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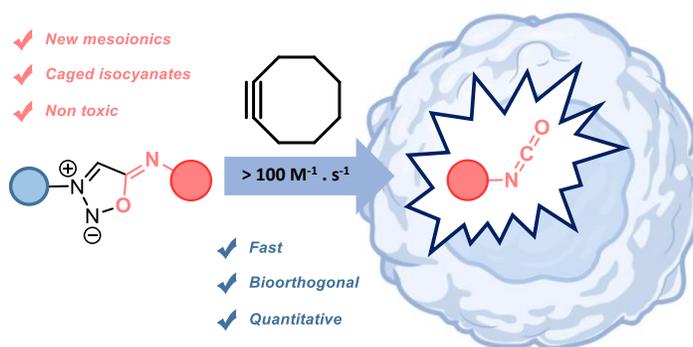
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Fast and Bioorthogonal Release of Isocyanates in Living Cells from IminoSydnones and Cycloalkynes.

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ABSTRACT: Bioorthogonal click and release reactions are powerful tools for chemical biology allowing, for example, the selective release of drugs in biological media, including inside animals. Here we developed two new families of iminosydnone mesoionic reactants that allow a bioorthogonal release of electrophilic species under physiological conditions. Their synthesis and reactivities as dipoles in cycloaddition reactions with strained alkynes have been studied in detail. Whereas the impact of the pH on the reaction kinetics was demonstrated experimentally, theoretical calculations suggest that the newly designed dipoles display reduced resonance stabilization energies compared to previously described iminosydnones explaining their higher reactivity. These mesoionic compounds react smoothly with cycloalkynes under physiological, copper-free reaction conditions to form a click pyrazole product together with a released alkyl- or aryl-isocyanate. With kinetic constants up to $1000 \text{ M}^{-1} \text{ s}^{-1}$, this click and release reaction is among the fastest described to date, and represents the first bioorthogonal process allowing the release of isocyanate electrophiles inside living cells offering interesting perspectives in chemical biology.



INTRODUCTION

Besides the numerous bioorthogonal tools available to efficiently link two (bio)molecules together, substantial effort is now devoted at developing bioorthogonal cleavage reactions.¹⁻⁵ Such reactions recently found considerable applications, in particular in the field of drug release.⁶ To this end, several cleavage reactions have been developed and optimized in order to improve their biocompatibilities, kinetics and efficiencies. Among them, the inverse-electron-demand Diels–Alder cycloaddition between tetrazines (Tz) and *trans*-cyclooctenes (TCO) carbamate derivatives is undoubtedly one of the most effective to achieve spatially and/or temporally controlled release of amine or alcohol-containing drugs inside living cells and even in animals.⁷⁻⁸ The biological activity of drugs can be temporarily masked by a Tz or TCO protecting group and subsequently deprotected by the corresponding cleavage trigger, restoring its activity. Improvements of this reaction⁹ both in terms of yields (up to 93%) and kinetics (up to $k = 23000 \text{ M}^{-1} \text{ s}^{-1}$) of drug release hold promise for clinical applications.¹⁰⁻¹² An impressive illustration is the development of a TCO-doxorubicin derivative, currently under clinical trials for solid tumor therapy in human patients, which is degraded when it comes in contact with a Tz-biopolymer implanted at the tumor site.¹³⁻¹⁴

Our group recently reported a new bioorthogonal cleavage reaction involving iminosydnones and cycloalkynes (SPICC: Strained-Promoted Iminosydnones Cyclooctyne Cycloaddition,

Figure 1).¹⁵ In the process, a first [3+2]-cycloaddition is occurring followed by a retro Diels–Alder step generating a pyrazole “click” product with the concomitant release of an isocyanate.

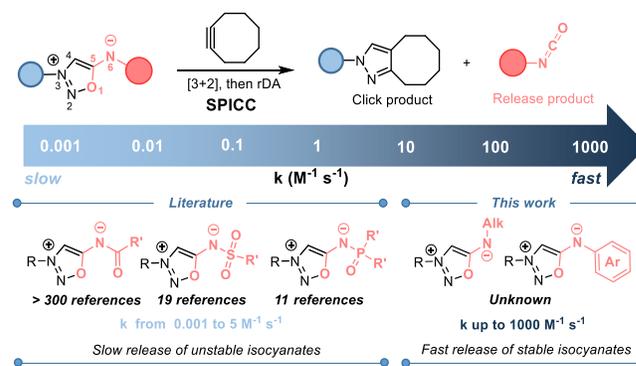


Figure 1. Structure and reactivity of iminosydnones toward click and release reactions with cyclooctynes.

