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Sterically shielded tetrazoles for fluorogenic photoclick reaction: Tuning cycloaddition rate and product fluorescence[†]

Peng An and Qing Lin*

Department of Chemistry, State University of New York at Buffalo, Buffalo, New York 14260, USA

Abstract

A panel of sterically shielded tetrazoles with different *N*-aryl groups were synthesized and subsequently evaluated in the photoinduced tetrazole-alkene cycloaddition reaction. It was found that increase in HOMO energy of the corresponding nitrile imines leads to faster cycloaddition reaction along with red shift in fluorescence emission of the pyrazoline cycloadduct.

Fluorescent molecules are indispensable tools for visualizing biomolecule localization and dynamics in living systems.¹ The commonly used fluorophores include rhodamine,² fluorescein,³ BODIPY,⁴ cyanines,⁵ coumarin,⁶ and their numerous derivatives with diverse photophysical properties.⁷ To introduce fluorophores selectively into biomolecules in living systems, the bioorthogonal chemistry approach has been widely adopted.⁸ Among many bioorthogonal reactions successfully employed in selective fluorescent labeling of biomolecules in living systems, fluorogenic reactions such as tetrazole-alkene cycloaddition reaction⁹ and tetrazine ligation¹⁰ are particularly valuable because they generate fluorescent adducts *in situ* without the use of excess amount of fluorescent reagents. During the optimization of this type of fluorogenic bioorthogonal reactions, it has been shown that both the reaction kinetics and the photophysical properties of the adducts are highly dependent on the reactant structures.¹⁰

In optimizing chemo-selectivity of the tetrazole-alkene cycloaddition reaction, we reported recently a sterically shielded tetrazole **1** (Chart 1) that reacts with the spiro[2.3]hex-1-en-lysine (SphK) encoded glutamine binding protein (QBP) and generates *in situ* an environmentally sensitive pyrazoline fluorophore.¹¹ To fully understand how the tetrazole structures affect the reaction kinetics and photophysical properties of the pyrazoline products, here we report the synthesis of a panel of eight new sterically shielded tetrazoles **2–9** (Chart 1), and the evaluation of their reaction kinetics with spiro[2.3]hex-1-ene as well as the fluorescent properties of the resulting pyrazolines.

We have shown previously that the diaryltetrazole reactivity is critically dependent on the *N*-aryl substituent.¹² To probe how electronic effect affects the reactivity of the sterically

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qinglin@buffalo.edu; Fax: +1 716 6456963; Tel: +1 716 645 4254.

Conflicts of interest

There are no conflicts to declare.

shielded tetrazoles, we designed a panel of tetrazoles **2–9** containing the same bulky C-aryl group but different N-aryl substituents with varying electronic properties (Chart 1). These new tetrazoles were readily synthesized through a two-step procedure: (i) formation of the dibromo-diaryltetrazole intermediates via Kakehi tetrazole synthesis or Cu^{II}-mediated cross-coupling; and (ii) installation of the di-*N*-Boc-pyrrole steric shield via Suzuki cross-coupling (Scheme 1). In brief, 2,6-dibromobenzaldehyde was treated with benzenesulfonyl hydrazide and various diazonium salts successively to give the corresponding dibromo-diphenyltetrazole **2a–6a** in 25–45% yields. After appropriate functional group interconversion to generate **5b**, **7a** and **8a**, the dibromo-diphenyltetrazoles were reacted with 2-(*N*-Boc-pyrrole)boronic acid via Suzuki cross-coupling to generate tetrazoles **2–8** in 35–75% yields (Scheme 1a). For bithiophene-substituted tetrazole **9**, 2,6-dibromo-benzonitrile was converted to tetrazole **9a** in 69% yield, which was followed by Cu-catalysed cross-coupling with the bithiophene iodonium salt to give the dibromo-tetrazole **9b** in 65% yield. The sterically shielded tetrazole **9** was then obtained in 58% yield through standard Suzuki cross-coupling with 2-(*N*-Boc-pyrrole)boronic acid (Scheme 1b).

