

## Computational Chemistry

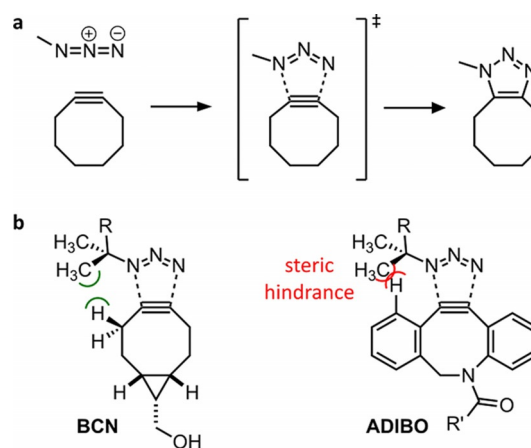
## Chemoselectivity of Tertiary Azides in Strain-Promoted Alkyne-Azide Cycloadditions

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**Abstract:** The strain-promoted alkyne-azide cycloaddition (SPAAC) is the most commonly employed bioorthogonal reaction with applications in a broad range of fields. Over the years, several different cyclooctyne derivatives have been developed and investigated in regard to their reactivity in SPAAC reactions with azides. However, only a few studies examined the influence of structurally diverse azides on reaction kinetics. Herein, we report our investigations of the reactivity of primary, secondary, and tertiary azides with the cyclooctynes BCN and ADIBO applying experimental and computational methods. All azides show similar reaction rates with the sterically non-demanding cyclooctyne BCN. However, due to the increased steric demand of the dibenzocyclooctyne ADIBO, the reactivity of tertiary azides drops by several orders of magnitude in comparison to primary and secondary azides. We show that this chemoselective behavior of tertiary azides can be exploited to achieve semiorthogonal dual-labeling without the need for any catalyst using SPAAC exclusively.

Bioorthogonal chemistry was introduced in 2000 by Saxon and Bertozzi, who showed that a modified Staudinger reaction can be used to ligate two compounds in a biological environment.<sup>[1]</sup> In the past two decades, the field has significantly

emerged and many additional reactions have been developed.<sup>[2–7]</sup> The strain-promoted alkyne-azide cycloaddition (SPAAC) and the inverse-electron demand Diels–Alder (DA) reaction of 1,2,4,5-tetrazines represent the most powerful and commonly used bioorthogonal ligations.<sup>[2,8]</sup> In SPAAC reactions, a cyclooctyne reacts with an organic azide in a 1,3-dipolar cycloaddition, affording a highly stable 1,2,3-triazole linkage (Figure 1 a). The pre-distortion of the cycloalkyne reduces the



**Figure 1.** a) Concerted mechanism of the strain-promoted alkyne-azide cycloaddition; b) Expected impact of the steric demand of tertiary azides on the transition state geometries when reacting with the cyclooctynes BCN and ADIBO.

energy required to achieve the transition state geometry and enhances orbital interactions between the distorted reactants. A similar electronic mechanism governs the trend of DA reactions involving cycloalkenes.<sup>[9]</sup> Cycloalkyne cycloadditions proceed with significantly lowered activation barriers enabling spontaneous reactions at room temperature without the need for a catalyst (second order rate constants of  $0.002\text{--}4.0\text{ M}^{-1}\text{ s}^{-1}$ ).<sup>[10,11]</sup> While tetrazine ligations show significantly higher reaction rates (up to  $3.3 \times 10^6\text{ M}^{-1}\text{ s}^{-1}$ ),<sup>[12]</sup> which is essential for rapid ligation in vivo,<sup>[13,14]</sup> SPAAC can be used in non-time critical applications, wherein the key advantages of the azide tag can be exploited: (i) high stability, (ii) small size, (iii) general orthogonality to biological processes, and (iv) metabolic incorporation into biomolecules.<sup>[15]</sup> Hence, SPAAC reactions have found broad application in many fields of research.<sup>[16–19]</sup>

An enormous effort has been made to optimize SPAAC mainly to improve reaction kinetics. Several studies focused on

