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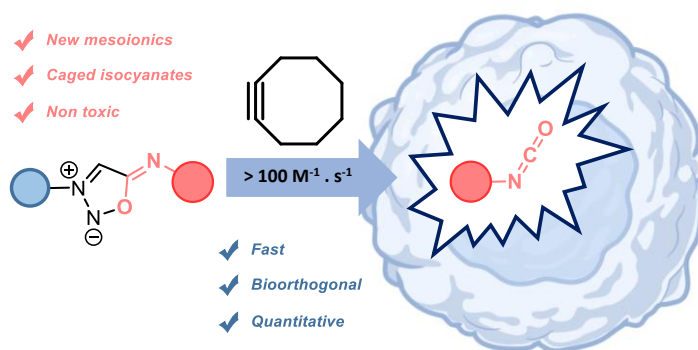
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Fast and Bioorthogonal Release of Isocyanates in Living Cells from IminoSydnones and Cycloalkynes.

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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.

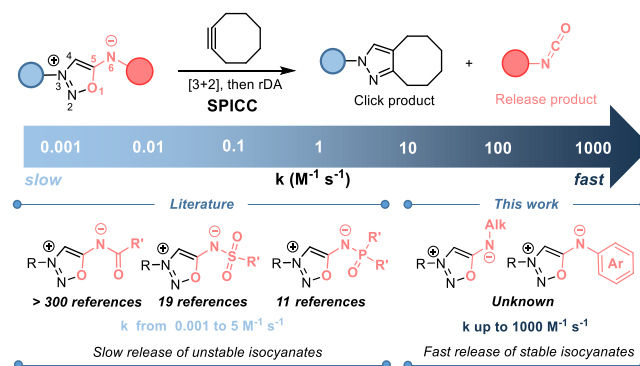


Figure 1. Structure and reactivity of iminosydnones toward click and release reactions with cyclooctynes.

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

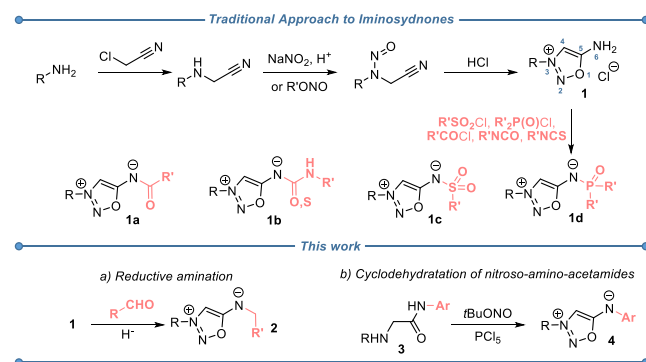
surprising that some members of the iminosydnone family have remained unexplored to date.

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

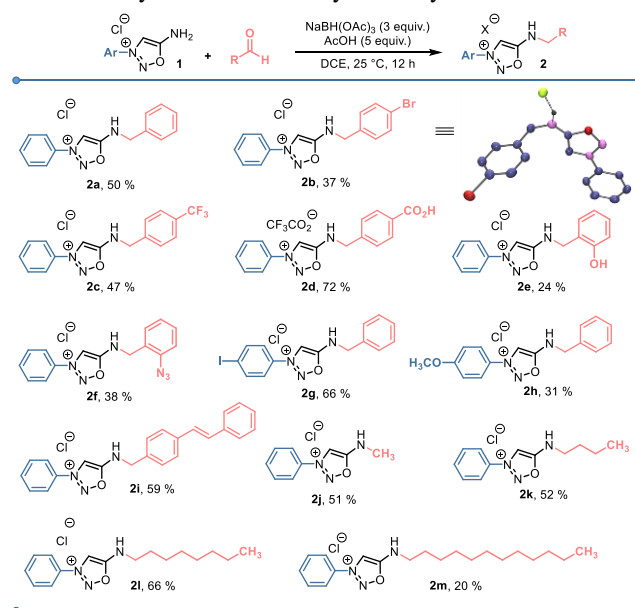
Synthesis. Since their discovery in the late 1950s by Brook and Walker,²⁴ the synthesis of iminosydnone has remained unchanged. The unique described synthetic route involves the formation of the iminosydnone salt **1** through nitrosylation of α -amino-nitriles and subsequent cyclization under acidic 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

We next investigated the importance of pH on kinetics of the SPICC reaction using iminosydnone **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 iminosydnone **4a** and **4o** (pKa = 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 (pKa = 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 iminosydnone **4a** and **4o** although to a lesser extent 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 iminosydnone 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**.

