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Strain-promoted cycloadditions of cyclic nitrones with cyclooctynes for labeling human cancer cells†

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Strain-promoted cycloadditions of cyclic nitrones with cyclooctynes proceed with rate constants up to 3.38 ± 0.31 M^{-1} s^{-1} in CD_3CN, or 59 times faster than the analogous reaction of azides. This highly specific modular labeling strategy can be applied for direct labeling of proteins and for live cell imaging of cancer cells.

Incorporation of fluorescent probes into proteins, DNA, RNA, lipids and glycans within their native cellular environments provides opportunities for imaging and understanding their roles *in vivo*. Labeling of specific functional groups on live cells *via* bioorthogonal chemical reporter strategies[1] have become increasingly powerful applications in cell biology.[2–4] Cycloaddition reactions are particularly attractive for this purpose because they are usually very selective, high yielding and proceed rapidly in aqueous media. Azide-based reactions[5] have been applied broadly to bioconjugation *in vivo* since the azide 1,3-dipole is stable and easily introduced into biomolecules or probe molecules without changing functional properties. The copper-catalyzed azide–alkyne cycloaddition (CuAAC)[6,7] has been applied mainly to cell lysates[8] but recently has also been applied to labeling the exterior of cell surfaces *in vivo*.[9,10] To avoid using potentially toxic copper catalysts and reducing agents,[11,12] Staudinger ligation with phosphine esters[13] and strain-promoted azide–alkyne cycloaddition (SPAAC)[14] have been implemented for labeling of biomolecules on the exterior of live cells[15] and within living animals[8,16] with minimal cytotoxicity. A number of cyclooctyne reagents have been synthesized for SPAAC applications including difluorinated cyclooctynes (DIFO) and derivatives,[15] 4-dibenzocyclooctynol (DIBO),[17] photochemically generated dibenzocyclooctynes,[18] aza-dibenzo-cyclooctyne (DIBAC),[19] biarylcyclooctynone (BARAC),[20] and bicyclononynes.[21] Other examples of bioorthogonal cycloadditions include reactions of strained alkenes with nitrile oxides,[22] tetrazines,[23,24] unstrained alkenes with photochemically generated nitrile imines[25] as well as olefin metathesis.[26] Strain promoted reactions of cyclooctynes with other 1,3-dipoles, including diazo compounds and nitrile oxides, have also been reported.[27,28]

In pursuit of fast and efficient metal-free bioorthogonal cycloadditions as a means for reducing the concentrations of labeling reagents, we have shown that cyclic nitrones display exceptionally fast kinetics in strain-promoted cycloadditions with cyclooctynes, proceeding up to 25 times faster than analogous reactions involving azides.[29] We attributed this rate increase to the additional strain associated with the cyclic nitrone moiety. A one-pot N-terminal modification of peptides *via* strain-promoted alkyne–nitrone cycloaddition (SPANC) was independently reported thereafter.[30] Here we demonstrate strain-promoted cycloadditions of cyclic nitrones with cyclooctynes, as well as direct protein modification and live-cell labeling of cell surface proteins. In addition to rapid reactivity, the synthetic accessibility and ease of functionalization of nitrones make them particularly attractive 1,3-dipoles for strain-promoted [3 + 2] cycloadditions with cyclooctynes. Nitrones can also undergo Kinugasa reactions with terminal alkynes.[31,32]

To probe the feasibility of SPANC reactions of cyclic nitrones as a tool for direct biological labeling, we examined the reaction scope and kinetics for reactions of a series of cyclic nitrones with dibenzocyclooctyne (**2a**).[29] In order to optimize the tradeoff between cyclic nitrone reactivity and stability for bioconjugation, we tested a series of cyclic nitrones differing in ring size, sterics and stereoelectronics to determine their respective contributions to nitrone reactivity in SPANC reactions.

Aldonitrones **1a–e** reacted significantly faster than the ketonitrones **1g–h** in SPANC reactions with **2a**. All nitrones employed yielded isoxazoline cycloadducts in excellent yields. The pyrroline N-oxides, **1a–e**, were found to be optimal, blending fast kinetics with enhanced stability under ambient conditions. The presence of the gem-disubstitution adjacent to nitrogen in nitrones **1a** and **1b** resulted in second-order rate constants of 0.63 ± 0.12 M^{-1} s^{-1} and 7.69 ± 0.19 M^{-1} s^{-1} respectively. Prolinol nitrone **1c** reacted with **2a** in good yield with a rate constant of 1.95 ± 0.60 M^{-1} s^{-1} (Table 1). The presence of the N-Boc group adjacent to the α-position in **1d** resulted in a slower reaction with **2a** relative to the analogous reaction of **1c**. Attempts to further activate the cyclic nitrone using gem-difluoro groups at the α-position in nitrone **1e**