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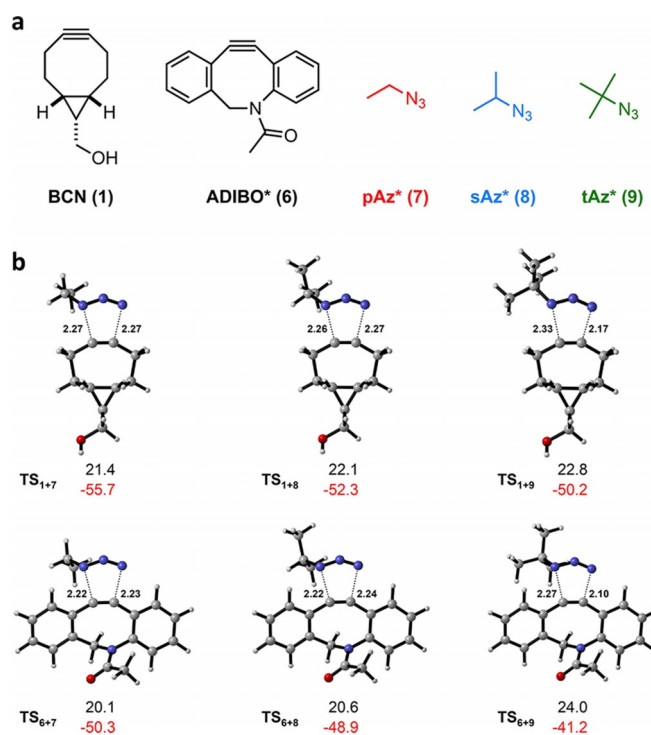
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the development and application of modified cyclooctynes as dipolarophiles.<sup>[11,17,20–24]</sup> The reactivities of new derivatives were investigated extensively both experimentally and theoretically,<sup>[25–27]</sup> leading to the development of bicyclo[6.1.0]non-4-yn-9-ylmethanol (BCN, **1**)<sup>[28]</sup> and azidobenzocyclooctyne (ADIBO, **2**),<sup>[29]</sup> currently the most commonly used cyclooctynes for SPAAC applications, with many commercially available reagents and tools (e.g., for labeling, bioconjugation, or molecular imaging). However, only a few reports focused on the structural modification of the azide to tune reaction kinetics. Hosoya and co-workers reported that sterically hindered 2,6-substituted phenyl azides unexpectedly exhibited a higher reactivity with dibenzo-cyclooctynes compared to sterically non-demanding phenyl azides.<sup>[30]</sup> In 2014, van Delft, Bickelhaupt, and co-workers showed that higher rates can be achieved by using electron-deficient aryl azides in inverse electron demand SPAAC reactions with BCN.<sup>[10]</sup>

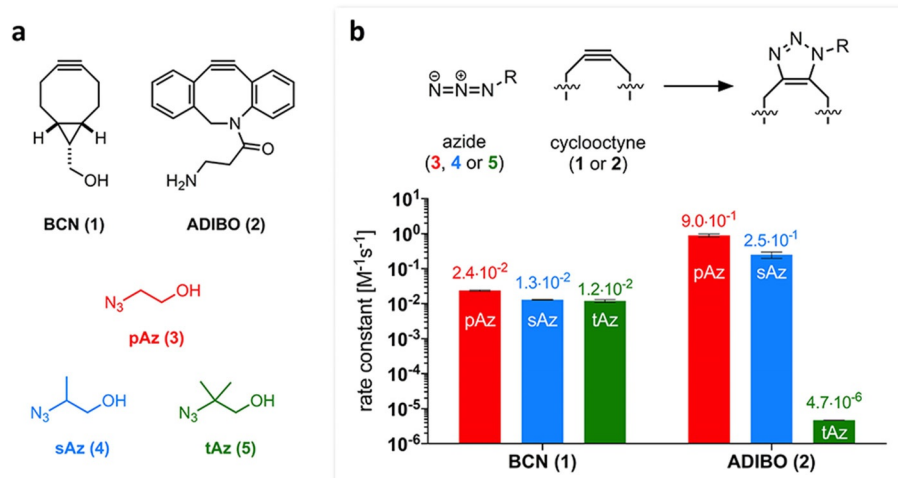
Herein, we report on the chemoselective reaction of tertiary azides with BCN (**1**) in the presence of sterically demanding ADIBO (**2**), which can thus selectively be reacted with primary or secondary azides. So far, only a few SPAAC reactions with secondary or even tertiary azides have been reported.<sup>[31–34]</sup> We hypothesized that primary, secondary, and tertiary azides exhibit similar reactivity with sterically non-demanding cyclooctynes, but expected a significant decrease in reactivity for tertiary azides when reacting with sterically demanding dibenzocyclooctynes (Figure 1 b).

