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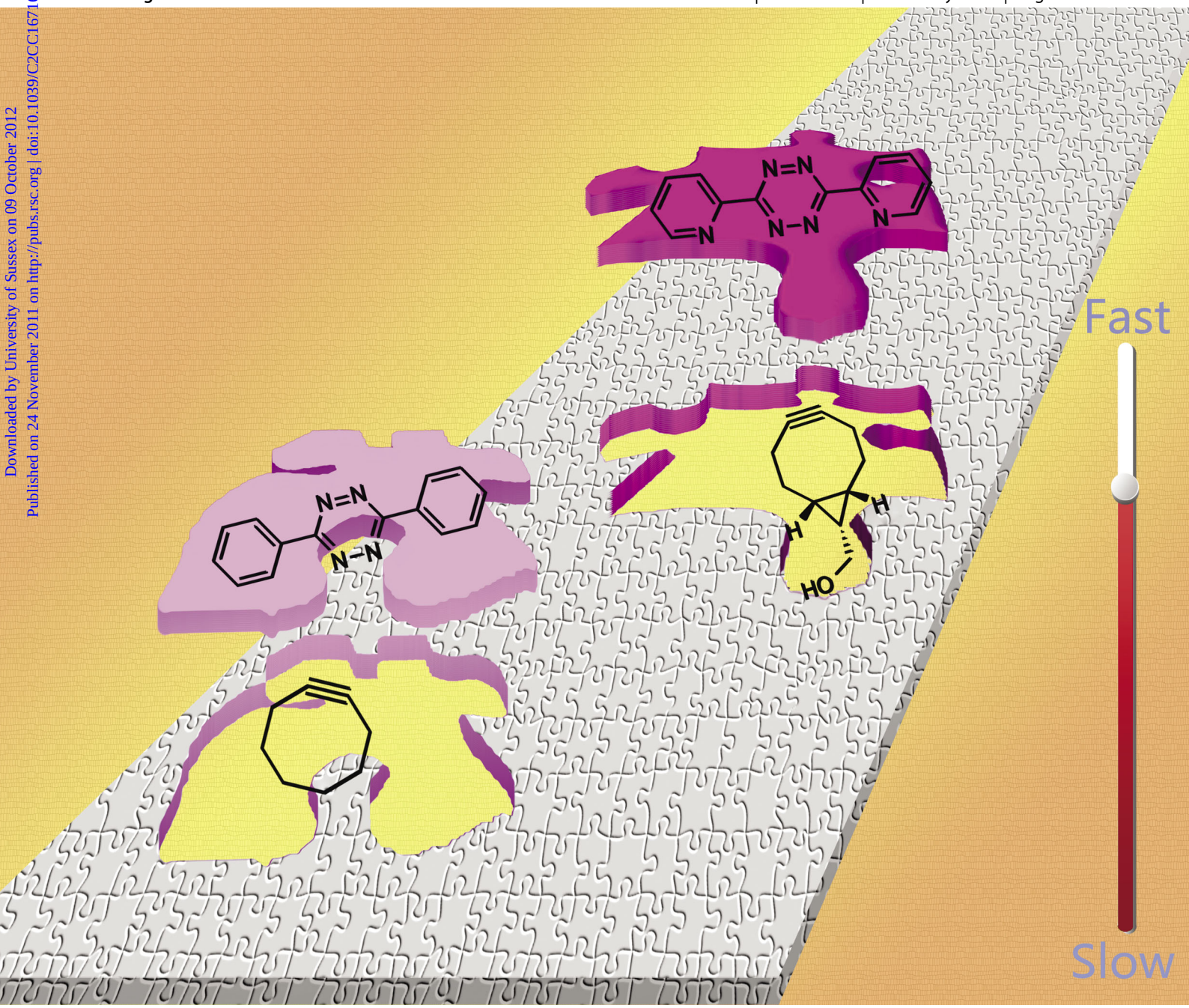

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Clicking 1,2,4,5-tetrazine and cyclooctynes with tunable reaction rates†

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Substituted tetrazines have been found to undergo facile inverse electron demand Diels–Alder reactions with "tunable" reaction rates.