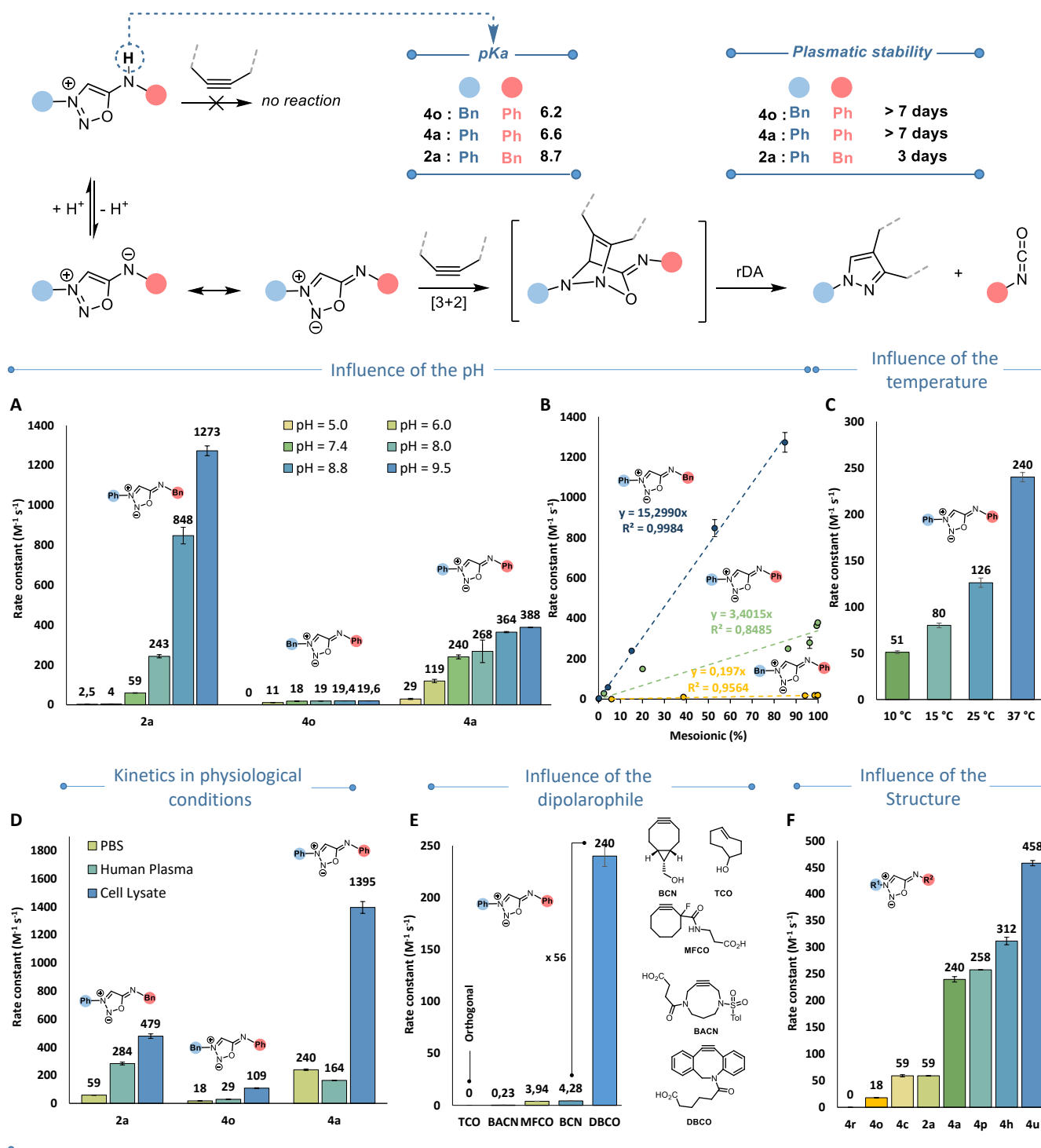


Figure 2. Cycloaddition reactions of iminosynones with DBCO. A) and B) Influence of the pH, C) Influence of the temperature, D) influence of the media, E) Influence of the structure of the cycloalkynes, F) Influence of the structure of iminosynones. All reactions were with 100 μ M of iminosynones and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