Iminosydnones are known for decades to display interesting biological activities, mostly through their capability to act as effective exogenous donors of nitric oxide.¹⁶⁻²⁰ Some derivatives are also used as medicines (Molsidomine, Sydnophen and Mesocarb). In view of these remarkable biological applications, it is

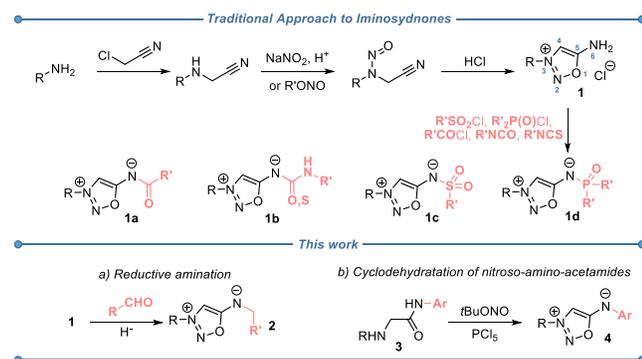
surprising that some members of the iminosydnone family have remained unexplored to date.

In 2017, based on computational estimations of the resonance stabilization energy (RSE) of several dipoles, one of us predicted that unsubstituted iminosydnone should be more reactive than regular sydnone.²¹ All derivatives described so far have an electron-poor carbonyl, sulfonyl or phosphoryl group substituting the exocyclic 6-*N* atom of the iminosydnone core (Figure 1). These reported derivatives are suitable substrates for the SPICC reaction. Their reaction with cyclooctynes, affords quantitatively the corresponding pyrazoles together with activated acyl-, sulfonyl- or phosphoryl-isocyanates, which are highly unstable in biological media. They undergo spontaneous hydrolysis to form the corresponding terminal amides, ureas, sulfonamides or phosphoramides products. This bioorthogonal reaction has been recently exploited to release the sulfonamide anti-inflammatory drug Celecoxib.²² However, despite a number of efforts to improve them, reaction kinetics using these iminosydnone were found quite moderate ($k < 10 \text{ M}^{-1} \text{ s}^{-1}$).²³ Based on the assumption that iminosydnone bearing electron-donating groups at the exocyclic nitrogen 6-*N* may be less resonance-stabilized and thus more active toward the SPICC reaction, we decided to investigate the synthesis of 6-*N*-alkyl and 6-*N*-aryl iminosydnone. Preliminary computations of their RSEs supported our hypothesis (see SI section VI). In addition to their expected improved reactivity, such compounds should release alkyl or aryl-isocyanates whose electrophilicity could be exploited. Herein, we report the synthesis of these unknown iminosydnone, their properties towards the SPICC reaction and their use to release alkyl- or aryl-isocyanates in biological media.

RESULTS AND DISCUSSION

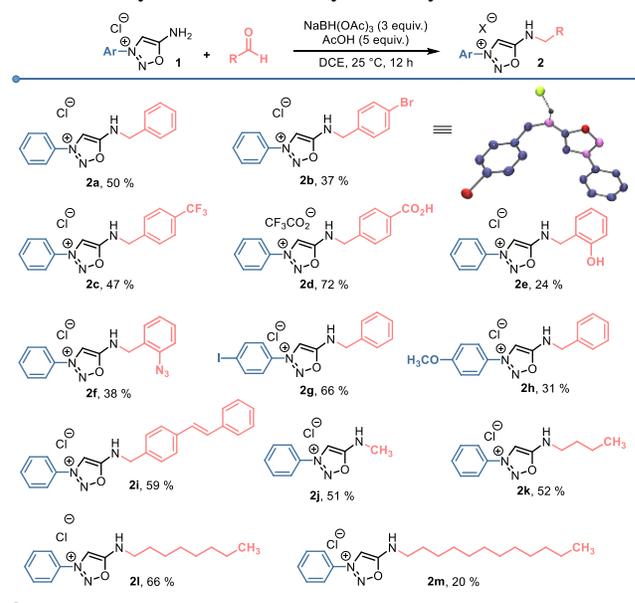
Synthesis. Since their discovery in the late 1950s by Brook and Walker,²⁴ the synthesis of iminosydnone has remained unchanged. The unique described synthetic route involves the formation of the iminosydnone salt **1** through nitrosylation of α -amino-nitriles and subsequent cyclization under acidic conditions (Scheme 1). Iminosydnone **1** can then react with strong electrophiles such as sulfonyl chlorides, phosphoryl chlorides, acid chlorides or isocyanates to afford iminosydnone **1a-d** (Scheme 1).²⁵⁻²⁷

Scheme 1. Conventional and proposed synthetic approaches to iminosydnone.



