NRC Research Press

ARTICLE

Kinetics studies of rapid strain-promoted [3+2] cycloadditions of nitrones with bicyclo[6.1.0]nonyne

Douglas A. MacKenzie and John Paul Pezacki

Abstract: Strain-promoted alkyne–nitrone cycloaddition (SPANC) reactions represent a bioorthogonal labeling strategy that is both very rapid and at the same time efficient and selective. Nitrones provide increased reaction rates as well as greater susceptibility toward stereoelectronic modification when compared with organic azides. We find that strain-promoted cycloadditions of cyclic nitrones with bicyclo[6.1.0]nonyne react with second-order rate constants as large as 1.49 L mol^{-1} s^{-1} at 25 °C. These reactions display rate constants that are up to 37-fold greater than those of the analogous reactions of benzyl azide with bicyclo[6.1.0]nonyne. We observed that reactions of nitrones with bicyclo[6.1.0]nonyne showed a stronger dependence on substituent effect for the reaction, as evidenced by a larger Hammett ρ value, than that for biaryl-aza-cyclooctanone. We demonstrate the ability to stereoelectronically tune the reactivity of nitrones towards different cyclooctynes in SPANC reactions. This ability to introduce selectivity into different SPANC reactions through substituent provides the opportunity to perform multiple SPANC reactions in one reaction vessel and opens up potential applications in multiplex labeling.

Key words: metal-free bioothrogonal cycloadditions, 1,3-dipolar cycloadditions, nitrones, click chemistry, bicyclo[6.1.0]nonyne, strain-promoted alkyne–nitrone cycloaddition (SPANC).

Résumé : La réaction de cycloaddition par catalyse forcée de nitrones à un alcyne (« strain-promoted alkyne–nitrone cycloaddition » ou SPANC) constitue une méthode de marquage à la fois efficace, sélective et très rapide. Les nitrones augmentent les taux de réaction amélioré et la sensibilité aux modifications stéréoélectroniques par comparaison avec les azotures organiques. Nous constatons que les réactions de cycloaddition par catalyse forcée de nitrones cycliques au bicyclo[6.1.0]nonyne ont lieu avec des taux de second ordre élevé de 1,49 L mol^{-1} s^{-1} à 25 °C. Ces réactions montrent des valeurs de taux jusqu'à 37 fois plus élevées que celles des réactions similaires de l'azoture de benzyle avec le bicyclo[6.1.0]nonyne. Nous avons observé que les réactions de nitrones avec le bicyclo[6.1.0]nonyne dépendaient davantage de substituant sur la réaction, comme l'a montré l'augmentation de la valeur de Hammett ρ, que celles avec la biaryl-aza-cyclooctanone. Nous avons démontré qu'il était possible d'ajuster stéréoélectroniquement la réactivité des nitrones avec différents cyclooctynes dans des réactions de SPANC. Cette aptitude à introduire une sélectivité dans différentes réactions de SPANC par l'intermédiaires de substituants rend possible la réalisation de multiples réactions de SPANC dans un réacteur unique et pourrait éventuellement être appliquée au marquage multiple. [Traduit par la Rédaction]

Mots-clés : cycloadditions bioorthogonales sans métal, cycloadditions 1,3-dipolaires, nitrones, chimie « click », bicyclo[6.1.0]nonyne, cycloaddition par catalyse forcée de nitrones à un alcyne (SPANC).

Introduction

The study of complex biological systems at the molecular level requires the use of a powerful and highly selective nonperturbing probe or reporter reactions to produce coherent results with minimum alteration of the native chemical environment. To this end, researchers have developed a number of rapid conjugation methods that are inert to naturally occurring functional groups and physiological conditions, thereby satisfying the requirements of bioorthogonality.[1] Copper(I) catalyzed azide alkyne cycloaddition (CuAAC) has been used for site-specific labeling of cell surface or purified proteins, primarily because of the synthetically accessible and cost-effective reagents and rapid kinetics.[2] Lowering the LUMO energy of the alkyne has lead to enhanced reactivity in CuAAC, while careful selection of ligands alters the cellular consequences resulting from the copper catalyst, which have limited the applications of CuAAC in living systems.[3–5] CuAAC has found widespread use in materials research where it has been employed as a grafting approach in polymer and dendrimer science,[6] in the fabrication of micron-scale surface chemical gradients,[7] in functionalizing poly(cyclic olefin) surfaces,[8] and in organometallic chemistry for the post-synthetic modification of metal organic frameworks,[9] for example. However, intracellular chemistry and biomolecular labeling and capture often demand the use of less perturbing labeling strategies, such as copper-free cycloaddition reactions.[10–12]

