Bioorthogonal Chemistry

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Fast Alkene Functionalization In Vivo by Photoclick Chemistry: HOMO Lifting of Nitrile Imine Dipoles**

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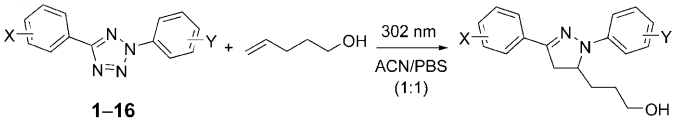
In combination with genetic encoding, bioorthogonal chemistry provides a powerful covalent strategy to probe biomolecular dynamics and function in living systems.[1] A number of bioorthogonal reactions have been successfully developed, including copper(I)-catalyzed cycloaddition ("click chemistry"),[2] strain-promoted cycloaddition,[3] Staudinger ligation,[4] photoinduced cycloaddition ("photoclick chemistry"),[5] and tetrazine ligation.[6] When a bioorthogonal reaction is applied to the study of biomolecular dynamics, it is imperative that the reaction proceeds in a time frame shorter than the half-lives of the biomolecules, particularly in the case of short-lived proteins, such as the endogenous tumor-suppressor protein p53, which has a half-life of only 40 minutes.[7]

We recently reported a photoinduced 1,3-dipolar cycloaddition reaction for the selective functionalization of an unactivated alkene in *Escherichia coli*.[5b] Although the reaction proceeded selectively, the cycloaddition rate was rather low ($k_2 = 0.00202 \pm 0.00007\ M^{-1}s^{-1}$). To accelerate concerted cycloaddition reactions, two effects have commonly been exploited: ring strain and the fluorine effect,[3] both of which lower the LUMO energy of the dipolarophile. Because enzymes involved in biosynthetic/metabolic pathways process nonnatural substrates that are similar in structure to the natural substrates, it is typically easier to encode small groups, such as the allyl group, into biomolecules than it is to incorporate bulky groups. Therefore, it is more desirable to increase the reactivity of the nitrile imine dipoles in our photoclick chemistry. Since the reaction rate is inversely related to free-energy gap between the highest occupied molecular orbital (HOMO) of the dipole and the lowest unoccupied molecular orbital (LUMO) of the dipolarophile for type-I 1,3-dipolar cycloaddition,[8] we envisioned that the cycloaddition reaction could be accelerated by raising the HOMO energy of the dipole. Herein, we report the identification of several simple, yet powerful tetrazole reagents for the photoclick reactions through systematic tuning of the HOMO energies of the nitrile imines. Extremely fast (<1 min) labeling of an alkene-containing protein in *E. coli* was observed with one of these reagents.

To examine the electronic effect of the nitrile imine on the cycloaddition reaction, we synthesized two series of tetrazoles with substituents on either the C5 phenyl ring (X) or the N2 phenyl ring (Y). With water-soluble 4-penten-1-ol as a model for the unactivated alkene dipolarophile, we measured the cycloaddition rate constants (k_2) in ACN/PBS buffer (1:1; Table 1).[9] In the first series (tetrazoles **1**–**5**), the exchange of an electron-withdrawing group (EWG; **1** and **2**) for an electron-donating group (EDG; **4** and **5**) as the X substituent led to a substantial increase in the reaction rate, whereas the reaction of the unsubstituted diphenyltetrazole **3** proceeded almost as fast as the reactions of **4** and **5**. Therefore, we decided to leave X as H, and varied the substitution of the N2 phenyl ring to generate the second series of substrates (tetrazoles **6**–**16**). We found that the presence of an EDG as the Y substituent (in **8**–**16**) generally led to a rate acceleration (relative to the reaction rate of **3**), whereas the presence of an electron-withdrawing substituent (in **6** and **7**) resulted in a rate decrease (Table 1). The substituent effect is clearly

Table 1: Photoinduced cycloaddition reactions of tetrazoles with 4-penten-1-ol.[a]