Anticipating that 6-*N*-alkyl iminosydnone **2** may have superior properties than **1a-d**, we started a synthetic program to access these unreported mesoionic from **1**. Due to the poor nucleophilicity of the exocyclic amine function of **1**, first attempts involving direct alkylation of **1** with alkyl-iodides or alkyl-triflates were unsuccessful and led mostly to decomposition. We then explored the reductive amination reaction of **1** in presence of benzaldehyde. After optimizations (Table S1), compound **2a** was obtained in 50% isolated yield by treatment with excess of $\text{NaBH}(\text{OAc})_3$ and AcOH in 1,2-DCE. The scope of the reaction was then investigated under the above-mentioned optimized conditions (Scheme 2). The reaction was compatible with several functional groups and worked in moderate to good yields with alkyl or aromatic aldehydes. Single-crystal X-ray diffraction analysis of compound **2b** highlighted a short exocyclic C-N bond (1.30 Å) of the mesoionic core, which is closer to a C=N double bond (1.28 Å) than a C-N single bond (1.38 Å).

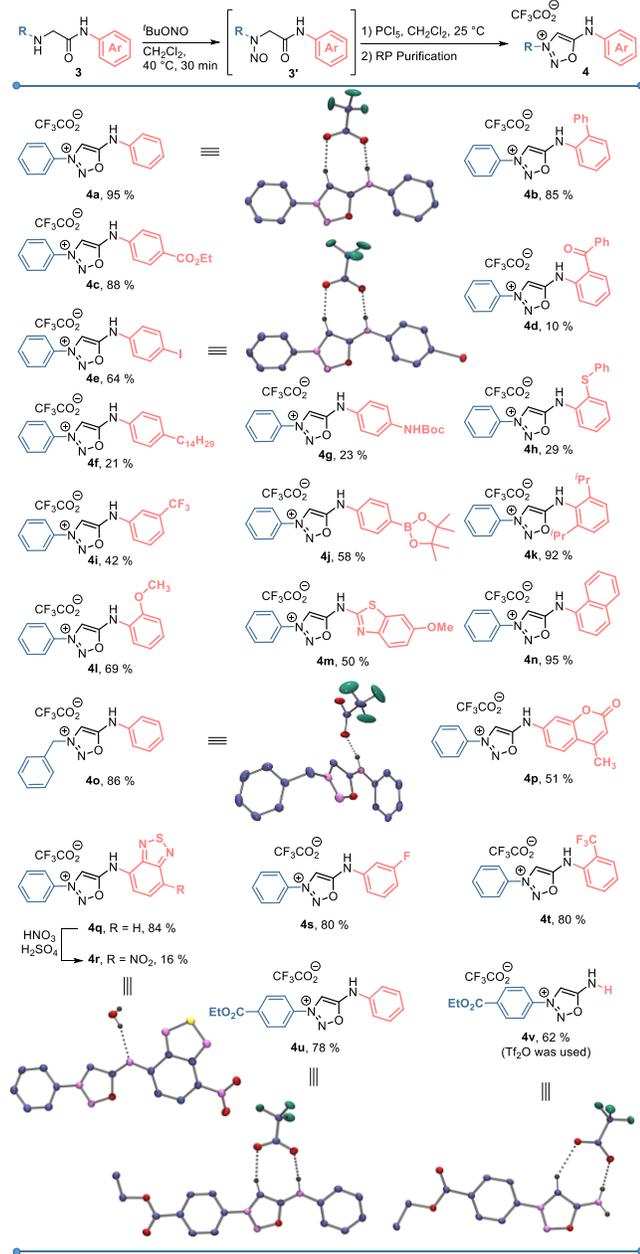
Scheme 2. Synthesis of 6-*N*-alkyl-iminosydnone



We then focused our attention on the synthesis of 6-*N*-aryl iminosydnone **4**. After multiple unsuccessful arylation attempts of compound **1** under Buchwald-Hartwig conditions, we investigated an alternative approach involving a cyclodehydration reaction of 2-nitroso-amino-acetamide derivatives **3'** as a possible route to mesoionics **4** (Scheme 3). *N*-phenyl-2-phenyl-aminoacetamide **3a** was selected as model substrate. After quantitative nitrosylation with *t*BuONO, **3a** was treated with a series of dehydrating agents (Table S2). PCl_5 was identified as the most effective, affording the desired iminosydnone **4a** in 95% yield. Mechanistic investigations conducted with an ^{18}O -labeled substrate indicated that the reaction proceeds through the attack of the oxygen atom of the *N*-nitroso function to the amide activated by PCl_5 (Scheme S1). The procedure was found quite general and afforded 6-*N*-aryl-iminosydnone **4a-v** in good yields (Scheme 3). All products were isolated as trifluoroacetate salts after reverse phase purification. Interestingly, this counter anion was found to form two H-bonds with positions 4-C-H and 6-*N*-H of the iminosydnone core according to X-ray analysis of compounds **4a**, **e**, **u** and **v**, but only one H-bond in

the case of 3-*N*-benzyl substituted **4o** (Scheme 3). All iminosydrones, except **4d**, were stable and could be stored on the bench for weeks at room temperature.