Staudinger ligation provides a means to covalently link an azide-containing molecule to a triarylphosphine through an amide bond under physiological conditions. Despite a comparatively slow rate constant (0.0077 L mol^{-1} s^{-1}),[13] this strategy has been used to deliver photo-crosslinking diazirene groups[14] and azobenzene photoswitches[15] to azide functionalized biomolecules. To circumvent the toxicity issues associated with copper catalysis in CuAAC reactions, copper-free strategies have been developed. Increased reaction kinetics have been obtained by the use of strain-promoted azide–alkyne cycloadditions (SPAAC).[16,17] The Bertozzi group has designed a number of cyclooctynes displaying rapid kinetics with benzyl azide, most of which make use of electron-withdrawing

Received 17 December 2013. Accepted 24 February 2014.

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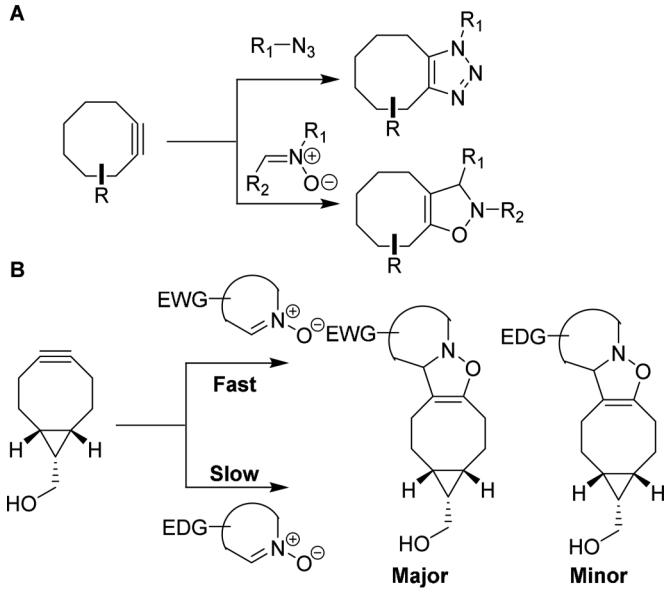
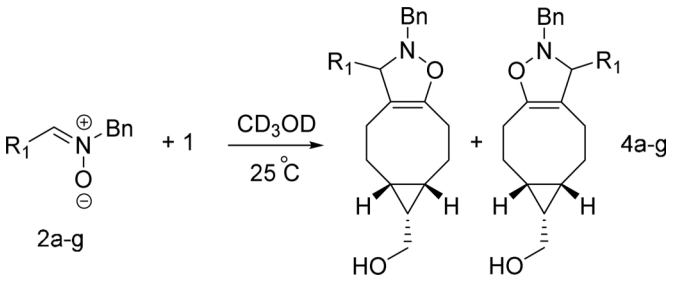
Fig. 1. (A) Overview of strain-promoted cycloadditions of cyclooctynes with azides and nitrones. (B) Overview of strain-promoted cycloadditions of electron-rich BCN (**1**) with stereoelectronically tuned endocyclic nitrones.



fluorine substituents or benzanulation to increase reaction rates.[18] Dommerholt et al. designed the relatively electron-rich bicyclo[6.1.0]nonyne (BCN) (**1**), making use of a fused cyclopropane ring to increase strain, leading to increased SPAAC rates.[19] Chin et al. have incorporated BCN as a dieneophile in unnatural amino acids for site-specific protein labeling in rapid and fluorogenic [4+2] cycloadditions with tetrazines.[20] Fluorogenic cycloadditions of tetrazoles and alkenes have also been demonstrated in live mammalian cells by Lin et al. using two-photon excitation to drive a "photoclick" conjugation.[21] Given the stereoelectronic differences in the current strained cycloalkynes that are applicable to bioorthogonal applications such as SPAAC reactions, there is an opportunity for developing new reactions by tuning the reactivity in copper-free ligation using stereoelectronically different dipoles other than alkyl azides.