With tetrazoles **1–9** in hand, we examined their reactivity in the photoinduced cycloaddition reaction with spiro[2.3]hex-1-ene (Sph)¹³ in mixed phosphate buffer/acetonitrile (1:1) solvent. Because the reaction produces fluorescent pyrazoline adduct,¹⁴ we followed the reaction progress by monitoring the time-dependent fluorescence change (Figure S1). We found that the cycloaddition rate was dictated by the electronic effect. Compared to the electron-neutral phenyl group in tetrazole **4**, the electron-deficient aryl groups in tetrazoles **2** and **3** decrease the rate whereas the electron-rich aryl groups in tetrazoles **1** and **5–9** increase the rate (Table 1). The fastest reaction was observed with tetrazole **7** containing *p*-aminophenyl group, with the second-order rate constant, k_2 , measured to be $7300 \text{ M}^{-1} \text{ s}^{-1}$, more than 36-fold faster than that of tetrazole **4**. To investigate whether the rate acceleration is a result of greater stability of the corresponding nitrile imine, the half-lives of all nitrile imines were determined by following our published procedure (Figure S2), and the data are collected in Table 1. Inspection of the data revealed there was no direct correlation between the rate constant and the nitrile imine half-life. However, the electron-rich nitrile imines generally exhibit shorter life-lives than the electron-deficient ones, with the three most electron-rich nitrile imines derived from tetrazoles **7–9** possessing $t_{1/2}$ less than 15 s (Table 1).

Since the nitrile imine-alkene cycloaddition represents type I 1,3-dipolar cycloaddition reaction, we calculated the highest occupied molecular orbital (HOMO) energies of the nitrile imines from the corresponding tetrazoles (Table 1) and probed the substituent effect by plotting $\log k_2$ vs. E_{HOMO} (Figure 1). A linear regression line can be obtained with the R^2 value of 0.77, suggesting that the rate enhancement observed for the electron-rich substituents is primarily due to the HOMO-lifting effect as we reported previously.¹²

Next, we assessed the effect of *N*-aryl substituents on the product fluorescence. We found that the fluorescence colour varied widely, ranging from blue for the CN group, to green for the OH group, and to brownish yellow for the NMe₂ group (Figure 2a). The pyrazoline from the acetamide-substituted tetrazole **6** gave the strongest fluorescence (Figure 2b). Since the pyrazolines are environmentally sensitive fluorophores,¹⁵ we further examined the

pyrazoline-6 fluorescence in various solvents. As expected, pyrazoline-6 showed weak fluorescence in polar solvents such as 9:1 PB/ACN, but drastically stronger fluorescence in nonpolar solvents such as dichloromethane and ethyl acetate (Figure 2c). Indeed, a turn-on ratio of 140-fold at emission wavelength of 486 nm was detected going from 10% acetonitrile in phosphate buffer to ethyl acetate, suggesting that tetrazole **6** could serve as an excellent reagent for *in situ* formation of an environment-sensitive fluorescence sensor with the alkene-containing proteins.¹¹

To probe whether the tetrazoles are capable of generating the pyrazoline fluorophores *in situ* with protein substrates, five tetrazoles (**3** and **5–8**) with distinct emission (442, 486, 501, 545 and 560 nm) were reacted with the non-fluorescent superfolder GFP mutants containing SphK¹³ or BocK (BocK = *N*^ε-Boc-lysine) at position 204 (sfGFP-Q204SphK/BocK)¹¹ (Figure 3a). In-gel fluorescence analysis of the reaction mixtures revealed the formation of fluorescent bands with sfGFP-Q204SphK but not sfGFP-Q204BocK on the SDS-PAGE gel (Figure 3b). Tetrazole **6** produced the strongest fluorescent band upon excitation with a 365-nm UV light, which was further verified by direct fluorescence measurement in solution (Figure 3c). The emission maxima and intensity trend within the protein samples match well with those observed in the small-molecule samples (Figure 2b).