Scheme 3. Synthesis of 6-*N*-aryl-iminosydrones



Reactivity toward the SPICC reaction. With these new iminosydrones in hands, we looked at their capacity to undergo SPICC reaction with cyclooctynes. We first selected compounds **2a** and **4a** as model substrates and compared their reactivity towards the cyclooctyne DBCO with known iminosydrones **1a** and **1b** (Table 1). SPICC reactions were conducted under physiological conditions in the presence of excess of 2-naphthohydrazide (NP) to trap the corresponding isocyanates formed during the retro-Diels-Alder step. To our delight, we observed very fast reactions with iminosydrones **2a** and **4a**, affording almost instantaneously pyrazoles **5** together with benzyl or phenylisocyanates, which were either trapped by NP to form adducts **6** or hydrolyzed to their corresponding amines **7**. On the

contrary, iminosydrones **1a** and **1b** reacted much slower and the released acyl-isocyanates were totally hydrolyzed into the corresponding amide and urea respectively (Table S9). The constants determined for **2a** and **4a** were 59 and $240 \text{ M}^{-1} \text{ s}^{-1}$ respectively, which are significantly higher than those obtained with known iminosydrones.

Table 1. Comparison of the reactivity of iminosydrones towards the SPICC reaction.^a

| ImSyd | R | k^b | 5 ^c | 6 ^c |
|-----------|---|-------|-----------------------|-----------------------|
| 1a | | 0.001 | > 98% (15 h) | <0.01% (15 h) |
| 1b | | 5 | > 98% (2 h) | <0.01% (2 h) |
| 2a | | 59 | > 98% (<10 min) | 28% (10 min) |
| 4a | | 240 | > 98% (<10 min) | 41% (10 min) |

^aReactions were conducted in a 20/80 DMSO/0.1 M PBS buffer (pH 7.4) using $300 \mu\text{M}$ of iminosydrones, 1.5 equiv. of DBCO and 10 equiv. of NP. ^bKinetic constant ($\text{M}^{-1} \text{ s}^{-1}$) for the formation of **5** was determined by absorbance monitoring. ^cCrude yields were determined by LCMS (time required to reach full conversion). See Figure S21 for calibration.

We next investigated the importance of pH on kinetics of the SPICC reaction using iminosydrones **2a**, **4a** and **4o** as model substrates (Figure 2). These compounds were isolated as cationic salts of trifluoroacetate which are not dipolar structures susceptible to react with cyclooctynes. To form the active mesoionic form, deprotonation of the exocyclic 6-NH function is required. We therefore determined the pKa values of this function for the three model substrates and found quite different values, depending on the substituent in position 6-*N*. While 6-*N*-aryl iminosydrones **4a** and **4o** ($\text{pK}_a = 6.6$ and 6.2 respectively) are mostly present as mesoionic forms at physiological pH, only 4 % of mesoionic **2a** are present at this pH according to the pKa value ($\text{pK}_a = 8.7$, Figure S9). Despite the low abundance of the mesoionic form at pH 7.4, the constant of the click and release reaction with **2a** is still $59 \pm 1 \text{ M}^{-1} \text{ s}^{-1}$. As expected, this value increases dramatically with pH, to reach $k = 1273 \pm 49 \text{ M}^{-1} \text{ s}^{-1}$ at pH 9.5. On the contrary, the constant is only $2.5 \pm 0.1 \text{ M}^{-1} \text{ s}^{-1}$ at pH 5, which can be a limitation as acidic pH can be found in some compartments of the cells, such as lysosomes. The increase of the speed of the SPICC reaction in line with the pH is also observed for iminosydrones **4a** and **4o** although to a lesser extend due to lower pKa values (Figure 2A). The results globally indicated a direct correlation between the kinetic constant values and the percentage of mesoionic forms of iminosydrones demonstrating that this form is the only active species for the SPICC reaction (Figure 2B). The slopes of these correlations enable a comparison of the intrinsic reactivity of these three mesoionics, showing that the most reactive is **2a**.