1–16

Tetrazole	X	Y	k_2 [$M^{-1}s^{-1}$]	E_{HOMO} [eV][b]
1	*p*-CO_2Me	H	0.004	−7.0193
2	*p*-CN	H	0.001	−7.1419
3	H	H	0.13	−6.7517
4	*p*-Me	H	0.18	−6.6447
5	*p*-OMe	H	0.15	−6.6529
6	H	*p*-CN	0.01	−7.2631
7	H	*p*-CO_2Et	0.02	−7.1403
8	H	*p*-Me	0.27	−6.5881
9	H	*p*-NHAc	0.26	−6.4098
10	H	*p*-OMe	0.52	−6.4735
11	H	*p*-NH_2	0.79	−6.1994
12	H	*o*-OMe	0.29	−6.7826
13	H	*m*-OMe	0.19	−6.8114
14	H	*o*,*p*-$(OMe)_2$	0.51	−6.6072
15	H	*m*,*p*-$(OMe)_2$	0.35	−6.7184
16	H	*m*,*m*-$(OMe)_2$	0.11	−6.8873

[a] A 200 μL mixture of the tetrazole (100 μM) and 4-penten-1-ol (10 mM) in a quartz test tube was irradiated with a handheld UV lamp at 302 nm. [b] HOMO energy of the nitrile imine intermediate. ACN = acetonitrile, PBS = phosphate-buffered saline.

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position-dependent, whereby an electron-withdrawing substituent at the *para* position produces the most pronounced rate enhancement (compare **10**, **12**, and **13**; **14**–**16**). The fastest rate was observed for tetrazole **11**, which contains a *para* NH_2 group. With a k_2 value of $0.79\,M^{-1}s^{-1}$, this reaction was nearly 200 times as fast as the equivalent reaction with tetrazole **1**, which we used previously.[5b] As expected, the yield of the cycloaddition product was highest with tetrazole **11** (ca. 50% after 75 s); the product of quenching by water (ca. 10%)[5a] and the dimerization product (ca. 15%)[10] were formed as the major side products.

We calculated the HOMO energies, E_{HOMO}, of the nitrile imine intermediates derived from tetrazoles **1**–**16** (Table 1)[9] and plotted E_{HOMO} against log(rate) to gauge the electronic effect on the reactivity of the nitrile imines (Figure 1). The good linear fit found ($R^2 = 0.86$) suggests that the observed rate enhancement is primarily due to the HOMO-lifting effect.

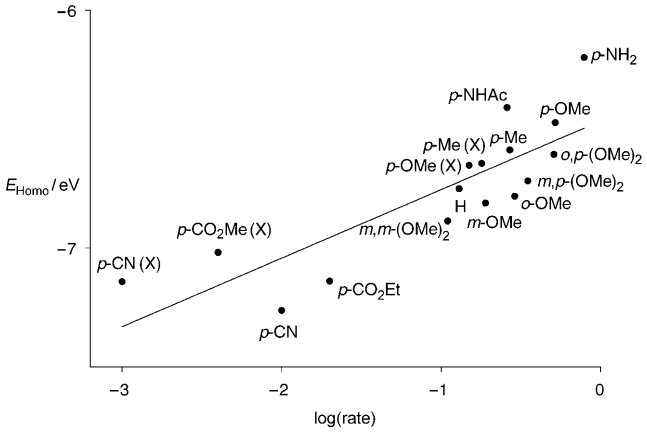

Figure 1. The cycloaddition rate is dependent on the HOMO energy of the nitrile imine intermediate. The HOMO energies of the nitrile imines were calculated by using the Hartree–Fock 3-21G model based on the AM1-optimized geometries (see the Supporting Information for details).