The reaction kinetics of SPAAC ligations of BCN (**1**) and ADIBO (**2**) with primary, secondary, and tertiary azides were studied by NMR using the homologous series 2-azidoethanol (pAz, **3**), 2-azidopropanol (sAz, **4**), and 2-azido-2-methylpropanol (tAz, **5**), respectively (Figure 2b). As expected, all three azides show similar reaction rates with BCN ( $0.012$  to  $0.024 \text{ M}^{-1} \text{ s}^{-1}$ ), while we observed a rate of only  $4.7 \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1}$  when reacting ADIBO with the tertiary azide **5**, which is thus five orders of magnitude less reactive than the primary azide **3** ( $0.90 \text{ M}^{-1} \text{ s}^{-1}$ ) and the secondary azide **4** ( $0.25 \text{ M}^{-1} \text{ s}^{-1}$ ; Figure 2b).

To investigate the reason for the low reactivity of **5** in the reaction with ADIBO, we performed density functional theory (DFT) calculations to unravel the underlying mechanisms. Simplified model structures ADIBO\* (**6**), pAz\* (**7**), sAz\* (**8**), and tAz\* (**9**) were used to mimic ADIBO and azides **2–5**, respectively (Figure 3a). Various density functionals (PBE-D3, M06-2X, BP86, BP86-D3, B3LYP, and B3LYP-D3) in combination with the 6-311 + G(d,p) basis set were benchmarked against experimental data using Gaussian 09.<sup>[35]</sup> Quasi-harmonic corrections were applied to the calculations of entropies.<sup>[36]</sup> B3LYP-D3 greatly outperformed all other functionals when correlating the calculated



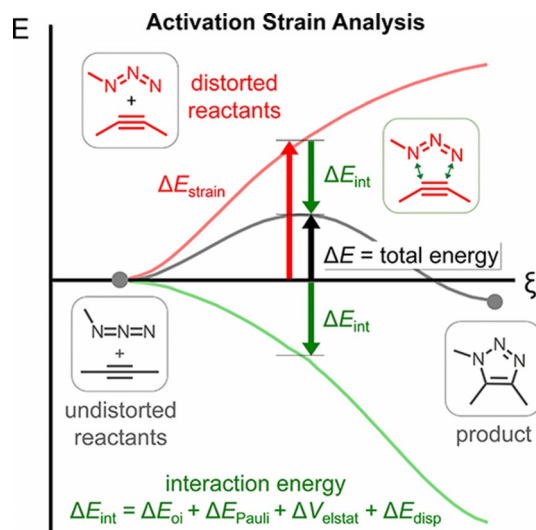
**Figure 3.** a) Model compounds used in DFT studies; b) Transition state geometries (distances in Å),  $\Delta G^\ddagger$  (black), and  $\Delta G^\ddagger_{\text{rxn}}$  (red) in kcal mol<sup>-1</sup> calculated at the B3LYP-D3/6-311 + G(d,p) level.



**Figure 2.** a) Azides and cyclooctynes used for reaction kinetics investigations by NMR; b) Second-order rate constants (25 °C in CDCl<sub>3</sub>, n = 3).

Gibbs free energies of activation ( $\Delta G^\ddagger$ ) and the measured reaction rates (see Supporting Information, Figure S1).