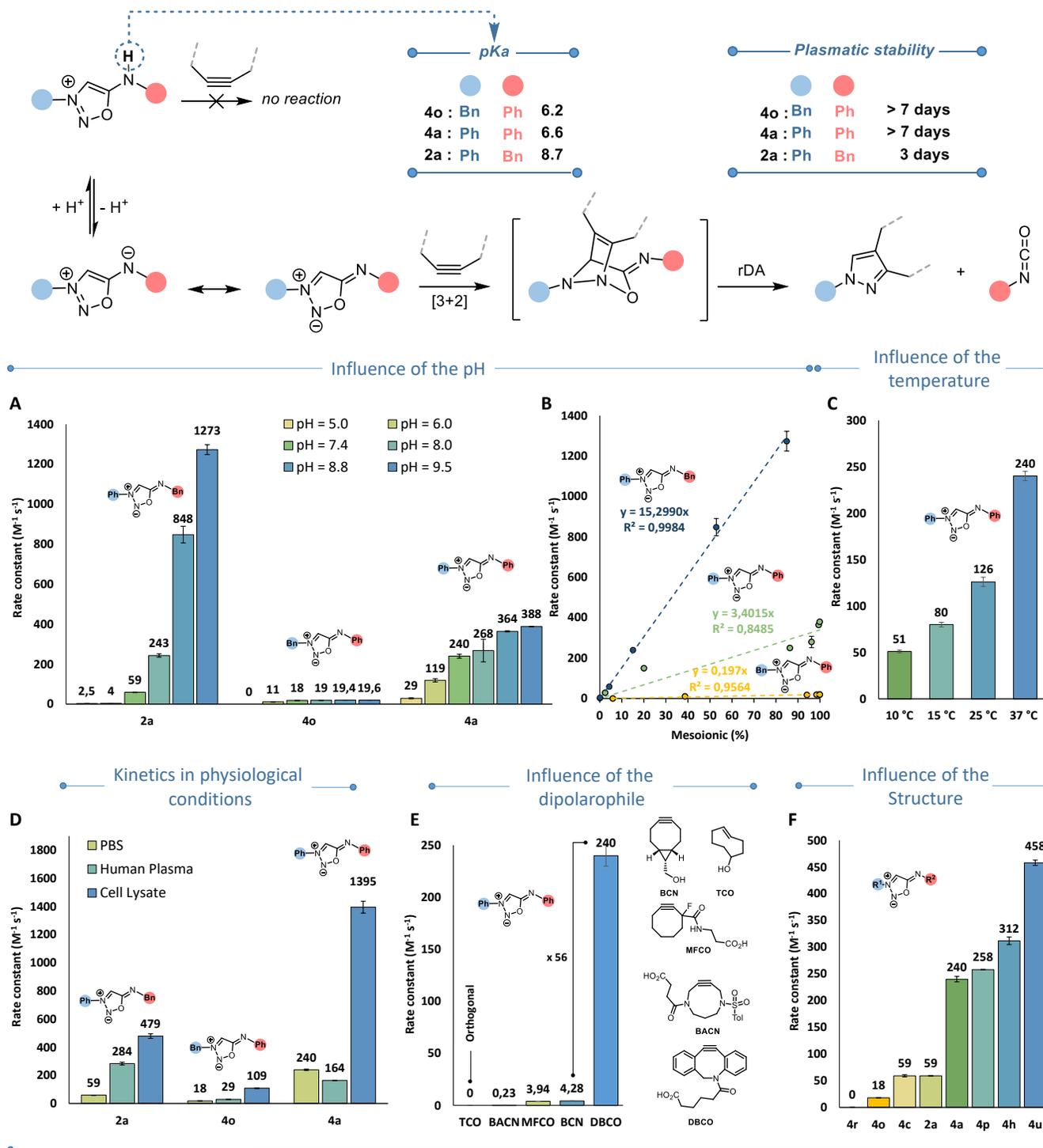


Figure 2. Cycloaddition reactions of iminosydones with DBCO. A) and B) Influence of the pH, C) Influence of the temperature, D) influence of the media, E) Influence of the structure of the cycloalkynes, F) Influence of the structure of iminosydones. All reactions were with 100 μM of iminosydones and 100 μM of DBCO. The evolution of the reactions was monitored by absorbance.

We then studied the compatibility of the reaction with biological media. All iminosydones were found stable in human blood plasma for several days (Figure S10). As shown in Figure 2D, their SPICC reactions with DBCO showed high efficiency both in cell lysate and in blood plasma, even significantly faster than in PBS buffer. Similar phenomenon has been described with the strained-promoted cyclooctyne-azide cycloaddition.²⁸ Not surprisingly, the reaction is almost five times faster at 37

$^{\circ}\text{C}$ than at 10 $^{\circ}\text{C}$ (Figure 2C). As expected the structures of both cycloalkyne and iminosydnone substrates had a strong impact on reaction rates. DBCO is almost 56 times more reactive than BCN. Cyclononyne BACN reacted also with **4a** but with a low kinetic constant ($0.23 \pm 0.1 \text{ M}^{-1} \text{ s}^{-1}$). Interestingly, TCO does not react with **4a** and thus could be used for bioconjugation with a Tz moiety in an orthogonal manner to iminosydones (Figure 2E). Electronic effects of 6-*N*-aryl iminosydones were also

found to play a significant role in the speed of the reaction, the best substrates being those having electron poor aromatic rings in position 3-*N* and electron-rich aromatic rings in position 6-*N* (Figure 2F).