SPANC have been presented as an alternative labeling strategy and have displayed rapid kinetics with strained cyclooctynes, showing second-order rate constants up to 58.8 L mol^{-1} s^{-1}.[22,23] SPANC reactions have proven useful in protein modification[24,25] and cell surface labeling of human breast cancer cells.[26] The use of endocyclic nitrones rather than the linear azide allows for the introduction of ring strain as well as three potential sites for stereoelectronic modification and (or) incorporation of a handle or linker to the dipolar component of the cycloaddition. With these possibilities for modification and the variation of the calculated LUMO energy levels in known strained cycloalkynes that are bioorthogonal, we envisioned that appropriate alkyne–nitrone pairs could be designed to provide mutually exclusive reactivity allowing for simultaneous duplex or multiplex SPANC chemistry.[27] Herein, we have studied SPANC reactions with the electron-rich bicyclo[6.1.0]nonyne and have shown that these reactions are more sensitive to substituent effects than those involving more electron-poor cyclooctynes. We demonstrate that SPANC reactions can be tuned so that they can be reacted simultaneously with minimal crossover products. These represent a proof of concept for the stereoelectronic tuning of SPANC reactions using endocyclic nitrones for the simultaneous duplex and possibly multiplex SPANC labeling reactions.

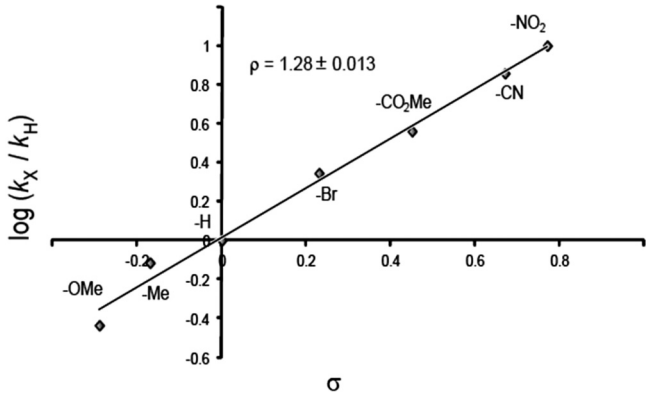
Table 1. Kinetics of SPANC reactions of acyclic nitrones and BCN.



Nitrone		k_2 (L mol^{-1} s^{-1})
2a	R_1 = *p*-OMeC_6H_4	0.0011
2b	R_1 = *p*-MeC_6H_4	0.0023
2c	R_1 = Ph	0.0029
2d	R_1 = *p*-BrC_6H_4	0.0065
2e	R_1 = *p*-CO_2MeC_6H_4	0.011
2f	R_1 = *p*-CNC_6H_4	0.021
2g	R_1 = *p*-$NO_2C_6H_4$	0.029

Note: Stock solutions of nitrones **2a–2g** were mixed in equimolar ratios with a stock solution of **1** at 25 ± 0.1 °C in CD_3OD. Rate constants k_2 were determined by ^{1}H NMR under second-order conditions.

Fig. 2. Hammett plot depicting the substituent effects on SPANC reactions of acyclic nitrones **2a–2g** and BCN.

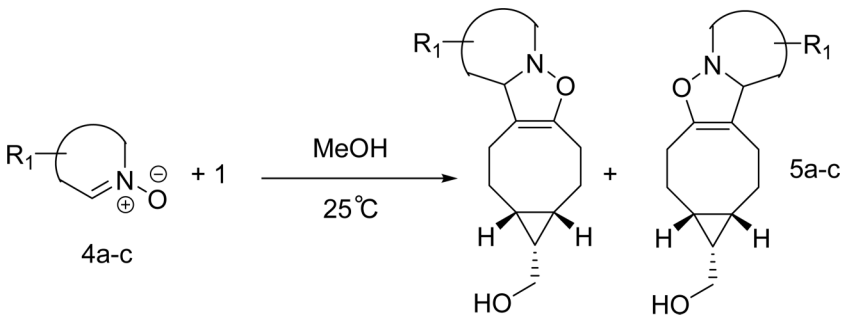


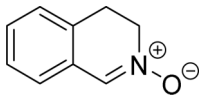
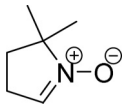
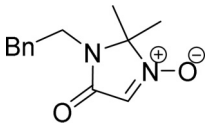
Results and discussion