B3LYP-D3/6-311+G(d,p) was used to calculate all geometries and energies. The factors controlling the reactivity trends were elucidated using the activation strain model as developed by Bickelhaupt and Houk (Scheme 1).<sup>[37–41]</sup> This analysis



**Scheme 1.** The activation strain model exemplified using a general azide alkyne cycloaddition.

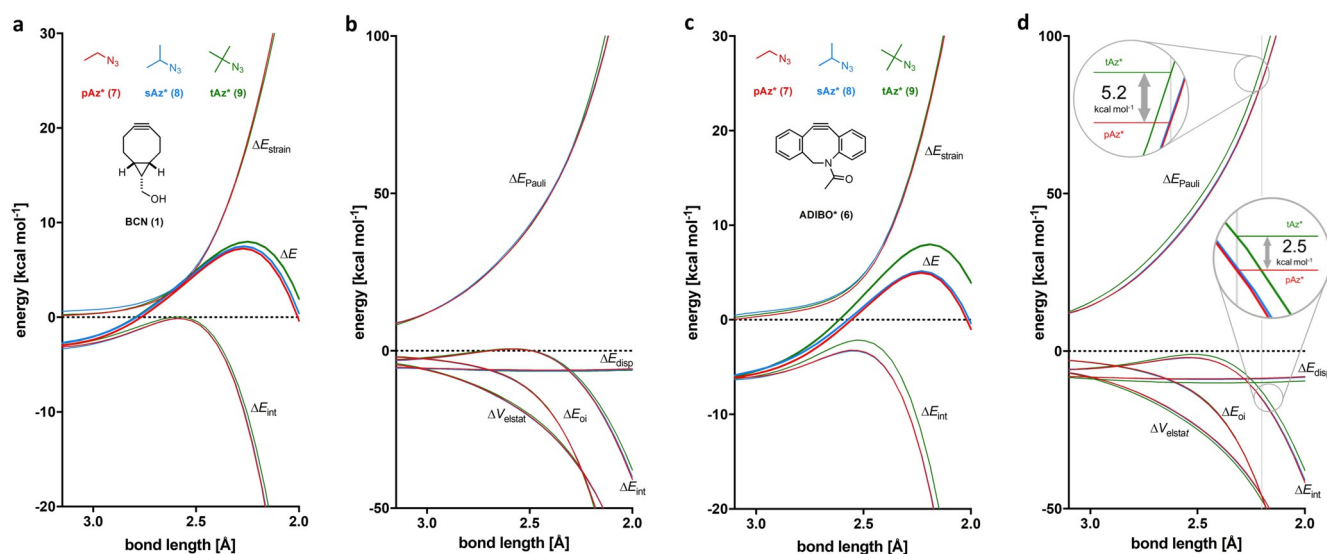
decomposes the electronic energy ( $\Delta E$ ) into two terms: the strain ( $\Delta E_{\text{strain}}$ ) resulting from the required distortion of the individual reactants, and the interaction ( $\Delta E_{\text{int}}$ ) between the distorted reactants along the reaction coordinate ( $\xi$ ) defined by the average length of the two forming C–N bonds.  $\Delta E_{\text{int}}$  was further decomposed into four terms by energy decomposition analysis:<sup>[42]</sup> (1)  $\Delta V_{\text{elstat}}$  corresponds to the classical electrostatic interactions, (2)  $\Delta E_{\text{Pauli}}$  is responsible for closed shell repulsions

(steric effects), and (3)  $\Delta E_{\text{oi}}$  accounts for charge transfer, mainly between frontier molecular orbitals (FMO), and polarization, and (4)  $\Delta E_{\text{disp}}$  which accounts for dispersion forces. The energy decomposition analysis was performed at the BLYP-D3/TZ2P<sup>[43]</sup>/B3LYP-D3/6-311+G(d,p) level using the Amsterdam density functional program (ADF).<sup>[44–46]</sup> Molecular structures were illustrated using CYLview.<sup>[47]</sup>

The B3LYP-D3/6-311+G(d,p) calculated free energies of activation range from 20.1 to 24.0 kcal mol<sup>−1</sup> for the reactions of azides 7–9 with ADIBO\* (6), and from 21.4 to 22.8 kcal mol<sup>−1</sup> for the reactions of 7–9 with BCN. In case of pAz\* (7) and sAz\* (8) the reaction proceeds via a concerted synchronous mechanism, while we observed highly asynchronous concerted transition states for tAz\* (9) (Figure 3 b).