To determine whether the HOMO-lifted tetrazoles provide fast labeling of the *O*-allyltyrosine-encoded Z-domain protein (alkene-Z), we first compared the reaction kinetics of tetrazoles **10** and **1** toward allyl phenyl ether (a small-molecule substrate in place of *O*-allyltyrosine) in PBS buffer.[11] Tetrazole **10** exhibited robust kinetics with a k_2 value of $0.95\,M^{-1}s^{-1}$ (Figure 2a): a 475-fold faster reaction rate than that of tetrazole **1** ($k_2 = 0.00202\,M^{-1}s^{-1}$).[5b] We then incubated tetrazole **10** with alkene-Z and followed the reaction progress over a 4-min period by measuring the relative fluorescence intensities of the corresponding pyrazoline–Z cycloadduct (Figure 2b). After photoirradiation for 30 s, conversion into the cycloadduct was greater than 40% with tetrazole **10**; with tetrazole **1** under identical conditions, conversion into the cycloadduct was only 5%. Thus, significantly faster kinetics for the labeling of alkene-Z protein in vitro was observed for tetrazole **10**.

To assess the yield and selectivity of the labeling of alkene-Z by tetrazoles, alkene-Z and the wild-type Z protein

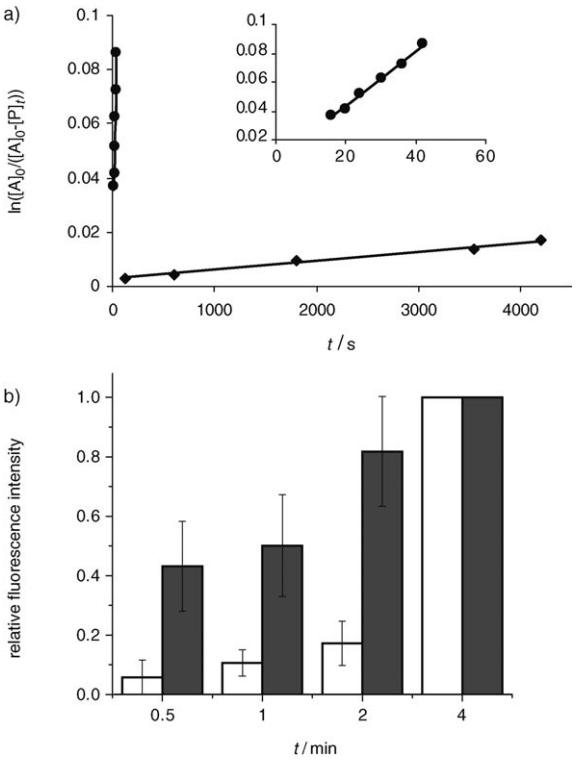

Figure 2. a) Kinetic study of the cycloaddition reactions of tetrazoles **1** (◆) and **10** (●) with allyl phenyl ether. The insert shows the kinetics of the reaction with tetrazole **10** in more detail. b) Kinetic study of alkene-Z labeling by tetrazoles **1** (white bars) and **10** (black bars). The fluorescence intensities at 4 min were treated as 100% for each reaction and used to derive the relative intensities. The concentrations of alkene-Z and the tetrazoles were 10 μM and 200 μM, respectively. Error bars show the standard deviation for three independent measurements.

were incubated separately with **17**, a tetrazole linked to monodisperse poly(ethylene glycol) (mPEG, MW ≈ 5 kDa),[9] for 2 hours after photoirradiation for 2 minutes (Figure 3a). A higher-molecular-weight band was observed more distinctly with alkene-Z (approximately 20% estimated yield as measured by densitometry) than with wild-type Z (Figure 3b, left).[12] This band coincides with a fluorescent band detected by in-gel fluorescence imaging of the same gel (Figure 3b, right). These results indicate both the formation of the fluorescent pyrazoline linkage in the PEGylated alkene-Z adduct (mPEG-Z)[5a] and the high selectivity of the labeling reaction. Furthermore, analysis of the alkene-Z reaction mixture by MALDI-TOF mass spectrometry confirmed the presence of mPEG-Z adducts, with the mass envelope centered at 13200 Da (expected: ca. 13 kDa; Figure 3c). The labeling of alkene-Z in bacterial cell lysates by tetrazole **10** was also found to proceed selectively, with efficiency comparable to that observed with purified alkene-Z proteins.[9]

To examine whether fast reaction kinetics in vitro translate into faster protein labeling in vivo, we incubated BL21 (DE3) cells expressing either alkene-Z or wild-type Z protein with tetrazole **10** (100 μM) for 30 minutes at room temperature. The cells were then irradiated with a handheld UV

