Published in final edited form as:

Angew Chem Int Ed Engl. 2009 ; 48(29): 5330–5333. doi:10.1002/anie.200901220.

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

Yizhong Wang, Wenjiao Song, Wen J. Hu, and Qing Lin *

^[*]Department of Chemistry State University of New York at Buffalo Buffalo, New York 14260 (USA)

Fax: (+01)716-645-6963 qinglin@buffalo.edu

Keywords

dipolar cycloaddition reaction; alkenes; proteins; dipole HOMO energy; biotechnology

Bioorthogonal chemistry has attracted intense interest recently because, combined with genetic encoding, it 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 Cu^I-catalyzed cycloaddition ('click chemistry'),[2] strain-promoted cycloaddition,[3] Staudinger ligation,[4] photoinduced cycloaddition ('photoclick chemistry'), [5] and tetrazine ligation.[6] In applying bioorthogonal reactions to study biomolecular dynamics, it is imperative that the reaction proceeds in a time frame shorter than the biomolecules' half-lives, which is especially important in case of short-lived proteins, such as the endogenous tumor suppressor protein p53 with a half-life of only 40 min.[7]

We recently reported a photoinduced 1,3-dipolar cycloaddition reaction for selective functionalization of an unactivated alkene in *Escherichia coli*. [5b] While the reaction proceeded selectively, the cycloaddition rate was rather low ($k_2 = 0.00202 \pm 0.00007 \text{ M}^{-1}\text{s}^{-1}$). To accelerate the concerted cycloaddition reactions, two strategies have been commonly employed in the literature: ring strain and fluorine effect,[3] both of which work by lowering the LUMO energies of the dipolarophiles. Because enzymes involved in the biosynthetic/metabolic pathways utilize unnatural substrates that are structurally close to the natural ones, it is typically easier to encode small groups such as allyl group into biomolecules than bulky ones. Therefore, it is more desirable if we can increase the reactivity of the nitrile imine dipoles in our photoclick chemistry. Since reaction rate is inversely related to free energy gap between dipole-HOMO and dipolarophile-LUMO for the type I HO-controlled 1,3-dipolar cycloaddition,[8] we envisioned that the cycloaddition reaction can be accelerated by lifting the dipole-HOMO energy. Herein, we report the identification of several simple, yet powerful tetrazole reagents for the photoclick chemistry by systematically tuning the HOMO energies of the nitrile imines, which afforded an extremely fast (< 1 min) labeling of an alkene-containing protein in *E. coli*.

[**] Q.L. is grateful to the National Institutes of Health (GM 085092) and New York State Center of Excellence in Bioinformatics and Life Sciences for financial support.

+ Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.

To examine the electronic effect of the nitrile imine on the cycloaddition reaction, we synthesized two series of tetrazoles carrying substituents either on the C^5 -phenyl ring (X) or on the N^2 -phenyl ring (Y). Using water-soluble 4-penten-1-ol as a model for the unactivated alkene dipolarophile, we measured the cycloaddition rate constants (k_2) in the ACN/PBS buffer (1:1) and the data are collected in Table 1.[9] In the first series (tetrazoles **1-5**), switching substituents from EWG (**1** and **2**) to EDG (**4**, **5**) led to substantial increases in reaction rate whereas the unsubstituted diphenyl-tetrazole **3** proceeded almost as fast as **4** and **5**. Thus, we decided to leave X as H, and varied substitutions on the N^2 -phenyl ring and generated the second series (tetrazoles **6-16**). Compared to **3**, we found EDG (**8-16**) generally led to rate acceleration while EWG (**6**, **7**) resulted in rate decrease (Table 1). The substituent effect is clearly position-dependent with the *para*-position producing the most pronounced rate enhancement (compare **10**, **12**, **13**; or **14**, **15**, **16**). In particular, tetrazole **11** carrying the *para*-NH₂ group afforded the fastest rate with k_2 value of $0.79 \text{ M}^{-1}\text{s}^{-1}$, nearly 200-fold faster than tetrazole **1** we used previously.[5b] As expected, tetrazole **11** also showed the highest yield (~50% after 75 sec) with the water-quenching product (~10%)**5a** and the dimerization product (~15%)[10] as the major side products. We further calculated the HOMO energies of the nitrile imines (Table 1)[9] and plotted E_{HOMO} vs. Log(rate) to gauge the electronic effect on the nitrile imine reactivity (Figure 1). The plot exhibited a good linear fit ($R^2 = 0.86$), suggesting that the observed rate enhancement is primarily due to the HOMO-lifting effect.

