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PAPER

Kinetics studies of rapid strain-promoted [3 + 2]-cycloadditions of nitrones with biaryl-aza-cyclooctynone†

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Strain-promoted cycloadditions of cyclic nitrones with biaryl-aza-cyclooctynone (BARAC) proceed with rate constants up to 47.3 M^{-1} s^{-1}, this corresponds to a 47-fold rate enhancement relative to reaction of BARAC with benzyl azide and a 14-fold enhancement over previously reported strain promoted alkyne–nitrone cycloadditions (SPANC). Studies of the SPANC reaction using the linear free energy relationship defined by the Hammett equation demonstrated that the cycloaddition reaction has a rho value of 0.25 ± 0.04, indicating that reaction is not sensitive to substituents and thus should have broad applicability.

Introduction

Reactions that do not interfere with biological functionality, termed bioorthogonal, have emerged as powerful tools for site-specific modification of biomolecules in cells and within living organisms.[1–4] Despite the multitude of developed organic reactions, only a handful of these are ideally suited for applications in biology.[5] Not only must the bioorthogonal reactive groups be mutually reactive, bio-inert and ideally non-toxic, the reaction must proceed with fast reaction rates to ensure formation of the product at low concentrations typically required in biological labelling experiments. A number of cycloaddition reactions have been developed that show fast kinetics, and high selectivity for functionalizing biomolecules. Cycloadditions that have been successfully applied to bioconjugation include the copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC),[6–9] strain-promoted azide alkyne cycloaddition (SPAAC),[4] strain-promoted nitrone–alkyne cycloaddition (SPANC),[10–12] and strain-promoted alkyne–nitrile oxide cycloaddition (SPANOC).[13,14] Diazo compounds have also been shown to react with cyclooctynes rapidly *via* strain promotion.[15] Also, cycloadditions of strained alkenes with tetrazines,[16] photochemical reactions of unstrained alkenes with tetrazoles,[17] olefin cross-metathesis,[18] and palladium-catalyzed cross-coupling reactions[19,20] have been reported specifically for bioorthogonal coupling or labelling reactions.

The CuAAC reaction has broad applicability and rapid kinetics.[9,21–24] The CuAAC reaction is also very popular since the azide and terminal alkyne moieties are small and non-perturbative of biological function and these reactions are very rapid and high yielding. Observed rate constants for CuAAC have been reported as high as 1 ×10^5 M^{-1} s^{-1} per mole of copper catalyst,[22] however, not all applications of CuAAC are tolerant to high concentrations of copper catalyst.[25] Also, CuAAC reactions can display complex dependencies on copper concentrations depending on what ligand is used and what solvent system is chosen.[22] Despite the fast kinetics of CuAAC, many of the copper catalysts used for CuAAC reactions are toxic in living systems and also have effects on cellular metabolism.[8,25–27] In order to improve upon the biocompatibility of azide–alkyne cycloadditions, Bertozzi and co-workers developed the copper-free variant, SPAAC, involving rate acceleration due to strain energy release in the alkyne moiety. A number of different cyclooctynes have been developed including difluorinated cyclooctyne (DIFO)[28] and derivatives,[29] Dibenzocyclooctynol (DIBO),[30] tetramethoxy-dibenzocyclooctynol (TMDIBO),[31] aza-dibenzocyclooctyne (DIBAC),[32] biaryl-aza-cyclooctynone (BARAC)[33,34] and bicyclononyne (BCN).[35]

Copper-free bioorthogonal reactions are bimolecular and bioconjugate formation can be represented by the following equation: [bioconjugate] = k_2 × [biomolecule] × [reagent] × t, where k_2 is the second-order rate constant, and the bioconjugate yield is proportional to the reagent concentration as well as k_2.[5] From a practical point of view, reactions with larger k_2 values and thus faster observed kinetics of bioorthogonal coupling reactions lead to shorter reaction times in biological systems. Also, larger k_2 values enable the reactions to reach completion at lower reagent concentrations as compared to slower reactions over the same reaction times. Thus, larger k_2 values can allow bioorthogonal labelling reactions to take place efficiently in a wider