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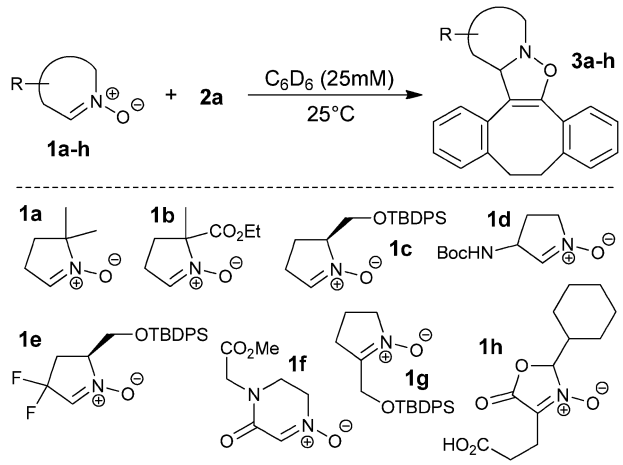
Table 1 Kinetics for strain-promoted cycloadditions of cyclic nitrones **1a–f** with **2a**[a]



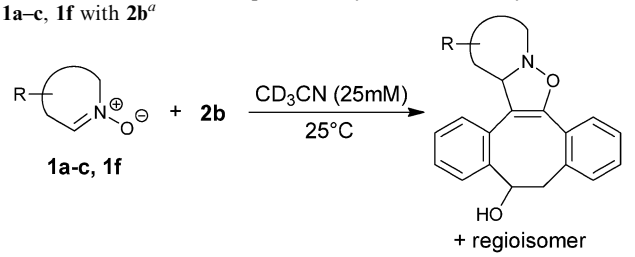
Nitrone	Product	Yield[b] (%)	t/min	k_2[c]/M^{-1} s^{-1}
1a	**3a**	91	15	0.63 ± 0.12
1b	**3b**	96	<2	7.69 ± 0.19
1c	**3c**	88	5	1.95 ± 0.60
1d	**3d**	90	18	0.62 ± 0.08
1e	**3e**	87	180	0.0215 ± 0.00038
1f	**3f**	84	3	1.41 ± 0.19
1g	**3g**	89	1380	n.d.
1h	**3h**	97	1440	n.d.

[a] Reagents were mixed 1 : 1 at 25 mM in C_6D_6 at 25 °C. [b] Isolated yield. [c] Rate constant, k_2, was determined by ^{1}H NMR under second order conditions.

resulted in slower rates for cycloaddition. This indicates that nitrones with electron withdrawing amide groups at the α-position react faster than those with inductively withdrawing fluoro-groups. Employing a six membered nitrone, **1f** containing the electron withdrawing α-amide group reacted with **2a** in 96% yield within 3 min at 25 °C; this corresponded to a rate constant of 1.41 ± 0.19 M^{-1} s^{-1}. The rate constant for reaction of **1f** with **2a** in toluene under pseudo first-order conditions was found to be 1.21 ± 0.06 M^{-1} s^{-1} by UV-visible spectroscopy, this value agrees with that determined by ^{1}H NMR. Despite its fast reactivity, nitrone **1f** was less stable and prone to dimerization at room temperature. Attempts to synthesize additional six membered nitrones that would be electronically and sterically similar to the five membered nitrones gave similar dimerization products as **1f** (Table 1).

Having established the five membered cyclic nitrones as optimal in terms of reactivity and stability in SPANC reactions with unfunctionalized **2a** in benzene, we measured the rate constants for the most reactive cyclic nitrones with **2b** in acetonitrile (Table 2). All cyclic nitrones tested gave excellent yields of isoxazoline products with comparable rates upon moving from benzene to acetonitrile. The rate constant for reaction of **1a** was 0.75 ± 0.04 M^{-1} s^{-1}. Although the value for reactions of **1a** with **2b** in acetonitrile is within experimental error with that obtained for its reaction with **2a** in benzene, it is still 13 times faster than analogous reactions of benzyl azide with **2b**.[18] Cyclic nitrone **1b** reacted with **2b** in 91% yield in 3 min with a rate constant of 3.38 ± 0.31 M^{-1} s^{-1}. This rate constant corresponds to a 44-fold enhancement relative to the reaction

Table 2 Kinetics of strain-promoted cycloadditions of cyclic nitrones **1a–c**, **1f** with **2b**[a]



Nitrone	Product	Yield[b] (%)	k_2[c]/M^{-1} s^{-1}
1a	**3i**	97	0.75 ± 0.04
1b	**3j**	96	3.38 ± 0.31
1c	**3k**	92	1.04 ± 0.17
1f	**3l**	95	0.46 ± 0.01

[a] Reagents were mixed 1 : 1 at 25 mM in CD_3CN at 25 °C. [b] Yield % was determined by ^{1}H NMR. [c] Rate constants, k_2, was determined by ^{1}H NMR under second order reaction conditions.

of benzyl azide with DIFO,[15] a 59-fold enhancement relative to reaction with DIBO[18] or a 3-fold enhancement relative to reaction with BARAC.[20] We also tested the prolinol nitrone, **1c** in reaction with **2b** and the rate constant was found to be 1.04 ± 0.17 M^{-1} s^{-1}, this corresponds to an 18-fold enhancement over analogous reaction of benzyl azide with **2b**. The rate constant for reaction of **1f** with **2b** determined by UV-visible spectroscopy under pseudo first-order conditions was found to be 0.56 ± 0.01 M^{-1} s^{-1} (see ESI†). This value agrees with that determined by ^{1}H NMR. Attempts to measure rate constants for the remaining nitrones in Table 2 by UV-visible spectroscopy were complicated by overlapping absorption spectra.