Figure 4a shows the activation strain analysis (ASA) of the reactions between BCN (1) and azides 7–9. The strain curves are nearly identical along the reaction coordinate and the small differences in the activation barriers are the result of different interaction energies. Figure 4b summarizes the decomposition of the interaction energy, which is slightly less stabilizing for reaction 1+9 compared to 1+7 and 1+8, due to a more destabilizing  $\Delta E_{\text{Pauli}}$ . The  $\Delta E_{\text{oi}}$ ,  $\Delta V_{\text{elstat}}$  and  $\Delta E_{\text{disp}}$  curves differ only minimally along the reaction coordinate and, thus, play a negligible role.

For the reactions of ADIBO\* (6) with azides 7–9, the difference in energy of activation between the primary/secondary and the tertiary azide is much more prominent. The respective ASA reveals that the higher activation barrier between 6 and 9 is due to a less favorable interaction energy compared to the reactions with 7 and 8, as the strain curves are nearly identical for all three reactions (Figure 4c). The energy decomposition of  $\Delta E_{\text{int}}$  reveals that the less stabilizing interaction of ADIBO\* (6) and tAz\* (9) primarily results from a strongly destabilizing  $\Delta E_{\text{Pauli}}$  (Figure 4d). The destabilizing nature of  $\Delta E_{\text{Pauli}}$  overcomes the more favorable  $\Delta E_{\text{disp}}$  and  $\Delta V_{\text{elstat}}$  during the reaction of tAz\* with ADIBO\*. It is noteworthy that (despite the asynchro-



**Figure 4.** a) Activation strain analyses and b) energy decomposition analyses for the reactions 1+7 (red), 1+8 (blue), and 1+9 (green); c) activation strain analyses and d) energy decomposition analyses for the reactions 6+7 (red), 6+8 (blue), and 6+9 (green). Differences in  $\Delta E_{\text{Pauli}}$  (5.2 kcal mol<sup>−1</sup>) and  $\Delta E_{\text{int}}$  (2.5 kcal mol<sup>−1</sup>) between reactions 6+7 and 6+9 are indicated for a bond length of 2.2 Å (vertical gray line).

ny in case of **6+9**) the  $\Delta E_{oi}$  along the reaction coordinate are virtually identical in all three cases. Therefore, the difference in reactivity is purely based on steric interactions (see Figure 1 b) and could be traced back to closed shell interactions between the *tert*-butyl group of **9** and ADIBO\* (see Supporting Information). We have previously found  $\Delta E_{Pauli}$  to be a decisive factor controlling the regioselectivity of 1,3-dipolar cycloadditions<sup>[48]</sup> and now identify this energy term to be responsible for the observed chemoselectivity of tertiary azides.

Having unraveled the chemoselectivity of tertiary azides we next focused on the application of our findings for the development of selective dual-labeling techniques. We aimed to implement such an approach without the need for other bioorthogonal reactions (e.g., tetrazine ligation)<sup>[49–52]</sup> that do not exhibit the advantages of SPAAC, such as the high stability and small size of the azide tag. So far, semiorthogonal labeling using azides has been achieved by combining SPAAC and copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC).<sup>[53,32]</sup> In contrast, we aimed to combine tertiary and primary azides with BCN and ADIBO to enable semiorthogonal SPAAC reactions without the need for any catalyst and by applying commercially available building blocks exclusively.