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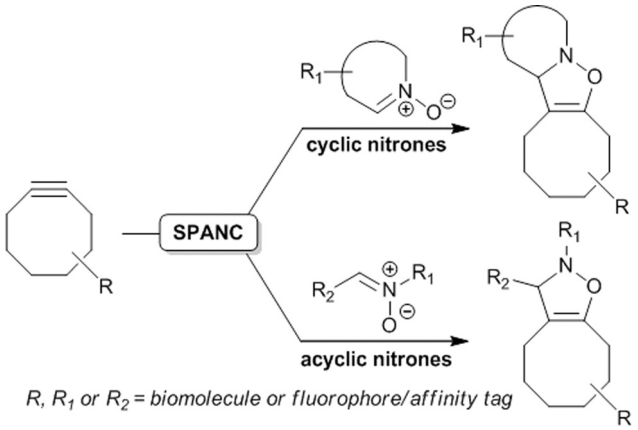


Scheme 1 Strain-promoted alkyne–nitrone cycloaddition (SPANC).

variety of living systems including live animals where the amounts of reagents required may be limiting.

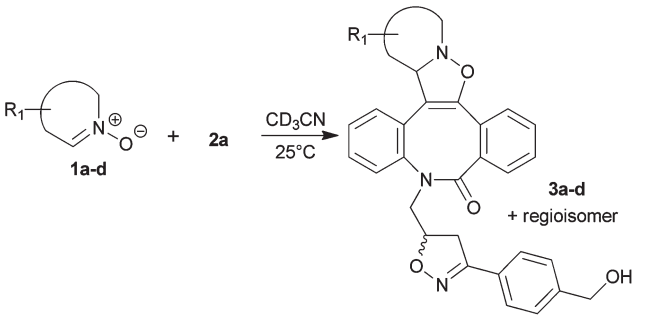
Nitrones have been shown to be useful dipoles in strain-promoted nitrone–alkyne cycloadditions (SPANC).[10–12] We recently reported the utility of cyclic nitrones in strain-promoted cycloadditions with dibenzocyclooctyne and 4-dibenzocyclooctynol (DIBO) and demonstrated that cyclic nitrones can serve as alternatives to azides with larger k_2 values generally observed with absolute values up to 3.38 M^{-1} s^{-1}.[11] We also showed that cyclic nitrones were generally more stable than their acyclic counterparts under aqueous conditions and could be employed to directly functionalize protein targets *in vitro* as well as on the surface of live human cancer cells.[11] The fast kinetics of SPANC reactions involving cyclic nitrones enables biological labelling at lower reagent concentrations. Recently, a more reactive aza-cyclooctyne, biaryl-aza-cyclooctynone (BARAC) was reported.[33] BARAC contains the highest number of sp^2-hybridized carbon atoms within the cyclooctyne ring, giving rise to greater ring strain than other reported cyclooctynes. Rate constants for SPAAC reactions involving BARAC were found to be significantly larger than for other cyclooctynes. However, BARAC has not yet been used for cycloaddition reactions with nitrones. Herein, we study reaction kinetics of SPANC reactions of BARAC[33] with cyclic and acyclic nitrones to illustrate the kinetic benefits of further ring strain to the SPANC reaction (Scheme 1).