DFT calculations:

In order to rationalize the difference experimentally observed in terms of kinetics depending on the nature of these novel mesoionics, we performed a theoretical study using DFT calculations at the M06-2X/6-31+G(d,p)/PCM(H₂O) // M06-2X/Def2TZVPP/PCM(H₂O) level of theory (see SI for computational details). Using iminosydnes **1a'**, **2a** and **4a**, the transition structures (TS) for their reactions with a DBCO model were located and their activation free energies ($\Delta G^\ddagger_{\text{water}}$) com-

puted (Figure 3). As shown in similar cycloadditions with sydnes,²¹ the cycloaddition step was confirmed as rate-determining in these systems as the retro-cycloaddition has a negligible barrier of activation (3.8 kcal mol⁻¹ for **2a**, see Figure S13). We thus compared the corresponding [3+2]-cycloaddition TSs for the three systems. The $\Delta G^\ddagger_{\text{water}}$ for **2a** and **4a** are 20.0 and 20.5 kcal mol⁻¹, respectively, significantly lower than that for **1a'** (26.0 kcal mol⁻¹). These differences in activation free energy between the three mesoionics are in line with the experimentally-observed rate enhancement. Indeed, the $\Delta\Delta G^\ddagger$ between **2a** and **4a** (0.5 kcal mol⁻¹) suggests a 2.3x faster rate of **2a** at 37.5 °C (exptl = 3.3 at pH 9.5). Similarly, the $\Delta\Delta G^\ddagger$ of 6.0 kcal mol⁻¹ between **2a** and **1a'** translates to a 1.7 x 10⁴ rate increase (at 37.5°C), in line with the relative reactivity of **1a** and **2a** (Table 1).

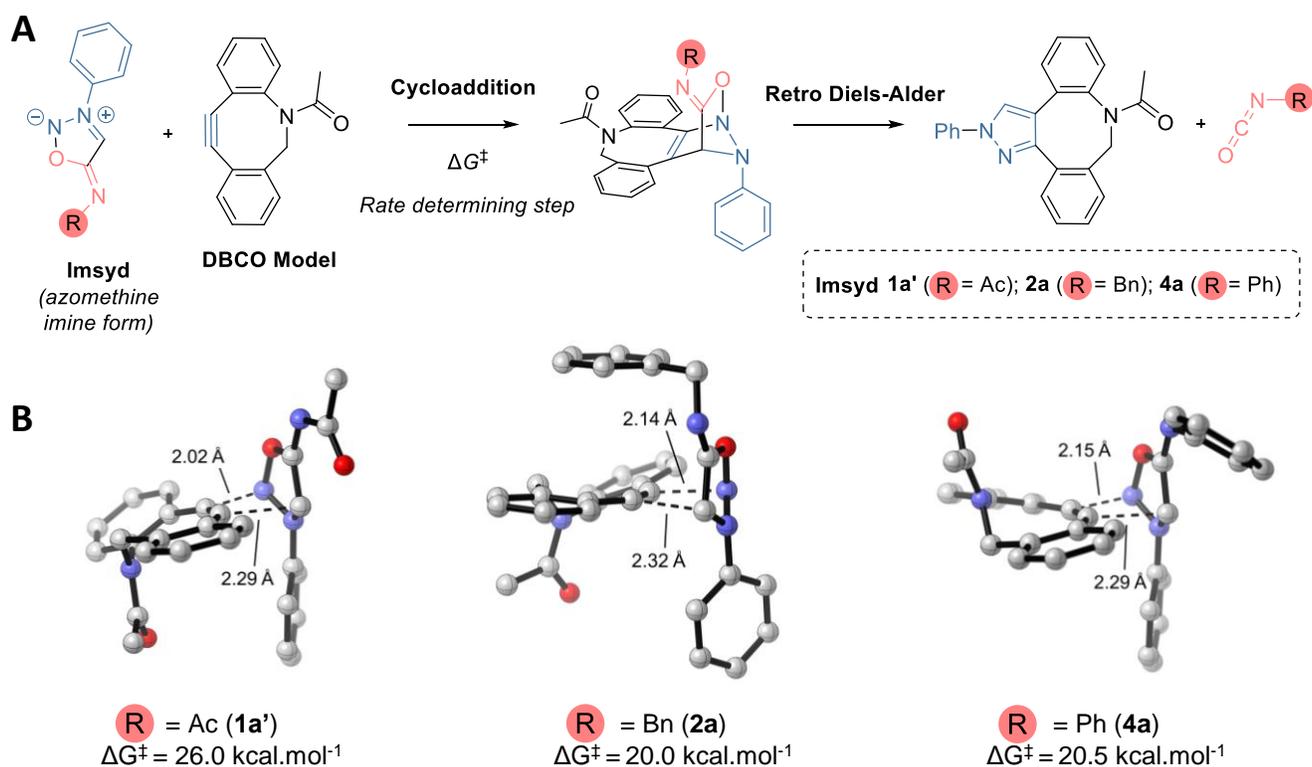


Figure 3: Theoretical study on the SPICC reaction. (A) Mechanism of the reaction and (B) lowest-energy transition structures involving Imsyd **1a**, **2a** and **4a** with a DBCO model.

