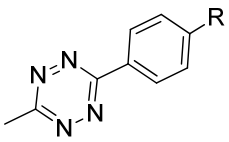
Click Chemistry Tools
Bioconjugate Technology Company

http://www.clickchemistrytools.com
tel: 480 584 3340
fax: 866 717 2037

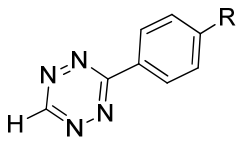
Tetrazine Selection Guide

The inverse electron demand cycloaddition reaction has recently emerged as powerful tool for exploring various aspects of biological systems. This reaction has gained popularity due to the potential for extremely fast cycloaddition kinetics with *trans*-cyclooctene (TCO) as the dienophile. Applications have included fluorescent labeling of cancer cells, *in vivo* cancer cell imaging with ^{111}In, ^{18}F radiolabeling, cancer detection applications, and bioconjugation.

Most commercially available tetrazines are either hydrogen or methyl substituted. Stability, kinetics, and solubility in aqueous media vary dramatically with slight changes in the nature of substituents. Therefore, it is increasingly important to be able to identify tetrazine candidates most ideally suited for a particular application.



Methyl substituted tetrazines



Hydrogen substituted tetrazines

Stability

In general, tetrazines with stronger electron withdrawing groups showed lower stability than hydrogen substituted tetrazines while the electron donating alkyl substituted tetrazines exhibited the highest stability. A recent study (Karver, M.K., et al, *Bioconjugate Chem*. **2011**, 22, 2263−2270) showed that hydrogen substituted tetrazines rapidly decompose in FBS at 37 °C, with less than 50% of intact tetrazines remaining in solution only after 10 hours. At the same time, the electron donating alkyl substituted tetrazines exhibited very little decomposition. Our studies revealed that methyl substituted tetrazines tolerate a much wider range of reagents and chemical transformations. In addition, proteins labeled with methyl substituted tetrazine retained reactivity toward TCO compounds over a long period of time. On average, we observe about 10-20% loss of reactivity toward TCO compounds after 4 weeks at 4°C, while proteins labeled with hydrogen substituted tetrazines lost most (>80%) of their reactivity toward TCO under identical conditions.

Kinetics

Hydrogen substituted tetrazines demonstrate exceptionally fast kinetics (up to 30 000 $M^{-1} s^{-1}$), generally at least 10 fold faster compared to methyl substituted tetrazines. Even relatively slow methyl substituted tetrazines (k ~1000 $M^{-1}s^{-1}$) react with TCO compounds at a much faster rate than any other bioorthogonal reaction pairs described to date. Despite demonstrating relatively slow kinetics, methyl substituted tetrazines were shown to be suitable for bioorthogonal use based on pre-targeted cancer cell labeling studies using flow cytometry and fluorescence microscopy (Devaraj, N. K., et al *Bioconjugate Chem.* 2008 19, 2297–2299).

We also studied the utility of this ligation pair (methyl substituted tetrazine-TCO) for protein-protein conjugation under highly dilute conditions. We demonstrated that goat IgG at 50 µg/mL (0.333 µM) activated with methyl-tetrazine can be converted into IgG-HRP conjugate with only 5 fold excess of TCO-activated HRP in 90 min with no detectable amount of unconjugated IgG remaining.

Solubility

In addition to these trends in kinetics and stability, other factors such as aqueous solubility should be considered before selecting tetrazines for a given application. Usually, 3,6-diaryl tetrazines demonstrate lower aqueous solubility than those with methyl or hydrogen substituents. For example, 3,6-diphenyl tetrazine is not soluble to any measurable amount in 100% water. The incorporation of hydrophilic PEG spacers dramatically increases solubility in aqueous media and reduces the aggregation of labeled proteins upon prolonged storage.

Making the Right Choice

The choice of a particular tetrazine depends on the individual application, and a one-size-fits-all approach might not be favorable. A wide range of applications can be distinctively separated into two groups, applications where extremely fast kinetics is required, and applications where chemical stability is critical.

In applications such as protein-protein crosslinking, or antibody-drug conjugations (ADC), where extremely fast cycloaddition kinetics may not be as critical and exceptional chemical stability would be more beneficial, methyl substituted tetrazines have the advantage. This is due to the fact that methyl substituted tetrazines are able to withstand more harsh chemical environments and can endure long-term storage in solution.

On the other hand, in applications such as *in vivo* cancer imaging or for pre-targeted cell labeling studies where rapid reaction kinetics are desired, a faster hydrogen substituted tetrazine with acceptable chemical stability would be a logical choice.

http://www.clickchemistrytools.com
tel: 480 584 3340
fax: 866 717 2037