The hydrolytic stability of cyclic nitrone **1a** was compared with an acyclic nitrone, 2-(methyloxidoimino)-*N*-(phenylmethyl)-acetamide[29] under acidic and basic conditions to determine whether the cyclic nitrones could offer enhanced stability toward hydrolysis (see ESI†). In 100 mM HCl solution, the acyclic nitrone underwent rapid hydrolysis to the corresponding aldehyde and *N*-methylhydroxylamine within 15 min, and in 100 mM NaOH solution, the acyclic nitrone was significantly hydrolyzed over 2 h. Alternatively, cyclic nitrone **1a** remained intact under both acidic and basic conditions.

To demonstrate direct protein functionalization by SPANC, we labeled bovine serum albumin (BSA) protein *in vitro* (see ESI†). An NHS-activated prolinol nitrone, **NHS-1c**, was prepared by desilylation of **1c** with TBAF and subsequent NHS-activation with *N*,*N*′-disuccinimidyl carbonate, and was coupled with lysine residues on BSA. BSA was modified with an average of four nitrone groups as confirmed by MALDI-MS. The nitrone modified BSA was treated with **2b** functionalized Alexa Fluor® 488 cadaverine for 0 to 60 min. Highly specific time-dependent labeling was detected by in-gel fluorescence scanning (Fig. S4, ESI†).

To illustrate the utility of SPANC for cellular imaging, we labeled epidermal growth factor receptors (EGFRs) that are overexpressed on the surface of human breast cancer cells (MDA-MB-468) (Fig. 1). In this approach, the human recombinant epidermal growth factor (EGF) was coupled with **NHS-1c** and targeted to EGFRs on the cell surface. Incorporation of two nitrone groups was confirmed by MALDI-MS (see ESI†).

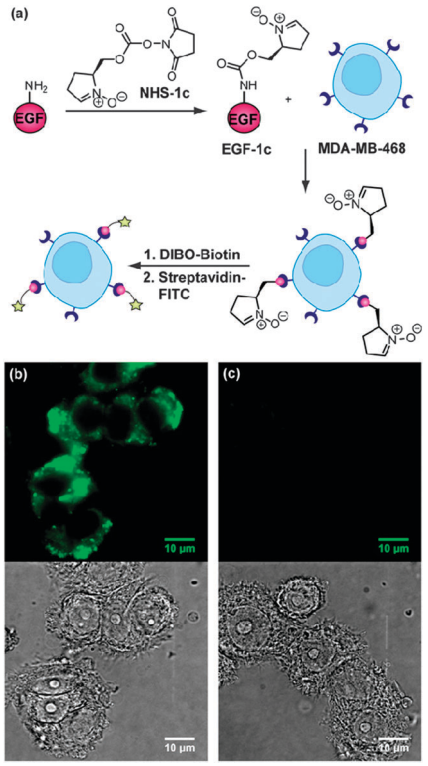


Fig. 1 *In situ* labeling of EGF–EGFR interactions *via* SPANC in MDA-MB-468 cells. (a) Cyclic nitrone modified EGF-**1c** bound to EGFR was labeled by SPANC with **2b**-biotin for 30 min prior to streptavidin-FITC fluorescent labeling. (b) Fluorescence (top) and bright field (bottom) image of *in situ* SPANC as described in (a). (c) Negative control, cells pretreated with EGF followed by treatment with **2b**-biotin and streptavidin-FITC, fluorescence (top) and bright field (bottom) image. Reagent concentrations: EGF-**1c** (10 μM), EGF (10 μM), **2b**-biotin (10 μM) and streptavidin-FITC (5 μg mL^{-1}).

Cellular labeling was accomplished by *in situ* SPANC reactions of cyclic nitrone modified EGF with DIBO-biotin and secondary labeling with streptavidin-FITC (Fig. 1b). For the negative control, MDA-MD-468 cells were pretreated with unmodified EGF followed by treatment with DIBO-biotin and streptavidin-FITC (Fig. 1c).

In summary, we have shown that five membered cyclic nitrones blend fast kinetics with enhanced stability in SPANC reactions with dibenzocyclooctynes. Reactions of cyclic nitrones proceeded with rate constants up to 3.38 ± 0.31 M^{-1} s^{-1} in CD_3CN, or 59 times faster than the analogous reaction of benzyl azide with DIBO. We have demonstrated highly specific direct labeling of BSA and EGF *in vitro* and have shown efficient labeling of EGFRs on the surface of cancer cells *via* SPANC. The SPANC bioconjugation approach presented here is modular and can be extended to other biological imaging applications, since cyclic nitrone functionalities can be easily conjugated to carboxylic acid or amine containing biomolecules.

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