The marked increase of reactivity of **2a**, **4a**, and other alkyl- or aryl-substituted iminosydnes can be explained by their reduced resonance stabilization energies (RSEs), as alluded to earlier. Indeed, such iminosydnes have RSEs that are significantly lower than regular sydnes and closer to that of an unsubstituted iminosydne. On the other hand, acyl-substituted iminosydnes such as **1a'** have RSEs that are larger than regular sydnes (Table S9). As RSE estimates the stabilization afforded by delocalization of the 1,3-dipole with the endo- and exocyclic substituents of mesoionics, it represents the contribution of the “aromatic” resonance structure of mesoionics to the global structure (see SI section VI for additional discussion on the structure and resonance hybrids of iminosydnes). **2a** and **4a** display structures that contain less contribution from that aromatic resonance form, and as such are more

reactive dipoles. RSE was indeed shown to correlate with activation energy in cycloadditions for mesoionics (Figure S15).²¹ Overall, these theoretical and experimental results demonstrated that the use of the newly designed iminosydnes accelerates significantly the SPICC reaction by favoring the active azomethine imine mesomeric form of iminosydnes through reduced RSEs.

SPICC bioconjugations of bio-nucleophiles and native biomolecules *in vitro* and *in living cells*. The above-described experiments demonstrated that 6-*N*-alkyl- and 6-*N*-aryl-iminosydnes can be considered as mild and bioorthogonal generators of alkyl- and aryl-isocyanate species in biological media. We thus investigated their use to trap endogenous biological nucleophiles. Amines are mostly protonated in biological media and therefore are considered as poor nucleophiles compared to biothiols. With concentrations up to 10 mM inside cells, glutathi-

ysis. The cells were lysed and an electrophoresis gel was performed. As expected, no labeling was observed for the probe **2n** and, more surprisingly, no protein labelling was observed either in the case of direct use of the isocyanate **8**. On the other hand, multiple fluorescent bands were observed in the case of in-cell formation of the isocyanate indicating that numerous proteins inside the cells have been efficiently labeled. Blank experiments carried out in absence of DBCO showed no labeling. All together, these results demonstrate the advantages of using

caged isocyanates to trap internal nucleophiles in cells. Complementary results were obtained with an alkynylated iminosydnone probe which demonstrated the possibility to alkynylate proteins inside living cells using the SPICC reaction (Figure S28). These results confirmed the possibility to use iminosydnone as tools for proteomic experiments. Their coupling to specific organite tracers may notably offer the possibility to identify proteins in specific compartments of the cells.