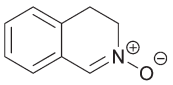
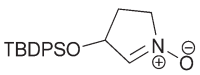
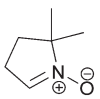
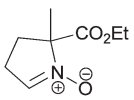
Results and discussion

Cyclic nitrones, **1a** and **1b**, were prepared by metal-free oxidation of the secondary amine using oxone in a biphasic medium,[36] 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO), **1c** was commercially available, and nitrone **1d** was prepared by an intramolecular alkyl-*N*-hydroxylamine/aldehyde condensation.[37] Acyclic nitrones, **1e–1l**, were efficiently prepared by micelle-promoted intermolecular condensation of alkyl-*N*-hydroxylamine and aldehyde.[38] Biaryl-aza-cyclooctynone (BARAC, **2a**) was synthesized according to literature protocol.[33]

In order to assess the utility of BARAC in SPANC, we first examined the efficiency of conversion to products. Previously, BARAC had been shown to react rapidly and with high conversion to products in cycloadditions with azides. Our expectation was that we would observe similar high conversions with cyclic nitrones that were shown previously to be stable and to work well in SPANC reactions. We performed reactions of compounds **1a–d** (~1 mM) with **2a** (~1 mM) in 99% MeCN–H_2O at r.t. We observed high yields of cycloaddition products and greater than 99% conversion after 20 min of reaction (Table 1), consistent with the previously reported rapid kinetics of SPAAC reactions involving BARAC. Next we sought to determine the bimolecular rate constants for SPANC reactions of a variety of nitrones with BARAC.

Table 1 Kinetics of SPANC reactions of cyclic nitrones with BARAC[a]



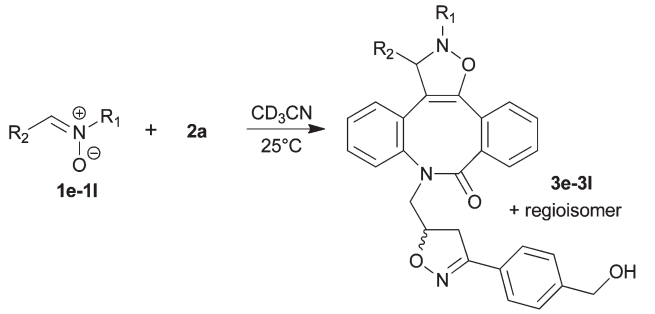
Nitrone		Product	% Conv.[b]	k_2[c] (M^{-1} s^{-1})
1a		**3a**	>99	22.4
1b		**3b**	>99	41.0
1c		**3c**	>99	39.2
1d		**3d**	>99	47.3

[a] Nitrone (**1a–1d**) and **2a** were mixed in a molar ratio 100 : 1 in 99% MeCN–H_2O at 25 ± 0.1 °C, the concentration of the excess nitrone was varied by ≥10% for an additional four trials. [b] Nitrone (**1a–1d**) and **2a** were mixed 1 : 1 at 25 mM final concentration, % conversion was determined by HPLC based on the amount of BARAC remaining after 20 min reaction. [c] k_2 was determined by UV-visible spectroscopy under pseudo-first order conditions.

Kinetics

In order to establish a kinetic profile for SPANC reactions of nitrones with BARAC, the associated bimolecular rate constant, k_2 was measured by UV-visible absorption spectroscopy under pseudo first-order reaction conditions (see ESI†). Firstly, we determined bimolecular rate constants of SPANC reactions of cyclic nitrones with BARAC (Table 1). Reactions of cyclic nitrones, **1a–d**, with **2a** yielded cycloadducts **3a–d** with excellent percent conversions within 20 min. While

Table 2 Kinetics of SPANC reactions of acyclic nitrones with BARAC[a]



Nitrone			k_2[b] (M^{-1} s^{-1})
1e	R_1 = Ph	R_2 = Ph	58.8
1f	R_1 = Bn	R_2 = Ph	2.8
1g	R_1 = Me	R_2 = Ph	6.8
1h	R_1 = Me	R_2 = *p*-$OMeC_6H_4$	4.9
1i	R_1 = Me	R_2 = *p*-MeC_6H_4	6.6
1j	R_1 = Me	R_2 = *p*-ClC_6H_4	7.9
1k	R_1 = Me	R_2 = *p*-CNC_6H_4	9.2
1l	R_1 = Me	R_2 = *p*-$NO_2C_6H_4$	10.1