We then studied the compatibility of the reaction with biological media. All iminosynones 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

°C than at 10 °C (Figure 2C). As expected the structures of both cyclooctyne and iminosynone 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 ± 0.1 M⁻¹ 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 iminosynones (Figure 2E). Electronic effects of 6-*N*-aryl iminosynones 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 iminosydones **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 sydrones,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).

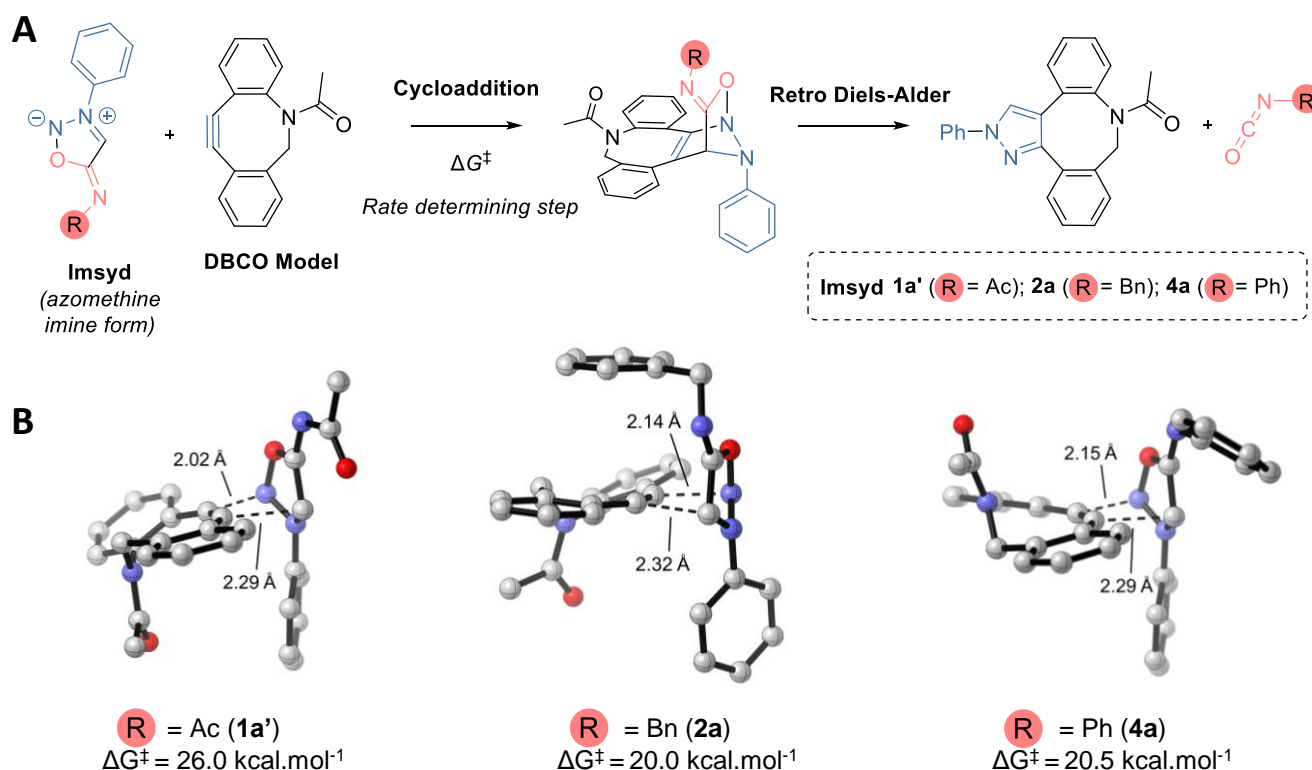


Figure 3: Theoretical study on the SPICC reaction. (A) Mechanism of the reaction and (B) lowest-energy transition structures involving Imsyd **1a**, **2a** and **4a** with a DBCO model.