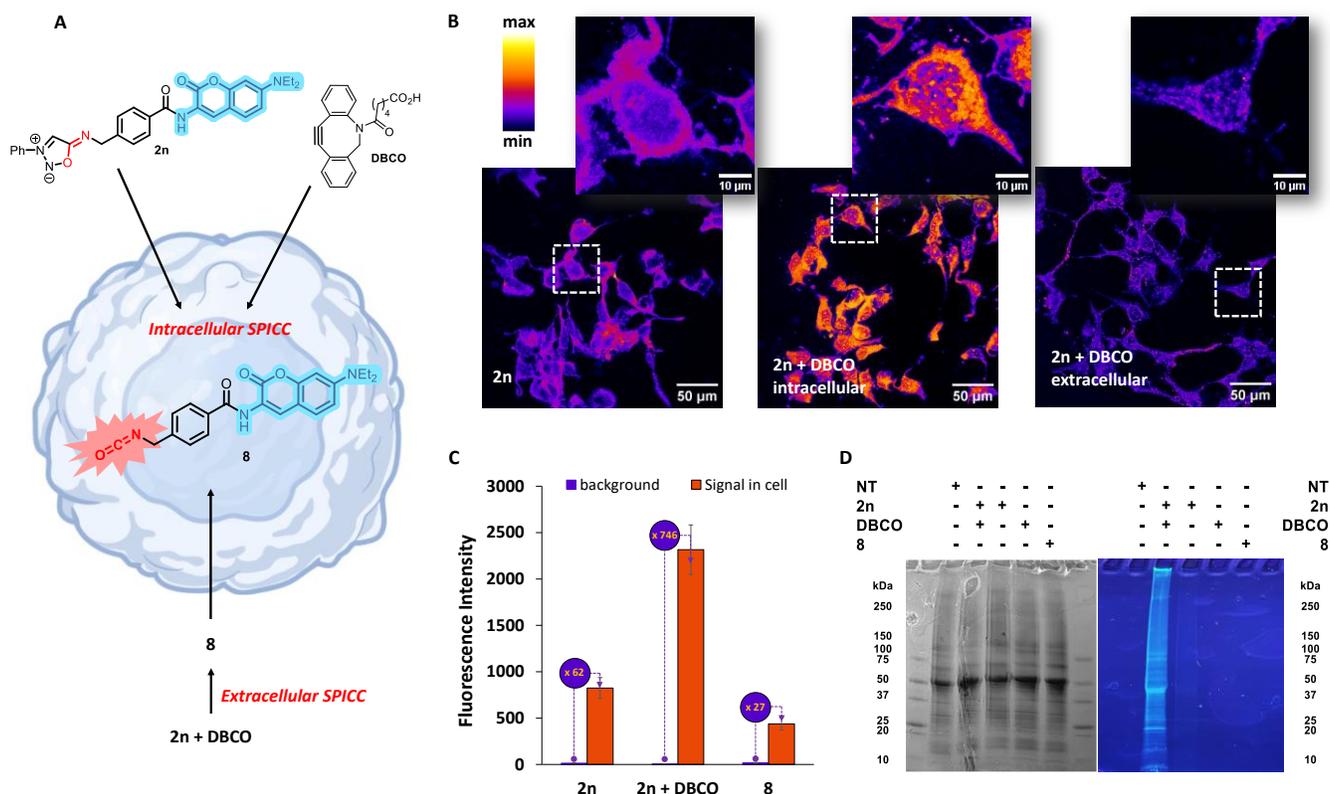


Figure 5. Experiments in cells. A) Schematic representation extra cellular versus intra cellular formation of the isocyanate B) Confocal microscopic images of live-HEK-293 kidney cells treated with compounds **2n** (left), **2n** + DBCO (middle) and **8** (right) at 10 μ M for 3 h at 37 $^{\circ}$ C using a 40x oil immersion objective. (Exc: 440 nm, Em: 470–550 nm), C) Fluorescence signal to background ratio in HEK-293 live-cell after washing with PBS buffer. Bar plot representing the fluorescence intensity obtained by measuring pixel mean using ImageJ software, D) SDS-PAGE of the lysate (60 μ g/well) Coomassie blue staining left picture and fluorescence at 312 nm right picture.

CONCLUSION

In summary, we developed two new families of iminosydnone that displayed improved reactivity toward bioorthogonal click and release reactions with cyclooctynes. Theoretical calculations have confirmed the initial working hypothesis that these new dipoles would exhibit reduced RSE compared to previously described iminosydnone and therefore accelerates significantly the kinetics of cycloaddition reactions and the related release. The SPICC reaction proved to be fast and compatible with biological media including cell lysate, blood and intracellular media. The described iminosydnone can be considered as caged *N*-alkyl and *N*-aryl isocyanates that can be released on demand through addition of cyclooctynes. A caged isocyanate nucleophile reactive fluorescent probe was developed that demonstrated low cytotoxicity, enabling internalization at high concentrations in cells. This probe was used to label proteins

inside living cells with preservation of native cellular environments during the nucleophile-trapping step minimizing disruptions caused during cell lysis. The SPICC reaction proceeds within minutes without the use of toxic metal catalysts and allows the generation of isocyanates in complex biological media through a simple, mild and practical process. Given the intrinsic advantages of temporal control, biocompatibility, exquisite chemoselectivity and high efficiency, the SPICC click and release reaction would provide a powerful and reliable chemical tool for applications in permanent cell labeling and protein fishing. We believe this strategy will be easy for researchers to implement in several fields of chemical biology.

ASSOCIATED CONTENT

Supporting Information. Detailed synthetic procedures and analytical data of presented compounds, NMR spectra, crystallographic data, kinetic studies, including S1-S22 Tables S1-S2 and

scheme S1. “This material is available free of charge via the Internet at <http://pubs.acs.org>.”

Accession Codes. CCDC 2150451–2150457 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

SPICC, Strained Promoted Iminosydnone Cyclooctyne Cycloaddition; BCN, Bicyclo-[6.1.0]-nonyne; DBCO, Dibenzocyclooctyne; 1,2-DCE, 1,2-dichloroethane; RP purification, Reverse Phase purification.

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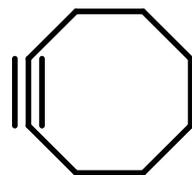
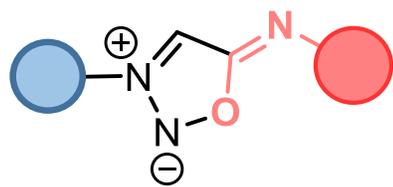
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✓ *New mesoionics*

✓ *Caged isocyanates*

✓ *Non toxic*



$> 100 \text{ M}^{-1} \cdot \text{s}^{-1}$

✓ *Fast*

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