[a] Nitrone **1e–f** and **2a** were mixed in a molar ratio 1 : 100 in 99% MeCN–H_2O at 25 ± 0.1 °C. Nitrone (**1g–l**) and **2a** were mixed in a molar ratio 100 : 1 in MeCN at 25 ± 0.1 °C. [b] k_2 was determined by UV-visible spectroscopy under pseudo-first order conditions.

dihydroisoquinoline *N*-oxide, **1a** reacted with **2a** with a rate constant of 22.4 M^{-1} s^{-1}, the five-membered pyrroline-*N*-oxides **1b–d** proceeded with rate constants of 41.0, 39.2 and 47.3 M^{-1} s^{-1}, respectively. Measuring rate constants allows one to infer effective rates at any concentrations of reactants. The reaction concentrations employed for measuring rate constants here are similar to the concentrations used in biological labelling experiments.[29,33] The five-membered nitrones demonstrated two-fold rate enhancement over the six-membered nitrones in reactions with **2a**. Cyclic nitrones reacted with BARAC up to 48 times faster than analogous reactions of benzyl azide, and 14 times faster than previously reported SPANC.

In order to examine the substrate scope and generality of SPANC reactions of nitrones with BARAC, we measured linear free energy relationships by changing substituents on the nitrones. We tested a series of acyclic nitrones bearing electron withdrawing and electron donating groups at the *para* position of the α-aryl substituent. We also varied the substitution at the N-position. Reaction of electron withdrawing *N*-phenyl substituted nitrone **1e**, with **2a** proceeded with an exceptionally fast rate (k_2 = 58.8 M^{-1} s^{-1}), whereas more electron donating methylene or methyl groups at the N-positions of nitrones **1f** and **1g** were well tolerated in reactions with **2a**, and demonstrated rate constants of 2.8 and 6.8 M^{-1} s^{-1}, respectively (Table 2). The presence of the *N*-phenyl electron withdrawing group in the cycloadduct **3e**, resulted in a thermally labile N–O bond, and it is well known that similar structures are prone to thermal rearrangement leading to azomethine ylides.[39,40] We find that smaller electron donating N-substituted nitrones proceed with sufficiently fast kinetics and provide stable products.

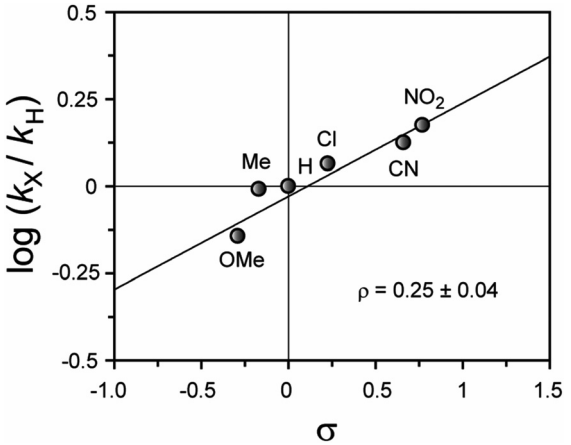
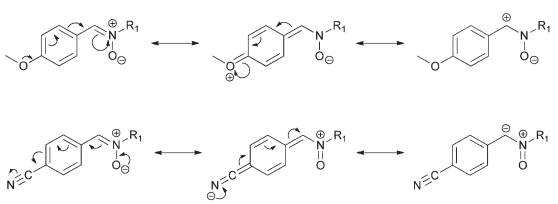


Fig. 1 Hammett plot depicting the substituent effects on the SPANC reactions of acyclic nitrones **1g–l** with BARAC.

Structure–reactivity relationships

Having established **1g** as optimal in terms of reactivity and stability we varied the substituents of the α-aryl groups in nitrones **1h–l**, to determine if electron withdrawing groups at this position would lead to rate enhancements. Modest rate accelerations were observed for acyclic nitrones containing electron withdrawing α-aryl substituents (Table 2).