The marked increase of reactivity of **2a**, **4a**, and other alkyl- or aryl-substituted iminosydones can be explained by their reduced resonance stabilization energies (RSEs), as alluded to earlier. Indeed, such iminosydones have RSEs that are significantly lower than regular sydrones and closer to that of an unsubstituted iminosydnone. On the other hand, acyl-substituted iminosydones such as **1a'** have RSEs that are larger than regular sydrones (Table S9). As RSE estimates the stabilization afforded by delocalization of the 1,3-dipole with the endo- and exocyclic substituents of mesoionic dipoles, 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 iminosydones). **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 mesoionic dipoles (Figure S15).²¹ Overall, these theoretical and experimental results demonstrated that the use of the newly designed iminosydones accelerates significantly the SPICC reaction by favoring the active azomethine imine mesomeric form of iminosydones 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-iminosydones 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-

one (GSH) represents the most abundant endogenous nucleophile of the cell and the main cell defense mechanism against electrophilic species.²⁹ GSH is well-known to react rapidly with isocyanates to form the corresponding thiocarbamates, which can further slowly react with free amine groups of proteins resulting in transcarbamoylation reactions.³⁰ To monitor the fate of isocyanates inside the cell, we synthesized the 6-*N*-alkyl-iminosydnone **2n** bearing a 7-diethylaminocoumarin reporter (Figure 4A).

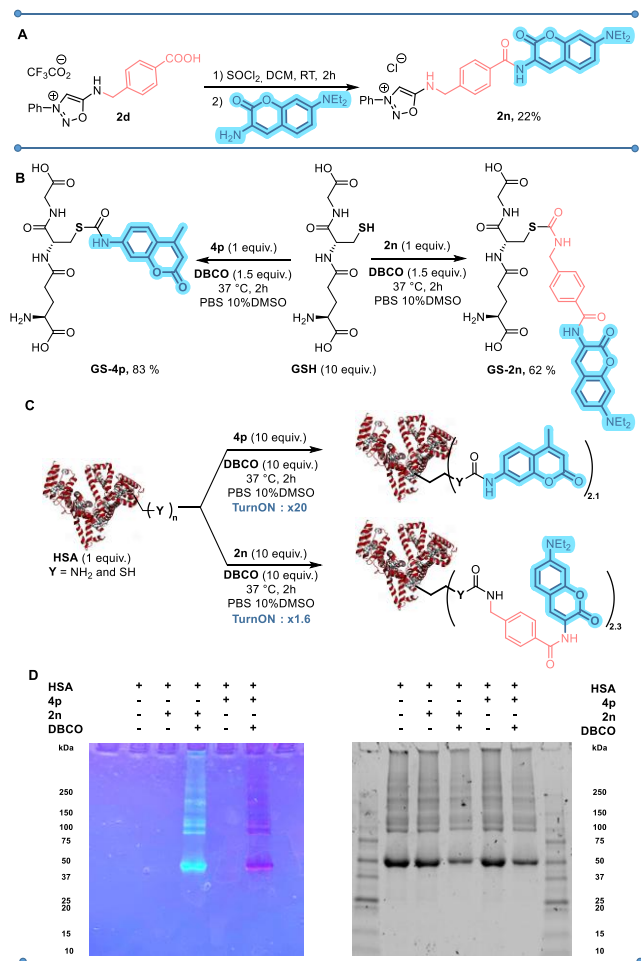


Figure 4. Release of a fluorescent isocyanates from iminosydnone **2n** and **4p** and their trapping with bio-nucleophiles *in vitro*. A) Synthesis of **2n** from **2d**; B) Reaction of GSH (1 mM) with **2n** or **4p** (100 µM) and DBCO (150 µM) in PBS at 37°C for 15 h; C) Reaction of HSA (2 mg/mL) with **2n** and **4p** (10 equiv.) and DBCO (10 equiv.) in PBS at 37°C for 2 h. D) SDS PAGE (10 µg/well). Right picture: coomassie blue staining; Left picture: fluorescence staining (312 nm).