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 tetrazole **10** to that of tetrazole **1** toward allyl phenyl ether (a small-molecule substrate in place of *O*-allyltyrosine) in PBS buffer.[11] Tetrazole **10** exhibited robust kinetics with k_2 value of $0.95 \text{ M}^{-1}\text{s}^{-1}$ (Figure 2a), 475-fold faster than tetrazole **1** ($k_2 = 0.00202 \text{ M}^{-1}\text{s}^{-1}$).[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 cycloadducts (Figure 2b). After 30 sec of photoirradiation, more than 40% cycloadduct was observed for tetrazole **10** while only 5% cycloadduct was observed for tetrazole **1** under identical condition, indicating significantly faster labeling kinetics of alkene-Z protein by tetrazole **10** *in vitro*.

To assess the yield and selectivity of the alkene-Z labeling reaction by tetrazoles, alkene-Z and wt-Z were incubated separately with a monodisperse poly(ethylene glycol) (mPEG, MW ~5 KDa)-linked tetrazole **22**[9] for 2 hr after 2-min photoirradiation (Figure 3a). A distinct higher MW band was observed prominently with alkene-Z (~20% estimated yield as measured by densitometry) over wt-Z (left panel, Figure 3b),[12] which coincides with a fluorescent band detected by in-gel fluorescence imaging of the same gel (right panel, Figure 3b), indicating both the formation of the fluorescent pyrazoline linkage in the PEGylated alkene-Z adduct (mPEG-Z)[5a] and high selectivity of the labeling reaction. Furthermore, MALDI-TOF mass spectrometry analysis of the alkene-Z reaction mixture confirmed the presence of mPEG-Z adducts with the mass envelop centered at 13,200 Da (expected ~ 13 KDa) (Figure 3c). Additionally, the labeling of alkene-Z in the bacterial cell lysates by tetrazole **10** was also found to proceed selectively, with efficiency comparable to that of the purified alkene-Z proteins. [9]

To examine whether fast reaction kinetics *in vitro* translates into faster protein labeling *in vivo*, we incubated BL21 (DE3) cells expressing either alkene-Z or wt-Z protein with 100 μ M tetrazole **10** for 30 min at room temperature. The cells were then irradiated with a handheld UV lamp for 30 sec before immediate image acquisition via fluorescence microscopy. Since tetrazole **10** derived pyrazoline showed maximum absorption at 360 nm and a broad emission band at 450–600 nm,[9] we chose the DAPI filter set (ex 365 nm, em 445 $\pm$ 25 nm) to monitor the cycloaddition reaction *in vivo*. In the DAPI channel, only the alkene-Z-expressing *E. coli* cells showed strong fluorescence while cells expressing wt-Z did not (compare Figure 4a to 4b), indicating that the tetrazole **10** mediated cycloaddition was indeed very fast and selective toward the alkene group. By comparison, the labeling reaction employing tetrazole **1** under identical conditions did not produce fluorescent cells.[9] There was no apparent cytotoxicity associated with the tetrazole **10** treatment based on the DIC images (Figure 4c, 4d).[13]

In conclusion, we have demonstrated a fast photoclick chemistry by systematically tuning the HOMO energies of the nitrile imines. One of the optimized tetrazoles, compound **10**, was found to label an alkene-encoded protein in less than 1 min inside *E. coli* cells. This dipole HOMO-lifting strategy is complementary to the existing dipolarophile LUMO-lowering strategy that when used together may provide robust reaction kinetics critical for the study of biomolecular dynamics in living systems.

Experimental Section

Live *E. coli* Cell Fluorescence Microscopy: *E. coli* cells expressing either alkene-Z or wt-Z domain proteins were sedimented by centrifugation for 5 min (6500 $\times$ g) at 4 $^{\circ}$ C. The cell pellets were washed twice with PBS, and the cells were then re-suspended in PBS buffer containing 5% glycerol and 100 μ M tetrazole **10** (or control tetrazole **1**). After incubation at 37 $^{\circ}$ C for 30 min, 5–20 μ L of the cell suspension was spotted on top of a glass slide. A glass cover slip was then placed on top of the mixture to sandwich the cells. The samples were irradiated with 302-nm UV light for 30 sec, and then immediately placed in the microscope sample chamber for image acquisition. All image acquisitions were performed under the identical conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