To probe the mechanism of SPANC, we investigated substituent effects of acyclic nitrones on the relative rates of SPANC reaction with BARAC using a Hammett plot (Fig. 1). The best fit was obtained using σ_P parameters owing to the direct resonance interactions between the *para* substituents and the nitrone functional group as illustrated for electron donating groups in eqn (1) and for electron withdrawing groups in eqn (2). Plotting $\log(k_X/k_H)$ against σ_P parameters, gives a reaction constant, ρ, value of 0.25 ± 0.04 (R = 0.94). The small value of ρ suggests either an early transition state or a concerted transition state with no charge build-up at the α-position of the nitrone during the transition state.



Previously we investigated the effects of substituents on the micelle-catalyzed cycloadditions of nitrones with cyclopentenone, according to eqn (3).[41] A ρ value of −0.94 was determined from the corresponding Hammett plot for these nitrone–alkyne cycloadditions. In this study the cycloadditions were slow, and relative rate constants were determined from the ratios of products for reactions in competition. The value of ρ for nitrone-cyclopentenone is consistent with a transition state that has modest positive charge buildup at the nitrone carbon atom in the transition state and is also consistent with a concerted rather than a stepwise mechanism for the micelle catalyzed cycloaddition. Since cyclopentenone can act as a Michael acceptor, it is possible that a transition state that is slightly non-synchronous with

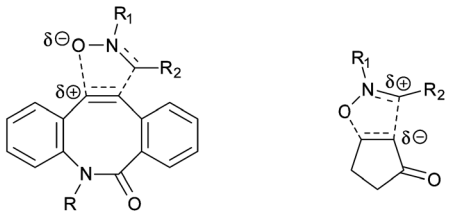
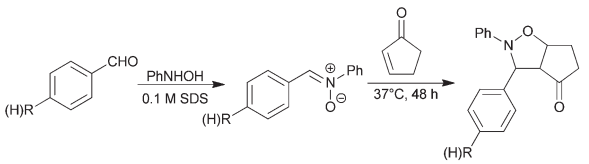


Fig. 2 Proposed transition states for cycloaddition reactions of substituted nitrones with BARAC and nitrones with cyclopentenone. The charge build-up in the transition states is depicted and is consistent with the differences in magnitude of the slopes of the Hammett plots for these two reactions.

more bond formation between the nitrone oxygen and the β-carbon of the α,β-unsaturated ketone and less bonding is formed between the nitrone α-carbon and the alkene exists. This would give rise to a modest positive charge build up in the transition state that would be stabilized by electron donating substituents.



(3)

Unlike the reactions of nitrones with cyclopentenone, the reactions of nitrones with BARAC are slightly accelerated by electron withdrawing substituents and show even less sensitivity to substituent effects. This suggests that the reactions of nitrones with BARAC have an even more synchronous transition state and follow a concerted mechanism. The ρ value for cycloadditions between nitrones and BARAC has the opposite sign to that for reactions of nitrones with cyclopentenone. This suggests that there is more bonding between the nitrone α-carbon and the cyclooctyne and less net positive charge on that α-carbon at the transition state for the reaction relative to the starting materials. Given that this is the case, the transition states for the two reactions can be represented by the structures in Fig. 2.

Structure–reactivity relationships for addition reactions onto nitrones have been studied extensively.[42–47] Generally, addition reactions that do not give rise to significant charge build up on the nitrone α-carbon atom have shown Hammett ρ values ranging from −1.0 to 0.76, consistent with the ρ value determined here. Interestingly, Durand *et al.* recently showed that Hammett σ_P values correlate with computed nitrone charge density as well as nitrone α-H NMR chemical shift values, suggesting that these parameters may also be predictive of reactivity of other nitrones.[48] For the nitrones tested here, we also observe a correlation between α-H chemical shift values and the corresponding σ_P constants, Fig. 3.