To evaluate the sensitivity of SPANC toward different cyclooctynes, we have studied the reactions of different acyclic and endocyclic nitrones with BCN (**1**) (Fig. 1). To study the reactivity, we conducted a Hammett study by changing the substituents on the nitrone component systematically using a homologous series of α-aryl nitrones containing functional groups of varying electron-withdrawing or -donating ability. The second-order rate constants of these acyclic nitrones were measured with BCN (**1**) by ^{1}H NMR in deuterated methanol. The rate constants for these reaction reactions are presented in Table 1. For methods and kinetics data, see the supporting information ("Supplementary material" section). Nitrones **2a** and **2c–2g** were prepared by micelle-catalyzed condensation of benzyl-*N*-hydroxylamine with the appropriate aldehyde,[22] while **2b** was prepared by condensation without catalysis.[28]

SPANC reactivity has largely been explored with electron-deficient alkynes containing high levels of conjugation and delocalized electron density, as is the case with biaryl-aza-cyclooctanone (BARAC) and dibenzocyclooctynol (DIBO). BCN (**1**) was chosen for this study based on the localization of the alkyne π-electrons (calculated alkyne LUMO energy of 38.2 kcal mol^{-1}) and the lack of electron-withdrawing functional groups.[27] Figure 2 shows the Hammett plot comparing the observed bimolecular rate constants with

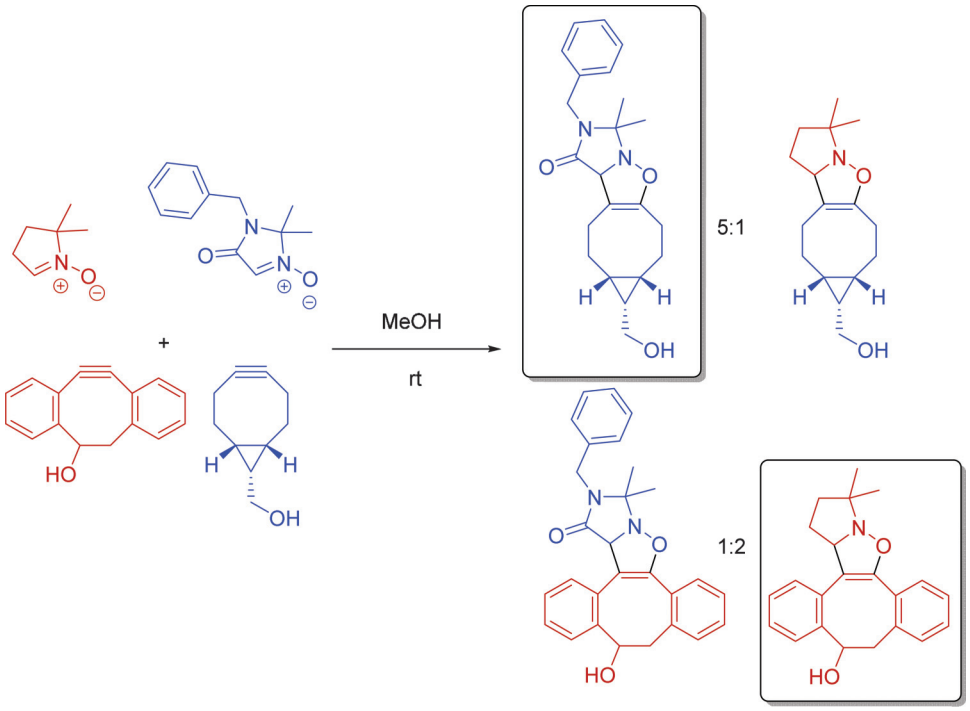
Table 2. Kinetics of SPANC reactions of cyclic nitrones with BCN.



Nitrone		Product	k_2 (L mol^{-1} s^{-1})	k_{rel}
4a		**5a**	0.65	16.2
4b		**5b**	0.05	1.24
4c		**5c**	1.49	37.0

Note: Nitrones **4a–4c** and **1** were mixed in 100:1 molar ratio in methanol at 25 ± 0.1 °C; the concentration of the excess alkyne was varied by ≥10% for an additional four trials. k_2 was determined by UV-Vis spectroscopy under pseudo-first-order conditions. k_{rel} is the second-order rate constant relative to the reaction of BCN with benzyl azide.