Compound **4p**, displaying interesting turn-on fluorescence properties upon SPICC reaction (Figure S16), was also selected to conduct preliminary *in vitro* experiments. Both probes display similar reactivity for SPICC reaction (**2n** : $k = 208 \pm 4 \text{ M}^{-1} \text{ s}^{-1}$, **4p** : $k = 258 \pm 3 \text{ M}^{-1} \text{ s}^{-1}$). Addition of DBCO to a PBS solution containing excess of GSH and probe **2n** or **4p** conducted to the formation of the thiocarbamate adducts **GS-2n** and **GS-4p** in 62% and 83% yields together with byproducts resulting from the hydrolysis of the released coumarin isocyanates (Figure 4b). No reaction with the amine function of GSH was observed according to MS/MS analysis (Figure S20). We then performed the same reaction with human serum albumin

(HSA), the most abundant protein in plasma, known to display a nucleophilic cysteine residue at position 34.³¹ Fluorescent staining of the SDS-PAGE confirmed efficient labeling of HSA upon SPICC reactions of **2n** and **4p** with DBCO (Figure 4C-D). Absorbance measurements indicated 2.1 and 2.3 equivalents of coumarin attached to HSA for **4p** and **2n** respectively, indicating that the fluorescent isocyanates reacted not only with cysteine-34 but also with amine functions of the protein. No labeling was observed without DBCO.

We then investigated the use of probe **2n** as a mild generator of fluorescent isocyanates inside the cell in order to evaluate the biocompatibility of the SPICC approach and its use for bioconjugation in living systems (Figure 5). The use of electrophiles for proteomic investigations inside cells is a well established approach in chemical biology³²⁻³⁴ which has been recently improved by the use of caged electrophiles activatable by external or internal stimuli. A caged bromomethyl ketone electrophile was notably developed in 2015, which provided electrophile activation upon UV-irradiation and allowed a global analysis of reactive cysteine in living cells by identifying 366 cysteine-containing proteins.³⁵ More recently, light-induced formation of nitroso-benzaldehyde species allowed efficient cyclization with amines within cells³⁶ and a bioorthogonal photocatalytic decaging reaction allowed the release of electrophilic quinone-methides able to react with endogenous nucleophiles.³⁷ These reactions were successfully applied respectively to protein fishing and mitochondria proteome profiling in living cells. Moreover, caged quinone methides electrophiles are routinely used for the localization of enzymatic activities inside cells through permanent labeling involving covalent linkage of fluorescent probes.³⁸⁻⁴³

To our knowledge no caged isocyanates have been developed so far for chemical biology and, considering the known reactivity of these species, it would be interesting to investigate their fate inside the cell. To this end, we first investigated the cell toxicity of iminosydnone and their corresponding isocyanates. Compounds **2a** and **4a** did not display any toxicity even at 100 µM concentration (Figure S22). More surprisingly, the release of aryl- or alkyl-isocyanates inside the cells was apparently not detrimental to cell viability, as no additional toxicity was observed when iminosydnone and DBCO were added to the cells. We first examined the intracellular distribution of the fluorescent compound **2n**. The probe has been incubated for 3h at a concentration of 10 µM in two cell models, a human embryonic kidney cell model (HEK293) and a lung cancer cell model (A549), and then imaged with confocal microscopy. No significant compartmentalization was observed (Figure S24) in either of the two cell models tested. Two additional colocalization experiments involving mitochondrial and lysosomal green fluorescent markers (Figures S25-27) confirmed these initial observations and allowed us to assert that probe **2n** is equally distributed within the cells, except the nucleus, and is not preferentially localized in a particular organelle. We then investigated the benefit of forming the isocyanate **8** in cellulo (**2n** + DBCO in cell) versus direct use of isocyanate **8**. The confocal microscopy images shown in Figure 5B indicates a significant contrast in fluorescence intensity in favor of in cellulo production of the isocyanate. The signal-to-noise ratio is particularly high in this case (Figure 5C) which may result from the fluorescent labeling of a certain number of intracellular proteins, resulting in a retention of the luminescent agent in the cell during the washing steps. In order to confirm this, we performed SDS-PAGE anal-

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.