In summary, we have synthesized a panel of eight sterically shielded tetrazoles by systematically tuning the electronic property of the *N*-aryl substituent. The reactivity of these new tetrazoles in the cycloaddition reaction with spiro[2.3]hex-1-ene was investigated, uncovering excellent correlation between the nitrile imine HOMO energy and the reaction rate. Moreover, these tetrazoles produced fluorescent pyrazoline adducts with a wide range of emission colours as well as solvent polarity dependency. In particular, the acetamide-substituted tetrazole **6** showed robust turn-on fluorescence with both the small-molecule spirohexene and the spiroalkene-encoded protein. On the basis of their fast kinetics and tunable fluorescence properties, this expanded set of tetrazoles may find wide use in fluorogenic labeling of proteins in complex biological systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Plot of $\log k_2$ of the cycloaddition reaction vs. HOMO energy of the nitrile imines in the photoclick reaction with Sph. For substituted phenyl rings, only *para*-substituents are shown.

Figure 2.

Characterization of the fluorescent properties of the *in situ* formed pyrazolines. (a) Fluorescent images of the pyrazolines from the reactions of 10 μ M tetrazoles **1–9** with 250 μ M Sph in 1:1 PB/ACN. The cuvettes were illuminated with a 365 nm UV light. (b) Fluorescence spectra of the pyrazolines. λ_{ex} = 360 nm for tetrazoles **2–4** and 405 nm for tetrazoles **5–9**; see Figure S3 in Electronic Supplementary Information for selection of appropriate λ_{ex} . For pyrazolines containing N-phenyl groups, only para-substituents are shown. (c) Solvent-dependent fluorescence spectra of the *in situ* generated pyrazoline **6**. λ_{ex} = 405 nm.

Figure 3.

Fluorogenic labelling of sfGFP-Q204SphK by the sterically shielded tetrazoles. (a) Reaction scheme: a mixture of 10 μ M sfGFP mutant encoding SphK at position-204 and 200 μ M tetrazole in PBS/ACN (1:1) was photoirradiated with a handheld 302-nm UV lamp for 30 sec. (b) SDS-PAGE/in-gel fluorescence analysis of the reaction mixtures. Top panel, Coomassie blue stained gel; bottom panel, in-gel fluorescence by illuminating the gel with a 365-nm UV light. The non-fluorescent sfGFP variant containing the T65G/Y66G mutations was used in this experiment. (c) Fluorescence spectra of the reaction mixtures. The emission maximum of sfGFP-Q204Pyr is marked on top of each spectrum. $\lambda_{\text{ex}} = 405$ nm.

Scheme 1.
 Synthesis of the sterically shielded tetrazoles **2–9**.

Chart 1.
Structures of sterically shielded tetrazoles **1–9**.

Table 1Characterization of the photoinduced cycloaddition of tetrazoles with spiro[2.3]hex-1-ene^a

tetrazole	Ar	k_2 [$M^{-1} s^{-1}$]	$t_{1/2}$ (s) ^b	E_{HOMO} [eV] ^c
1	<i>p</i> -OMe-Ph	2800 ± 200	102	-4.4292
2	<i>p</i> -CN-Ph	80 ± 20	59	-5.1114
3	<i>p</i> -CO ₂ Et-Ph	100 ± 40	87	-4.9508
4	Ph	200 ± 30	80	-4.6654
5	<i>p</i> -OH-Ph	2000 ± 190	32	-4.4743
6	<i>p</i> -NHAc-Ph	1700 ± 300	74	-4.8090
7	<i>p</i> -NH ₂ -Ph	7300 ± 570	<15	-4.0842
8	<i>p</i> -N(Me) ₂ -Ph	5200 ± 700	<15	-4.0257
9	bithiophene	4100 ± 800	<15	-4.4289

^aA solution of 10 μ M tetrazole and excess Sph in PB/ACN (1:1) in a quartz cuvette was photoirradiated with a handheld 302-nm (for tetrazoles 1–8) or 365-nm (for tetrazole 9) UV lamp. PB = phosphate buffer; ACN = acetonitrile.

^bThe half-lives of the nitrile imines were measured by following the published procedure in ref. 11.

^cHOMO energies of the nitrile imines were calculated using Gaussian09 program at B3LYP/6–31G (d) level.