Click chemistry is playing an increasingly important role in a variety of areas of research[1–8] including bioorthogonal labeling.[9–13] Critical to this field is the availability of reactions and reagents that allow for facile and specific reactions. Many reagents have been identified that serve this purpose very well in different situations. These include difluorinated cyclooctyne (DIFO),[14] dibenzocyclooxtyne (DIBO),[5,15] aza-dibenzocyclooctyne (DIBAC),[16] and biarylazacyclooctynone (BARAC).[17] All these belong to one type of reaction: 1,3-dipolar cycloaddition involving an azido and an alkynyl group. However, this field needs more such reactions in its arsenal for reasons of multiplexing, labeling diversity, biocompatibility under different conditions, and compatibility with other functional groups, as well as reaction conditions for reagent preparation. In addition, rate difference among various click reactions can also be exploited for selective labeling.[18] Thus having click reactions with sufficiently different reaction rates will be very useful. Fox *et al.*[19,20] and Weissleder[6] have developed a reaction using tetrazine and a strained *trans*-alkene. The reaction is very fast with a second order rate constant of 2000 to 22 000 $M^{-1}s^{-1}$, making it very useful for biolabeling applications. In our DNA labeling work,[21–23] we are interested in developing fast click reactions with tunable reaction rates and reactants that can be easily prepared. We thus turned to inverse-electron demand Diels–Alder reactions (IEDDA) involving an alkyne and tetrazine for several reasons. First, Schuster *et al.*[24] have demonstrated that un-substituted tetrazine can react very quickly with alkenes and alkynes. Second, many strained alkynes have been reported, giving us a set of "tools" for the intended studies. Third, unlike azido compounds, tetrazine's reactivity can be tuned by manipulating its electron deficiency through the introduction of functional groups at the 3,6-position. In our case, we need reactions to be fast enough so that at 10 μM concentrations, the half-life is no more than 2 h. This requires the second order rate constant to be higher than 20 M^{-1} s^{-1} at ambient temperature. This rate is higher than that of most strained promoted azido-alkyne cycloadditions (SPAAC) reported thus far.

The reaction between un-substituted tetrazine and cyclooctyne (**1**) has been reported with a stated second order rate constant of 27.1 M^{-1} s^{-1}. However the highly volatile 1,2,4,5-tetrazine is not suitable for any labeling work.[24] In our initial studies, we found the second order rate constant for the reaction between cyclooctyne (**1**) and diphenyltetrazine (**2**) to be 0.07 M^{-1} s^{-1}. This rate is similar to that of a typical SPAAC reaction[8] and is slower than what we need. Thus we undertook the effort to explore ways to enhance the reaction rates by performing modifications guided by computational work.

The reaction between cyclooctyne (**1**) and a substituted tetrazine (**2**) leads to significant changes in the UV-vis spectrum of tetrazine due to its conversion to a 1,2-diazine product (Fig. 1). Hence, the reaction can be easily monitored. We first studied the reaction between cyclooctyne (**1**) and 3,6-diphenyl-1,2,4,5-tetrazine (**2**) in dry methanol as a reference. The rate constant for the reaction between diphenyltetrazine and cyclooctyne was found to be 0.07 M^{-1} s^{-1} (Table 1), which is far smaller than that of the reaction between un-substituted tetrazine and cyclooctyne. Since the reaction between 1,2,4,5-tetrazines and a cyclooctyne depends on the $LUMO_{diene}$–$HOMO_{phil}$ gap,[25–27] we were interested in exploring ways to lower the LUMO of the diene or elevate the HOMO of the dienophile in order to facilitate the reaction. In such a case, it is reasonable to expect that electron donating substituents, as well as high strain energy will increase the HOMO energy of the dienophiles, and electron withdrawing substituents will decrease the LUMO energy of the diene, leading to a decrease in the $LUMO_{diene}$–$HOMO_{phil}$ gap and consequently an increase in the reaction rate.

We then conducted quantum mechanics (QM) calculation using known strained alkynes and *trans*-cyclooctene and some modified tetrazines. From Fig. 2, one can see that relative to cyclooctyne (**1**), the HOMO of BCN[28] (**4**) is 1.5 kcal mol^{-1} higher, giving this a chance to have increased reactivity. Installing electron withdrawing groups on cyclooctynes decreases the HOMO energy substantially. For example, computational results indicate that the HOMO energy decreases by 6.1 kcal mol^{-1} for DIFO[14] (**7**) and 11.5 kcal mol^{-1} for fluorocyclooctyne[29] (**8**) relative to cyclooctyne. Correspondingly, di-pyridine substituted 1,2,4,5-tetrazine **9** lowers the LUMO energy by 2.3 kcal mol^{-1} relative to 3,6-diphenyl-1,2,4,5-tetrazine (**2**) (Fig. 2). Such calculations suggest that the BCN–tetrazine pair could have significantly improved reactivity.