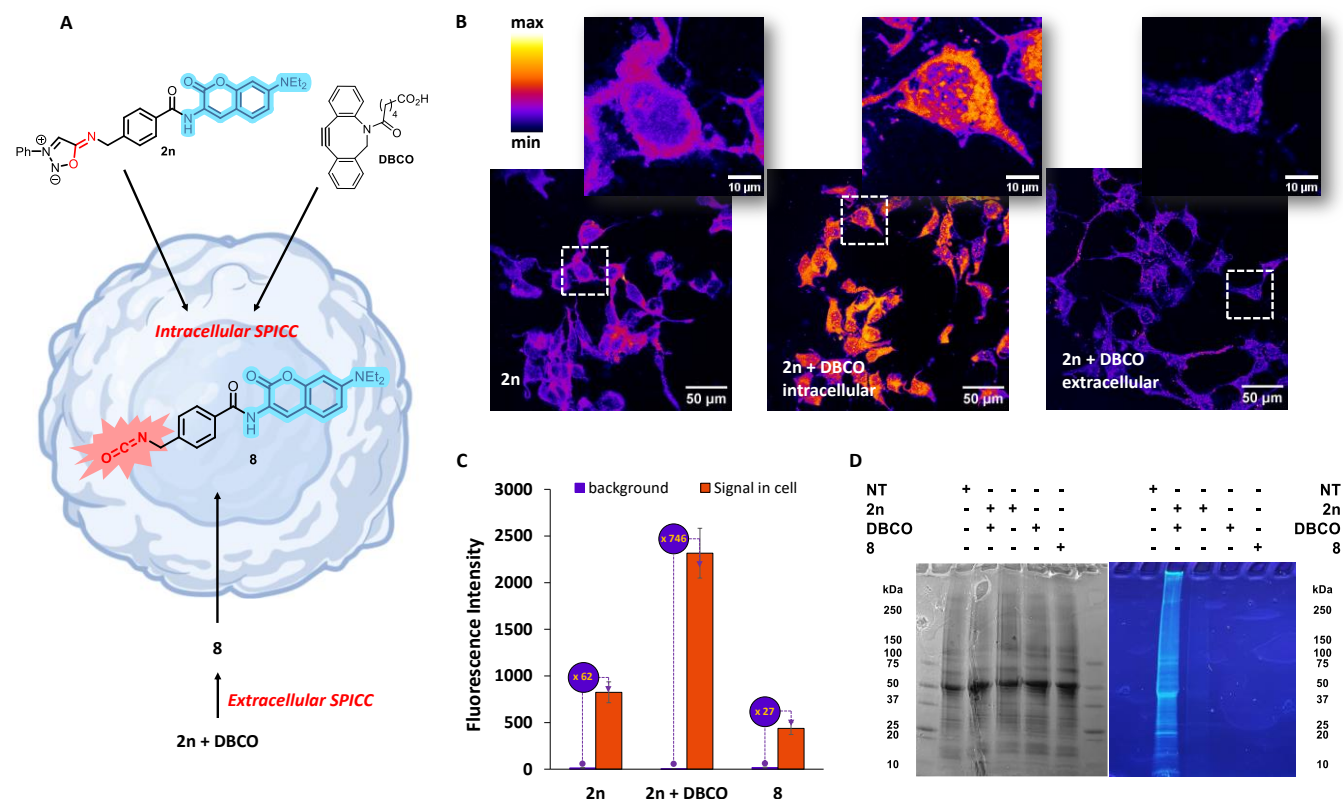


Figure 5. Experiments in cells. A) Schematic representation extra cellular versus intra cellular formation of the isocyanate B) Confocal microscopic images of live-HEK-293 kidney cells treated with compounds **2n** (left), **2n** + DBCO (middle) and **8** (right) at 10 μ M for 3 h at 37 $^{\circ}$ C 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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The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript.

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Notes

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ABBREVIATIONS

SPICC, Strained Promoted Iminosynone 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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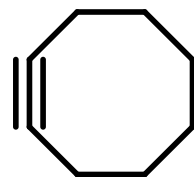
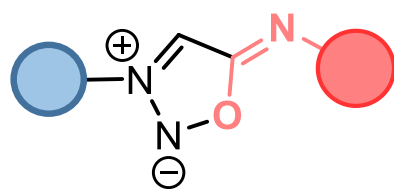
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✓ *New mesoionics*

✓ *Caged isocyanates*

✓ *Non toxic*



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

✓ *Fast*

✓ *Bioorthogonal*

✓ *Quantitative*