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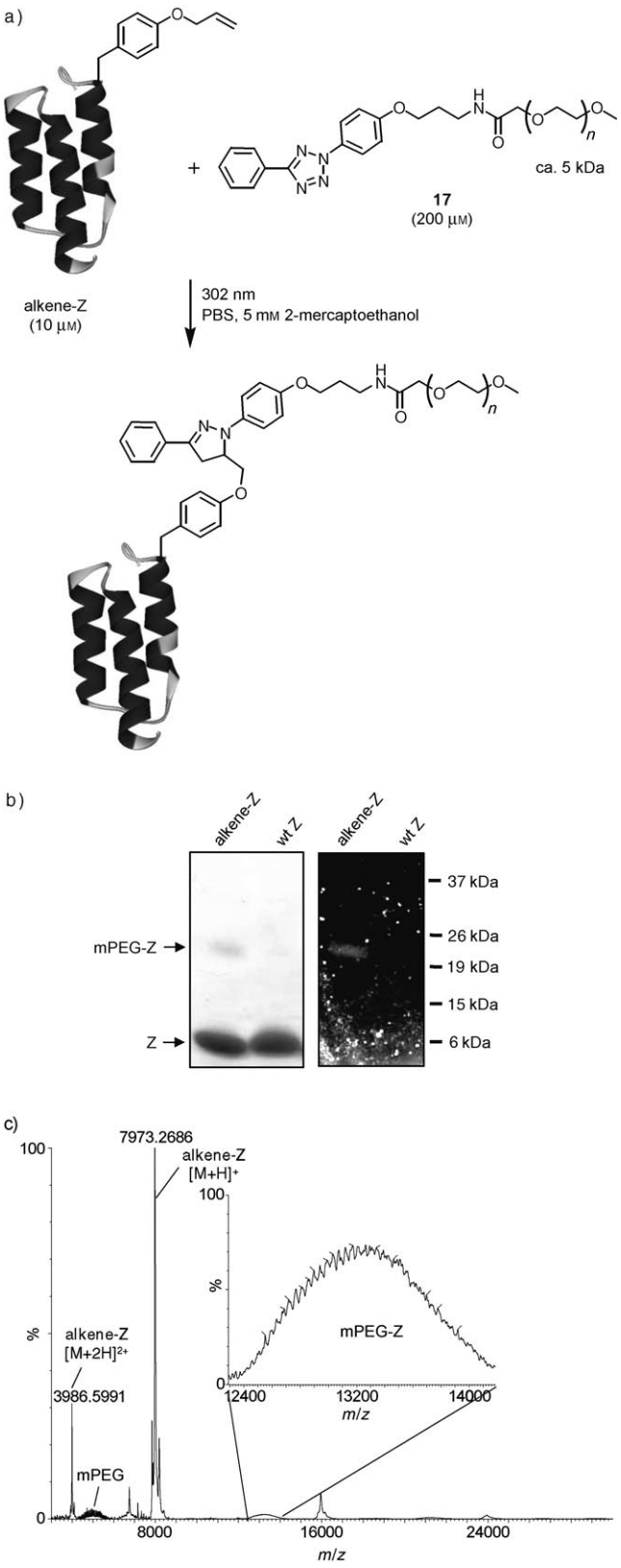

Figure 3. Selective PEGylation of alkene-Z by mPEG–tetrazole **17**. a) Reaction scheme. b) Coomassie blue staining (left) and in-gel fluorescence imaging (right, $\lambda_{ex} = 365$ nm) of alkene-Z upon photoirradiation for 2 min and additional incubation for 2 h in the presence of **17**. The experiment was repeated twice with similar results. wt = wild type. c) MALDI-TOF mass spectrum of the reaction mixture from the labeling of alkene-Z with **17**. The mass envelope for mPEG-Z is magnified in the insert.