- [1] a). Prescher JA, Bertozzi CR. Nat. Chem. Biol 2005;1:13. [PubMed: 16407987] b) Barglow KT, Cravatt BF, B. F. Nat. Methods 2007;4:822. [PubMed: 17901872]
- [2]. Rostovtsev VV, Green LG, Fokin VV, Sharpless KB. Angew. Chem. Int. Ed 2002;41:2596.
- [3] a). Agard NJ, Prescher JA, Bertozzi CR. J. Am. Chem. Soc 2004;126:15046. [PubMed: 15547999] b) Baskin JM, Prescher JA, Laughlin ST, Agard NJ, Chang PV, Miller IA, Lo A, Codelli JA, Bertozzi CR. Proc. Natl. Acad. Sci. USA 2007;104:16793. [PubMed: 17942682] c) Codelli JA, Baskin JM, Agard NJ, Bertozzi CR. J. Am. Chem. Soc 2008;130:11486. [PubMed: 18680289] d) Ning XH, Guo J, Wolfert MA, Boons G—J. Angew. Chem. Int. Ed 2008;47:2253.
- [4] a). Saxon E, Bertozzi CR. Science 2000;287:2007. [PubMed: 10720325] b) Hangauer MJ, Bertozzi CR. Angew. Chem. Int. Ed 2008;47:2394.
- [5] a). Song W, Wang Y, Qu J, Madden MM, Lin Q. Angew. Chem. Int. Ed 2008;47:2832. b) Song W, Wang Y, Qu J, Lin Q. J. Am. Chem. Soc 2008;130:9654. [PubMed: 18593155]
- [6]. Blackman ML, Royzen M, Fox JM. J. Am. Chem. Soc 2008;130:13518. [PubMed: 18798613]
- [7]. Dornan D, Wertz I, Shimizu H, Arnott D, Frantz GD, Dowd P, O' Rourke K, Koeppen H, Dixit VM. Nature 2004;429:86. [PubMed: 15103385]

- [8]. Houk KN, Sims J, Watts CR, Luskus LJ. J. Am. Chem. Soc 1973;95:7301.
- [9]. See the Supporting Information for details.
- [10]. Wang Y, Vera CI, Lin Q. Org. Lett 2007;9:4155. [PubMed: 17867694]
- [11]. We did not choose tetrazole **11** for the subsequent studies because the corresponding pyrazoline was found to be unstable during the silica gel flash chromatographic purification.
- [12]. The greater than 5 KDa increase in apparent MW is typically observed during electrophoresis of the PEGylation adducts, see:Deiters A, Cropp TA, Summerer D, Mukherji M, Schultz PG. Bioorg. Med. Chem. Lett 2004;14:5743. [PubMed: 15501033]and references therein
- [13]. Subsequent culture of the treated bacterial cells, however, revealed a reduced viability (~12% based on the colony formation assay; see Supporting Information), which can be attributed exclusively to UV damage and potentially preventable by including antioxidants in the culture medium (see:Yip C—W. J. Biol. Ed 2007;42:40.

Figure 1.

Cycloaddition rates are dependent on the nitrile imine HOMO energies. The HOMO energies of the nitrile imines were calculated using Hartree-Fock 3-21G model based on the AM1 optimized geometries (see Supporting Information for details).

Figure 2.

(a) Kinetic study of the cycloaddition reactions between allyl phenyl ether and tetrazole **1** (◆) and **10** (●), respectively. A close-up view of the tetrazole **10** kinetics was rendered in the insert; (b) Kinetic study of the alkene-Z labeling by either tetrazole **1** (open bar) or **10** (filled bar). The fluorescent intensities at 4 min were treated as 100% for each reaction and used in deriving the relative intensities. The concentrations of alkene-Z and tetrazoles were 10 μM and 200 μM , respectively. Error bars represent the standard deviations from three independent measurements.

Figure 3. Selective PEGylation of alkene-Z by mPEG-tetrazole **22**: (a) reaction scheme; (b) Coomassie blue staining (left panel) and in-gel fluorescence imaging (right panel, $\lambda_{\text{ex}} = 365 \text{ nm}$) of alkene-Z upon photoirradiation for 2 min and additional incubation for 2 hr in the presence of mPEG-tetrazole **22**. The experiment was repeated twice and similar results were obtained; (c) MALDI-TOF mass spectrum of the PEGylation mixture of the alkene-Z labeling by mPEG-tetrazole **22**. A close-up view of the mass envelope representing mPEG-Z was rendered in the insert.

Figure 4.

Fast, selective labeling of alkene-Z by tetrazole **10** in *E. coli* cells: DAPI channel (top row) and DIC channel (bottom row) images of bacterial cells expressing either alkene-Z (a, c) or wt-Z (b, d) after 30-sec photoirradiation in the presence of 100 μM tetrazole **10**; scale bar = 5 μm .

Table 1
Photoinduced Cycloaddition Reactions of Tetrazoles with 4-Penten-1-ol^a

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

^aThe reaction was performed by irradiating a 200-μL mixture of 100 μM tetrazole and 10 mM 4-penten-1-ol in a quartz test tube with a handheld UV lamp at 302 nm.

^bHOMO energies of the nitrile imines.