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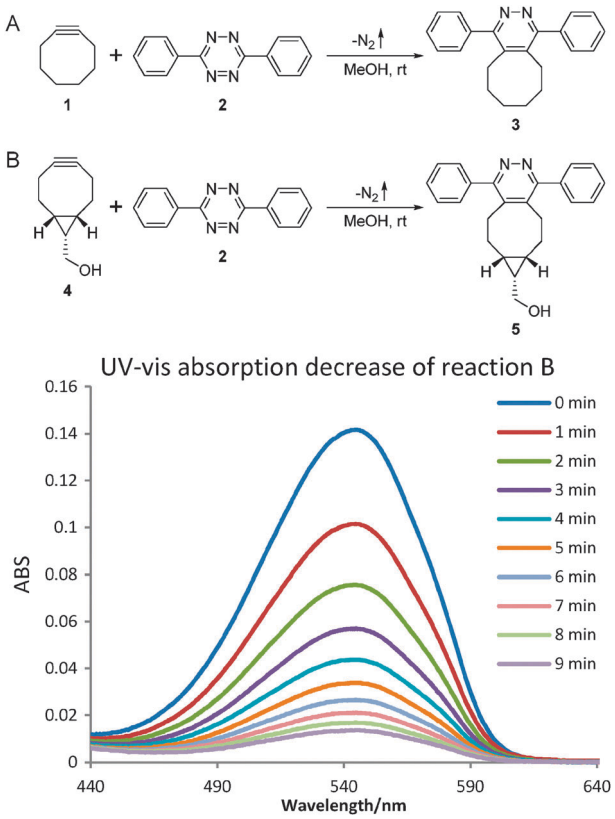

Fig. 1 Strain-promoted [4+2] cycloadditions of 1,2,4,5-tetrazine **2** and cyclooctynes **4**. Tetrazine: 1 mM, alkyne: 10 mM in dried MeOH.

Table 1 Second order rate constants of cyclooctynes with tetrazines

	1	4
2	$(7.0 \pm 0.7) \times 10^{-2}$ M^{-1} s^{-1}	3.3 ± 0.4 M^{-1} s^{-1}
9	2.0 ± 0.3 M^{-1} s^{-1}	44.8 ± 4.9 M^{-1} s^{-1}
10	ND	40.9 ± 13.8 M^{-1} s^{-1}

Indeed, when we tested the reactivity of diphenyltetrazine (**2**) with **4** at a 1 : 10 ratio (tetrazine: 1 mM; alkyne: 10 mM), the reaction finished within 10 minutes (Fig. 1), with a second order rate constant of 3.3 M^{-1} s^{-1} (Table 1). This represents a 47-fold improvement in reaction rate compared to the reaction between cyclooctyne and diphenyltetrazine. To further accelerate the reaction, two electron-withdrawing groups were attached to the tetrazine ring to give **9**. The second order rate constant for the reaction between 3,6-di-2-pyridyl-1,2,4,5-tetrazine (**9**) and alkyne **4** in dry MeOH at ambient temperature was found to be 44.8 M^{-1} s^{-1}, which represents a 640-fold improvement

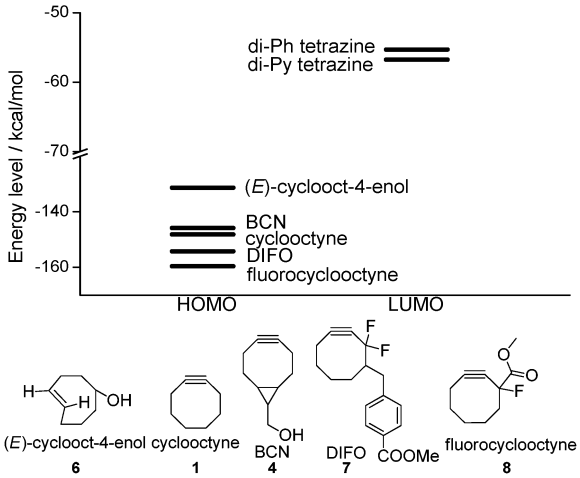

Fig. 2 Orbital energy of cyclooctynes and tetrazines.

in reaction rate over the tetrazine–cyclooctyne pair. On the other hand, **7** and **8** did not react with tetrazine **9** to an appreciable degree, as expected. To achieve a deeper understanding of the improved reaction rates, Density Functional Theory (DFT) calculations at the 6-31G** level of theory were performed to examine the possible transition state(s) and activation energies. Schematic representation of the energy profiles for the tetrazine alkyne reactions are shown in Fig. 3. All of the QM energies are in kcal mol^{-1} and are relative to the reactants (**1** and **2** at the top of Fig. 3, and **4** and **2** at the bottom of Fig. 3). The activation energy for the first step of the IEDDA reaction between **2** and **4** *via* transition state **TS1′** is 17.6 kcal mol^{-1} as compared to 23.3 kcal mol^{-1} for the reaction between cyclooctyne **1** and tetrazine **2**. The computational results support the idea that the increased HOMO energy in BCN (**4**) compared with cyclooctyne (**1**) translates into lowered activation energy and thus increased reaction rate. The intermediate **IN1** from the first step of the **1–2** reaction pair quickly loses nitrogen gas to yield product **3** by passing through a low activation barrier of 8.6 kcal mol^{-1}, with the reaction being strongly exergonic by −91.4 kcal mol^{-1}. Because of the low barrier for the second step, the reaction rate is entirely controlled by the first step of the reaction. For the reaction between BCN (**4**) and tetrazine **2**, the situation is similar except that the conversion of the intermediate **IN1′** to the final product has almost no activation barrier, with the overall reaction being strongly exergonic by −88.1 kcal mol^{-1}. All these results indicate that the IEDDA reaction and the subsequent elimination reaction take place spontaneously and irreversibly toward the pyridazine products.