lamp for 30 s before immediate image acquisition by fluorescence microscopy. Since the pyrazoline derived from tetrazole **10** showed maximum absorption at 360 nm and a broad emission band at 450–600 nm,[9] we chose the 4′,6-diamidino-2-phenylindole (DAPI) filter set ($\lambda_{ex} = 365$ nm, $\lambda_{em} = 445 \pm 25$ nm) to monitor the cycloaddition reaction in vivo. In the DAPI channel, only *E. coli* cells expressing alkene-Z showed strong fluorescence; cells expressing wild-type Z did not (Figure 4a,b). Thus, the cycloaddition mediated by tetrazole **10** was indeed very fast and selective toward the alkene group. By comparison, the labeling reaction with tetrazole **1** under identical conditions did not produce fluorescent cells.[9] On the basis of differential interference contrast (DIC) images, there was no apparent cytotoxicity associated with treatment of the cells with tetrazole **10** (Figure 4c,d).[13]

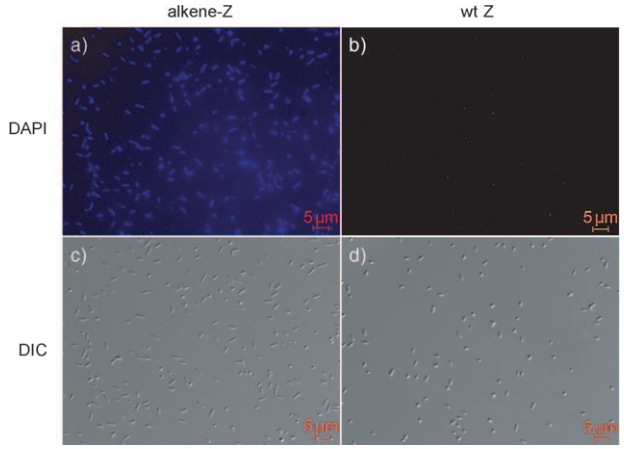

Figure 4. Fluorescence images showing the fast, selective labeling of alkene-Z by tetrazole **10** in *E. coli* cells: DAPI-channel (top) and DIC-channel images (bottom) of bacterial cells expressing either alkene-Z (a, c) or wt Z (b, d) after photoirradiation for 30 s in the presence of tetrazole **10** (100 μM).

In conclusion, we have demonstrated the development of a fast photoclick reaction by systematic tuning of the HOMO energies of the nitrile imine intermediates. One of the optimized tetrazoles, compound **10**, was found to label an alkene-encoded protein in less than 1 minute inside *E. coli* cells. The raising of the HOMO energy of the dipole is complementary to the existing strategy by which the LUMO of the dipolarophile is lowered. A combination of these strategies may lead to the robust reaction kinetics critical for the study of biomolecular dynamics in living systems.

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Experimental Section

Fluorescence microscopy of living *E. coli* cells: *E. coli* cells expressing either alkene-Z or the wild-type Z-domain protein were sedimented by centrifugation for 5 min (6500 × *g*) at 4 °C. The cell pellets were washed twice with PBS, and the cells were then resuspended in PBS buffer containing 5% glycerol and tetrazole **10** (or the control tetrazole **1**; 100 μM) and incubated at 37 °C for 30 min. A portion of the cell suspension (5–20 μL) was spotted on a glass slide. A glass cover slip was then placed on top of the mixture to sandwich the cells. The samples were irradiated with UV light at 302 nm for 30 s and then immediately placed in the microscope sample chamber for image acquisition. All image acquisitions were performed under identical conditions.

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[11] We did not choose tetrazole **11** for these studies, as the corresponding pyrazoline was found to be unstable during purification by flash chromatography on silica gel.
[12] A greater than 5 kDa increase in apparent molecular weight is typically observed during electrophoresis of the PEGylation adducts; see: A. Deiters, T. A. Cropp, D. Summerer, M. Mukherji, P. G. Schultz, *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5743, and references therein.
[13] Subsequent culture of the treated bacterial cells, however, revealed a reduced viability (viability ≈ 12%, based on the colony-formation assay; see the Supporting Information). The reduction in viability can be attributed exclusively to UV damage and may be preventable by including antioxidants in the culture medium; see: C. W. Yip, *J. Biol. Educ.* **2007**, *42*, 40.

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