Fig. 3. Stereoelectronically tuned alkyne–nitrone pairs in a one-pot competition experiment displaying potential for mutually exclusive reactivity with minimal cross-reactivity.



σ_p values. Interestingly, we observed a Hammet ρ value of 1.28 ± 0.01, which is significantly larger than the previously measured ρ value of 0.25 ± 0.04 observed with an analogous series of α-aryl nitrones reacted with BARAC. This suggests that SPANC reactions involving BCN (**1**) are more sensitive to electronic changes than BARAC and the transition state is more strongly stabilized by electron-withdrawing groups appended to the nitrone component of the cycloaddition.[23]

Next, we sought to determine whether SPANC reactions involving BCN (**1**) and endocyclic nitrones would display a similar reactivity and sensitivity to substitution pattern. To this end, nitrones **4a–4c** were synthesized as previously described (see supporting information) and the bimolecular rate constants were measured in reactions with **1** under pseudo-first-order conditions by UV-visible absorption spectroscopy as described in the Materials and methods (see supporting information).[29,30] **4a** was prepared by metal-free oxidation of the secondary amine using oxone in a biphasic medium,[29] **4b** was commercially available, and nitrone **4c** was prepared by oxidation of the secondary amine using m-CPBA.[30] Table 2 summarizes the results of these kinetic studies, with nitrone **4b** showing a 30-fold rate enhancement over **4c** and a 37-fold rate enhancement over benzyl azide in reactions with **1**. Strained BCN (**1**) displays a bimolecular rate constant with electron-deficient **4b** that is suitable for biological labeling applications (1.49 L mol^{-1} s^{-1}) while showing sluggish kinetics with electron-rich **4c** (0.05 L mol^{-1} s^{-1}), which may provide further opportunity for selective one-pot reactions involving different nitrones and strained cycloalkynes in simultaneous SPANC reactions.

We hypothesized that by choosing appropriate alkyne–nitrone pairs, duplex reactions could be conducted in one pot with minimal cross-reactivity in SPANC reactions and that these duplex reactions would ultimately have applications for the purpose of simultaneous labeling of distinct biomolecules. To evaluate this hypothesis, solutions of BCN (**1**) and DIBO (**6**) were combined in an equimolar ratio with solutions of nitrones **4b** and **4c** (Fig. 3). DIBO was chosen for this study based on the similarity in second-order rate constant of DIBO with benzyl azide in methanol to that of BCN (0.0567 versus 0.0403 L mol^{-1} s^{-1}, respectively) as well as the lower calculated energy of the alkyne LUMO (36.1 versus 38.2 kcal mol^{-1}, respectively) and the structural similarity to BARAC.[27,31] This set up a one-pot competition experiment that was monitored by LCMS (see supporting information). Interestingly, a 5:1 selectivity of **1** for nitrone **4b** over **4c** and a 2:1 selectivity of **6** for nitrone **4c** over **4b** were observed. These results support our hypothesis that SPANC reactions can be tuned by adjusting both the cyclooctyne structure and the nitrone structure so that mutually exclusive bioorthogonal SPANC reactions can be developed. Cyclooctynes BCN (**1**) and DIBO (**6**) in reactions with nitrones **4b** and **4c**, respectively, represent the first examples of SPANC reactions developed for one-pot duplex labeling with minimal crossover products in the metal-free SPANC reactions.

Conclusion

Strain-promoted cycloadditions between alkynes and nitrones provide a rapid and bioorthogonal labeling strategy. Reactions of nitrones with BCN (**1**) can achieve rate constants significantly higher than those involving the corresponding alkyl azide. Exploitation of the variety in stereoelectronic properties of bioorthogonal strained alkynes and endocyclic nitrones can lead to reaction pairs that can undergo SPANC chemistry in one pot with minimal cross-reactivity. Efforts to obtain higher selectivity and reduced cross-reactivity through the stereoelectronic modification and design of strained alkynes and nitrones are currently underway in our laboratory.

Supplementary material

Supplementary data for this paper are available on the journal web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjc-2013-0577.

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