivity profile

could potentially be exploited for ‘orthogonal’ bioorthogonal cycloaddition development.^{35–37}

To test these hypotheses, we synthesized a panel of substituted 1,2,4-triazines using the reaction sequence pictured in Scheme 1. In brief, glyoxal was condensed with aminoguanidine to afford 3-amino-1,2,4-triazine (**1**). Bromination of this scaffold provided a convenient handle for diversification, and triazine **2** ultimately underwent Suzuki couplings with a variety of commercially available boronic acids (Schemes 1 and S1, top). Subsequent deamination of the products afforded triazines **4–9**. To access triazines containing nucleophilic substituents (**10** and **11**), the reverse sequence—deamination/Suzuki coupling—was employed (Schemes 1 and S1, bottom). This short reaction scheme can be used to access 6-substituted triazines with a broad array of functionality. By contrast, traditional syntheses of tetrazines are typically not compatible with free amino groups owing to the harsh oxidants employed, although milder conditions have recently been reported.^{38,39}

Scheme 1. Synthesis of Functionalized 1,2,4-Triazines

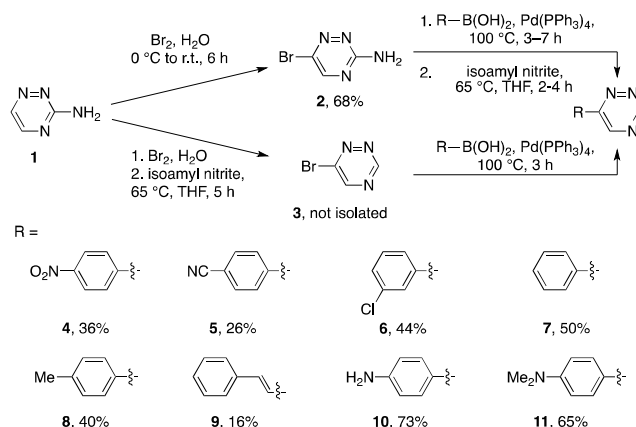


Table 1. Second-Order Rate Constants for the Triazine-TCO Ligation

Entry	R =	k_2 (× 10 ⁻² M ⁻¹ s ⁻¹)	Entry	R =	k_2 (× 10 ⁻² M ⁻¹ s ⁻¹)
1	Me ₂ N-Ph	1.2 ± 0.1	5	Ph	3.4 ± 0.5
2	H ₂ N-Ph	1.3 ± 0.2	6	p-Cl-Ph	4.7 ± 0.2
3	Ph-CH=CH ₂	2.3 ± 0.1	7	p-NC-Ph	6.4 ± 0.2
4	Me-Ph	3.4 ± 0.4	8	p-O ₂ N-Ph	7.5 ± 2.0

With the panel of triazines in hand, we analyzed their reactivities with TCO (Table 1 and Figures S3–S5). The reactions were monitored by ¹H-NMR, and oxidized cycloadducts were observed (Figure S6). As expected, the most electron-poor triazine **4** exhibited the fastest

rate (Table 1, entry 8), consistent with the inverse-electron-demand nature of the reaction. No reactivity was observed when electron-rich scaffolds **1** and **2** were incubated with TCO (Figures S7-S8). The triazine-TCO reaction is significantly slower than many of the tetrazine-TCO ligations,¹⁸ but on par with several copper-free click chemistries,^{40,41} and some IED-DA reactions with stabilized tetrazines.^{20,39} Hammett analysis of the triazine-TCO rate constants gave a slope of $\rho = 0.49$ (Figure 2). This value is consistent with concerted IED-DA reactions and suggests that only partial charge accumulates during the reaction.

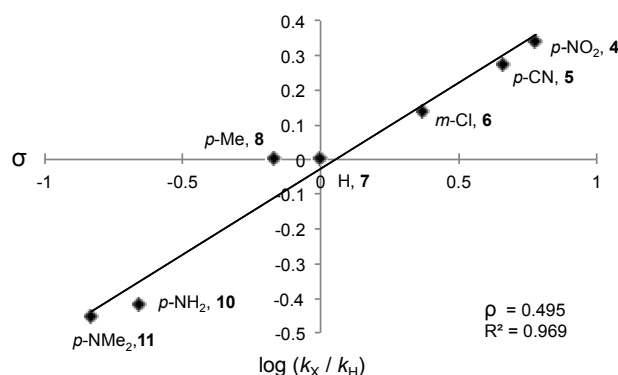
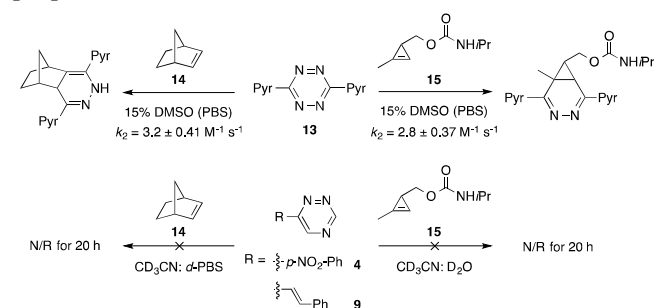


Figure 2. Hammett plot for the reactions between TCO **12** and a panel of 1,2,4-triazines. Sigma (σ) values derive from ref 42

As predicted by our DFT calculations, no ligation was observed between the more reactive triazines (**4** and **9**) and other strained alkenes, including norbornene (**14**) and cyclopropene (**15**) (Scheme 2, Figures S9-S12). These alkenes do react robustly with the common tetrazine reagent **13** (Figure S5), suggesting that triazines and combinations of other bioorthogonal reagents can be used in tandem.