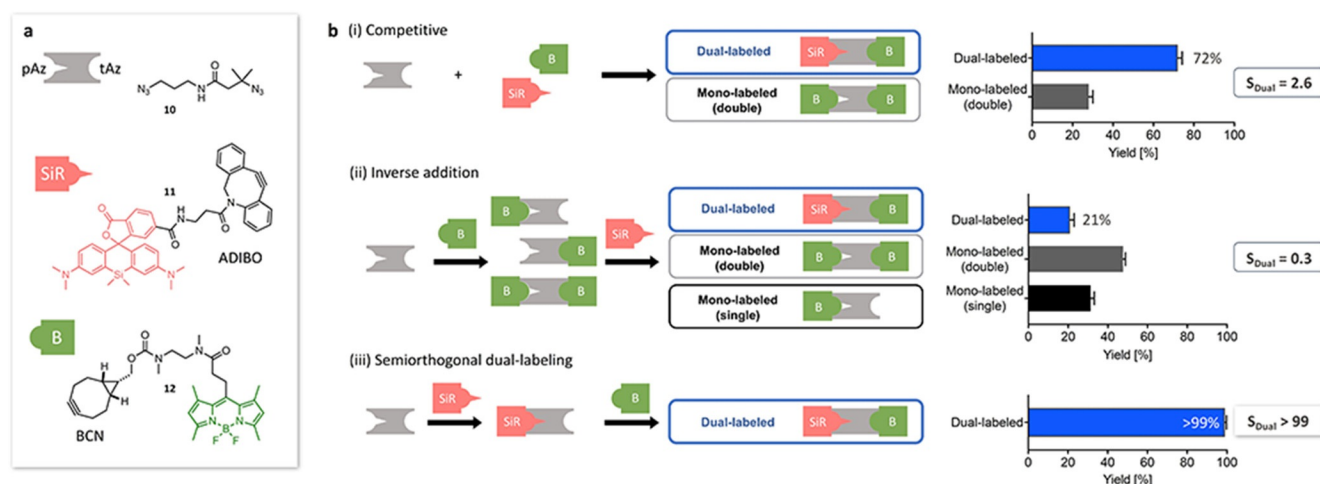
Compound **10** was used as a model substrate bearing a pAz and a tAz tag. ADIBO was labeled with a silicon rhodamine (SiR) dye affording compound **11**, and a boron-dipyrromethene (BODIPY) dye was attached to BCN to obtain conjugate **12** (Figure 5 a). Based on our previous findings we hypothesized that SiR-ADIBO (**11**) will react selectively with the pAz tag of **10** (considering the low reactivity of the tertiary azide with dibenzocyclooctynes), and that subsequent addition of BODIPY-BCN (**12**) will selectively afford a dual-labeled conjugate. To investigate such an approach, different labeling experiments were conducted and product mixtures were analyzed by LC-MS (Figure 5 b). Competitive reaction of **11** and **12** with **10** afforded a mixture of 72% dual-labeled product (containing both the SiR and BODIPY dye) and 28% of mono-labeled conjugate (containing two BODIPY units) resulting in a selectivity

for dual-labeling ( $S_{Dual}$ ) of 2.6. Inverse addition of both cyclooctyne dye conjugates, that is, reaction of **10** with BODIPY-BCN followed by SiR-ADIBO, afforded 21% of the dual-labeled product ( $S_{Dual}$  of only 0.27), due to formation of mono-labeled conjugates (48% with two BODIPY units, and 31% containing one BODIPY unit due to reaction of the pAz tag with **12**). To implement the envisaged semiorthogonal approach **10** was reacted with **11** followed by addition of **12**. In this order of addition, ADIBO selectively reacted with the pAz tag in the first step, and subsequent reaction of the remaining tertiary azide with BODIPY-BCN (**12**) afforded the dual-labeled product only (with no detectable mono-labeled byproducts;  $S_{Dual} > 99$ ), clearly demonstrating the ability of the pAz/tAz-ADIBO/BCN system to achieve semi- and bioorthogonal dual-labeling.

In conclusion, the reactivity of primary, secondary, and tertiary azides in the strain-promoted alkyne-azide cycloaddition was investigated in detail, using a combined experimental and computational approach. Tertiary azides exhibit decreased reactivity towards the dibenzocyclooctyne ADIBO due to more demanding steric interactions, in particular, increased Pauli repulsion, as shown by applying the activation strain model and energy decomposition analysis. The observed chemoselectivity was used to achieve highly selective bioorthogonal dual-labeling of azide-modified compounds without the need for any catalyst.

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**Figure 5.** a) Compounds used in dual-labeling experiments; b) model compound **10** was reacted with SiR-ADIBO (**11**) and BODIPY-BCN (**12**) in three different modes: i) competitive (i.e., simultaneous addition with **11** and **12**), ii) inverse addition (**12** followed by **11**), and iii) semiorthogonal (**11** followed by **12**). A high selectivity for dual-labeling ( $S_{Dual}$ ) was observed in the semiorthogonal approach applying the chemoselectivity of the tertiary azide tag.

on the Vienna Scientific Cluster (Austria) and the Cartesius supercomputer at SURFsara in Amsterdam.

## Conflict of interest

The authors declare no conflict of interest.

**Keywords:** activation strain model · bioorthogonal chemistry · click chemistry · computational chemistry · kinetics

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