Nitrones are also often used as spin traps for free radicals and tend to react with hydroxyl and alkoxyl radicals as well as superoxide radical anions. Therefore, applications of SPANC reactions in living systems, particularly when those systems are under oxidative stress, the rates of cycloaddition would have to outcompete reactions with toxic free radicals. The rate constants for the spin trapping by DMPO, range from (2.7–3.6) × 10^9 M^{-1} s^{-1} for HO· depending on solvent and from 1.2 to 310 M^{-1} s^{-1} depending on solvent for $O_2^{\cdot -}$.[49,50] Since the magnitudes of the rate constants for addition of superoxide radical anions and for SPANC reactions involving reactive nitrones and BARAC are comparable, we expect that SPANC labelling should not be hampered by the presence of superoxide. Given that these free radicals are present at very low concentrations and also react rapidly with other cellular components, the rapid rates of cycloadditions between nitrones and cyclooctynes such as BARAC are expected to be sufficient to dominate so long as the concentration of BARAC is significantly higher than the concentrations of the reactive oxygen species, especially for hydroxyl radicals and alkoxyl radicals.

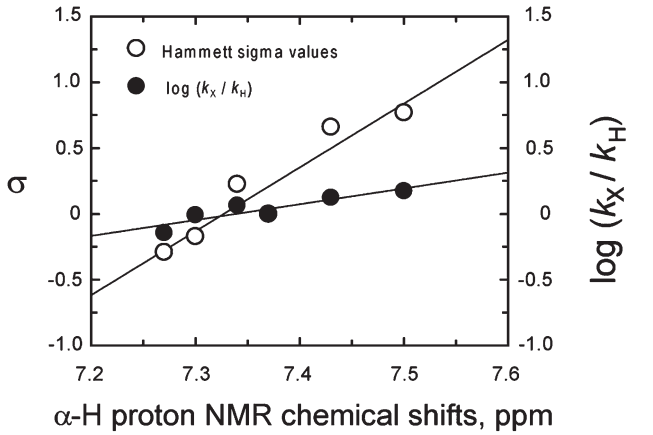


Fig. 3 Plots of nitrone α-H proton chemical shift values *vs.* σ_P constants with linear least squares fit showing positive correlation (R = 0.94) and nitrone α-H proton chemical shift values *vs.* log(k_X/k_H) values for the cycloaddition reactions between nitrones **1g–l** and BARAC (R = 0.95).

Overall, we observe a positive ρ value that is close to zero for cycloadditions of **1e–l** with **2a** and this indicates that the SPANC reactions with BARAC are not sensitive to substituent at the nitrone α-aryl carbon so that no significant rate enhancement can be obtained through substitution at this centre. The lack of sensitivity of SPANC to substituents suggests that the reaction is tolerant to a broad range of functionalized nitrones, which is desired for bioorthogonal labelling reactions. The nitrone α-carbon is therefore an ideal position for incorporation of bio-recognition elements, labels, or affinity tags. On the other hand, significant rate enhancements can be obtained by changing the substituents at the nitrone nitrogen atom (R_1 in Table 2). However, substituents at the nitrone nitrogen atom that accelerate the cycloaddition unfortunately also destabilize the reaction product by weakening the N–O bond. The optimal combination of rate enhancement and reactant and product stabilities is found with cyclic nitrones such as **1a–d** (Table 1).

Conclusion

We have demonstrated that both acyclic and cyclic nitrones serve as rapid alternatives to azides in strain-promoted cycloadditions with BARAC. SPANC reactions of cyclic nitrones proceeded with rate constants up to 47.3 M^{-1} s^{-1}, which corresponds to a

47-fold enhancement relative to reaction of benzyl azide and a 14-fold enhancement relative to previously reported SPANC. According to our studies of the effects of substituents, the SPANC reaction is sensitive to substituents on the nitrone nitrogen but not to substituents on the nitrone α-carbon atom. Thus, for chemical biology applications, nitrones should be further functionalized at the nitrone carbon atom. We are currently exploring metabolic and genetic routes for introducing nitrone reporters into biomolecules in living systems and detection *via* SPANC using functionalized BARAC and other strained alkynes.

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