Scheme 2. Comparison of Tetrazine and Triazine Cycloadditions with Norbornene or 1,3-Disubstituted Cyclopropene^a



^a The rate constant for **15** + **13** is from ref 8

The triazine scaffold also excels in a key aspect of bioorthogonality: stability. When mono-substituted triazines were dissolved in a mixture of *d*-PBS and CD₃CN, they remained stable for over one week at 37 °C (Figures S13-S16). Triazine scaffolds were also inert to cysteine over a similar time period (Figures S17-21). These

results are in sharp contrast to mono-substituted tetrazines that have been observed to hydrolyze and/or react with cysteine under similar conditions (Figure S22).^{17-20,39}

The remarkable stability of the triazine scaffold suggested immediate application in environs that have been difficult to access with bioorthogonal reagents, including recombinant protein production in intracellular environments. Disubstituted tetrazines and cyclopropenes have been previously incorporated into recombinant proteins and tagged with TCO or tetrazine probes, respectively.^{9,43} However, mono-substituted tetrazines and TCO have been more difficult to incorporate directly into proteins, due to the length of time required for protein production and the instability of the scaffolds.^{19,43} Mono-substituted triazines offer unique advantages in terms of their size and stability.

To demonstrate that mono-substituted triazines are compatible with protein labeling and sufficiently stable for genetic code expansion, we synthesized triazine amino acid **16** (Figure 3A). We screened a panel of seven *Methanocaldococcus jannaschii* tyrosyl tRNA synthetase (RS)/tRNA_{CUA} pairs for permissivity toward **16**, while maintaining fidelity against canonical AAs (Figure S23).^{43,44} The *M. jannaschii* (RS)/tRNA_{CUA} pairs were previously evolved to incorporate non-canonical amino acids (ncAAs) of similar structure (**S10**, Figure S23) in response to an amber codon.⁴⁵ One of the seven RS/tRNA_{CUA} pairs efficiently incorporated **16** in response to an amber codon-disrupted GFP gene, resulting in expression of 18.8 mg/L of GFP-**16** in the presence of **16** (Figure 3B, lane 3).

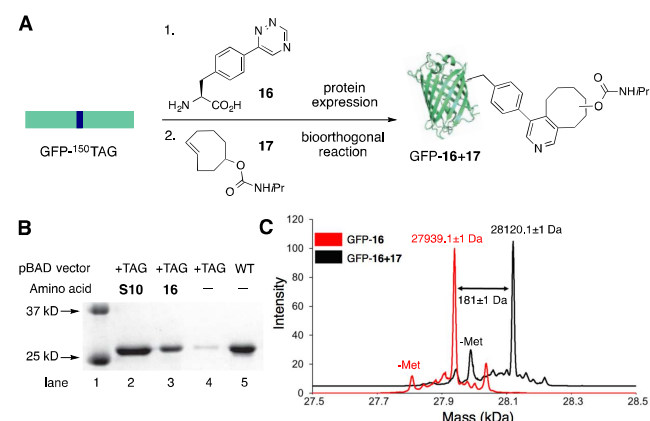


Figure 3. Triazines are suitable for recombinant protein production. (A) Genetic incorporation of **16** into proteins and reaction with TCO (B) SDS-PAGE analysis of site-specific incorporation in response to amber codon 150 in GFP. (C) MS analysis of GFP-**16** shows a single major peak at 27939.1±1 Da. Reaction of GFP-**16** with TCO shows a single major peak at 28120.1±1 Da, consistent with the expected mass increase from selective reaction with TCO.

To verify that **16** is stable in complex media and can be incorporated into recombinant proteins, we compared the masses of GFP-**16** to GFP-wt using ESI-Q mass

analysis. Native GFP-wt has a mass of 27826.0 ± 1 Da and GFP-16 exhibited the expected increase to 27939.1 ± 1 Da, verifying that 16 is incorporated at a single site (Figures 3C and S24). To determine whether the triazine/TCO ligation is also quantitative on proteins, pure GFP-16 (10 μ M) was incubated with TCO 17 (1 mM) in PBS (pH 7.0). ESI-Q mass analysis confirmed quantitative conversion of GFP-16 to GFP-16+17 (expected 28120.7 Da; observed 28120.1 ± 1 Da, Figure 3C). These results demonstrate that triazines are stable in cells and can be incorporated into proteins efficiently and with high fidelity using genetic code expansion. Furthermore, the triazine/TCO ligation is suitable for site-specific protein labeling applications.

In summary, we identified 1,2,4-triazines as a new class of bioorthogonal reagents. These scaffolds are remarkably stable in aqueous buffers, in the presence of biological nucleophiles, and in cells. Triazines can be easily assembled and decorated with diverse functional groups to tune reactivities. Triazines also react efficiently and selectively with TCO. These features render triazines suitable for a variety of intracellular applications, and we showed that a triazine amino acid can be efficiently incorporated into recombinant proteins and labeled site-specifically with TCO. Triazines are also compatible with other strained alkenes, and will enable different types of IED-DA reactions to be performed in tandem in cellular environments.

ASSOCIATED CONTENT

Experimental details, full spectroscopic data for new compounds, and computational details. This material is available free of charge via the Internet at <http://pubs.acs.org>

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Notes

The authors declare no competing financial interest.

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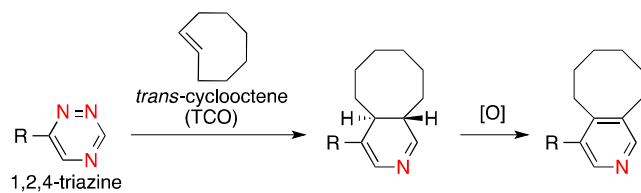
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- inert to cysteine
- reacts selectively with TCO
- suitable for recombinant protein production

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