With these exciting findings and resulting tunable reaction rates, we turned our attention to developing the chemistry needed for bioconjugation. In order to do so, a "handle" needs to be installed on the tetrazine system. Therefore 4-(6-(pyrimidin-2-yl)-1,2,4,5-tetrazin-3-yl)benzoic acid[30] was prepared. As a model, isopropyl amine was chosen for conjugation with the benzoyl chloride tetrazine derivative. The reaction between isopropyl amide tetrazine **10** and BCN (**4**) gave a second order rate constant of 40.9 M^{-1} s^{-1}, with half-lives of 24 seconds and 0.68 hour at mM and 10 μM concentrations, respectively. This is among the fastest click reactions, and can be used for a variety of labeling work.

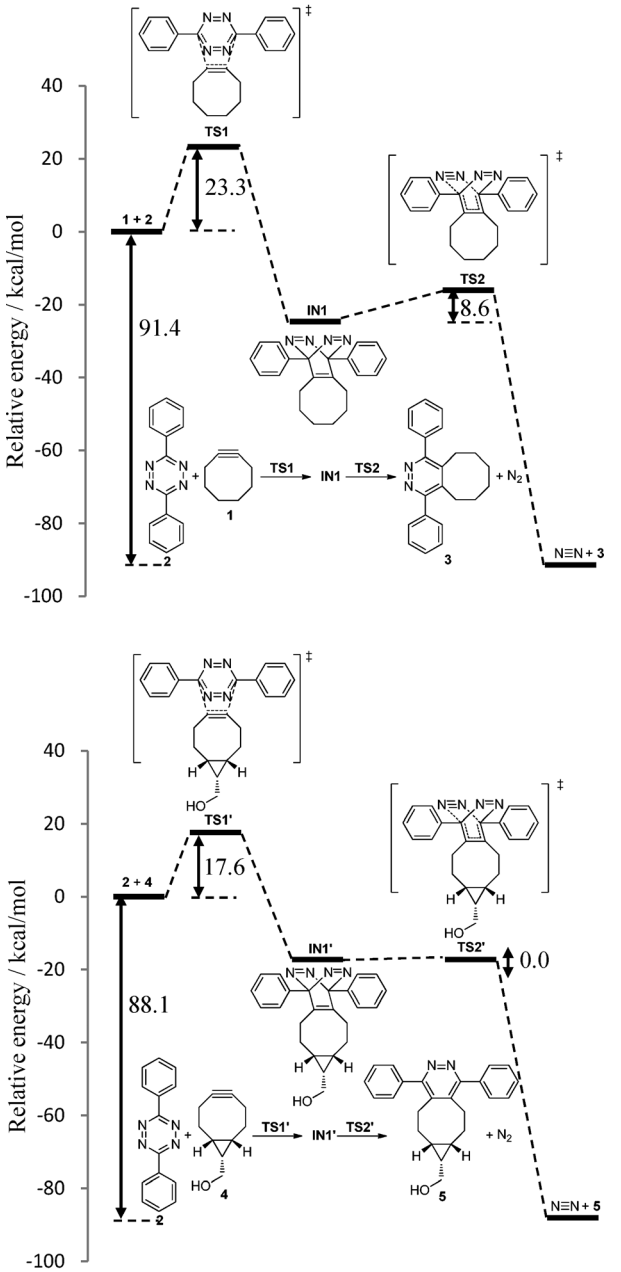

Fig. 3 Schematic representations (energy *vs.* reaction coordinate) of the reaction between diphenyl tetrazine and strained alkynes.

In summary, the reaction rate of inverse electron-demand Diels–Alder reactions involving 1,2,4,5-tetrazines and cyclooctynes can be tuned to allow for facile conjugation. The reaction rates found are among the fastest involving a tetrazine and alkyne. Furthermore, the wide range of reaction rates (over 640-fold difference) that can be achieved suggests possibilities of using such chemistry for mutiplexing click labeling. Work is underway to utilize BCN–tetrazine conjugation reactions for DNA and other labeling work.

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