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Fluorogenic iminosydnones: bioorthogonal tools for double turn-on click-and-release reactions†

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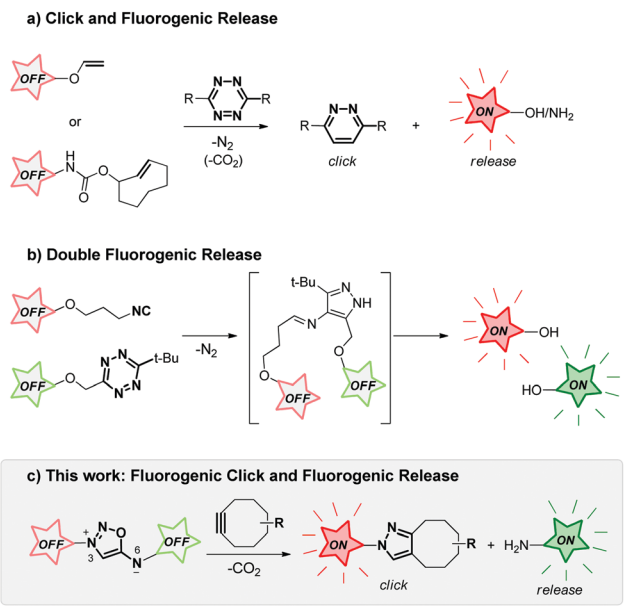
In this article, we report the synthesis and use of iminosydnone-based profluorophores as bioorthogonal cleavable linkers for imaging applications. These linkers react with cycloalkynes *via* subsequent [3+2] cycloaddition and retro Diels–Alder reactions, allowing simultaneous release of two dyes in biological media.

Bioorthogonal fluorogenic probes[1] and linkers[2] are exquisite tools for imaging, bioconjugation and chemical proteomic applications.[3] Such compounds typically bear a profluorophore moiety attached to a clickable handle which both quenches the fluorescence and allows efficient ligation. In particular, a series of azide-[4] and tetrazine-quenched fluorogenic dyes[5] have been developed as probes for cell imaging and as linkers for bioconjugation applications. Upon reaction with cyclooctynes and *trans*-cyclooctenes respectively the dye is unquenched leading to high turn-on fluorescence enhancement. In addition to these bioorthogonal handles, our group[6] and others[7] recently showed that mesoionic sydnones can also be used as a suitable moiety for efficient fluorogenic probes development.

Besides these ligation tools, recent efforts have been made to develop efficient bioorthogonal click and release reactions finding important applications in chemical biology, notably for the controlled delivery of drugs.[8] To better study these systems in a cellular context, cleavable fluorogenic substrates need to be developed in order to localize and track the products of the click and release reactions. To our knowledge, only few examples are described in literature. The first was developed by C. R. Bertozzi *et al.*[9] who designed a FRET-based phosphine probe functionalized with a quencher, which upon Staudinger ligation with azide-labelled glycans, exalted fluorescence. Still used nowadays,[10] this strategy suffers from the low reaction rates of Staudinger ligations. Fluorogenic decaging reactions involving the inverse electron demand Diels–Alder reaction (Scheme 1a) have also been successfully developed, using either terminal vinyl ethers or *trans*-cyclooctenes bearing a carbamate in allylic position.[11] Very recently, Franzini *et al.* showed the applicability of the reaction between tetrazines and isonitriles for the decaging of two separate molecules. The reaction yields 4-iminopyrazoles, which upon hydrolysis and subsequent elimination release two different products. The authors successfully applied the reaction to the release of two distinct fluorophores in zebrafish embryos (Scheme 1b) and demonstrated the interest of such double turn-on probes for the monitoring of multiplexed drug release.[12] However, such probes allowing multiple release of fluorophores remain sporadic in the literature and would deserve further development.[13] Herein, we describe double turn-on probes allowing optical reporting of the two products generated by click and release reactions involving iminosydnones and cyclooctynes (Scheme 1c).



Scheme 1 Fluorogenic click and release reactions.

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Our group recently developed the reaction of mesoionic iminosydnones with cyclooctynes as a new bioorthogonal click and release tool with applications in target fishing,[14] bioconjugation[15] and drug release.[16] The reaction works *via* a two-step process to form two products: a pyrazole click product and an urea or sulfonamide released product. Based on our previous finding on fluorogenic sydnones,[6] we envisaged that fluorophores connected in position N3 and N6 of the iminosydnone might be both quenched by the mesoionic core and efficiently released and turned-on following bioorthogonal cleavage by cyclooctynes (Scheme 1c).

At the outset, we first hypothesized that the strong structural modifications occurring at the iminosydnone core during the cycloaddition might affect the optical properties of fluorophores connected in position N3. To test this hypothesis, we first synthesized 4-styryl-iminosydnones protected in position N6 by a Boc group. Boc-protected 4-iodophenyl iminosydnone **1** was selected as a common synthon for its synthetic availability on a mutigram scale (Fig. 1a).[17] This building block could be derivatized by Heck coupling to access the styryl derivatives **3–5**. Aldehyde **2**, obtained by Suzuki cross-coupling from **1** in 96% yield, underwent a condensation reaction with *N*-methyl-4-picoline to obtain pyridinium **6** in 39% isolated yield.

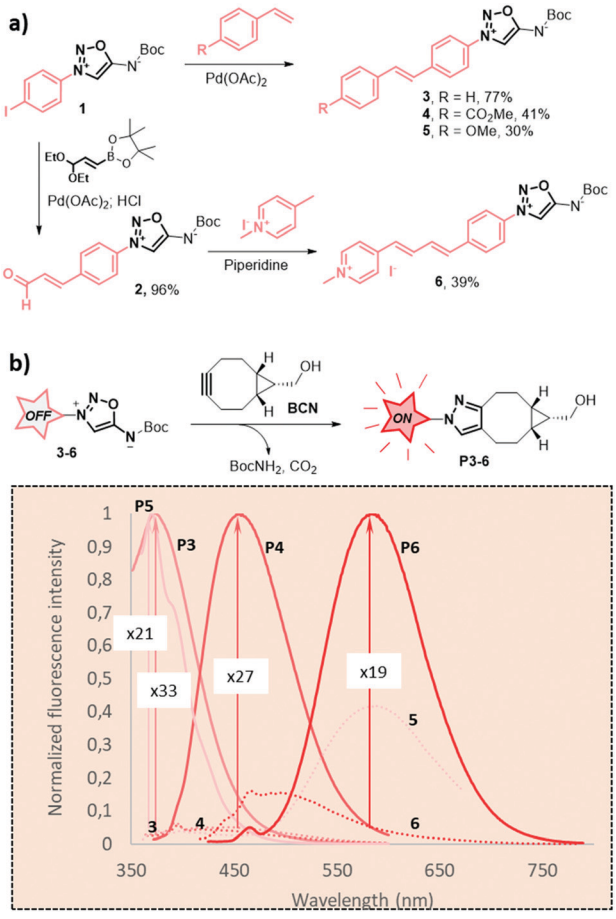

Fig. 1 (a) Synthetic routes to N3-iminosydnones profluorophores. (b) Fluorescence turn-on during the click and release reaction with BCN. Iminosydnones' fluorescence are in dotted lines, pyrazoles' are in plain lines. Solvent: DMSO.

Once those products in hand, we performed bioorthogonal cycloadditions with the model cycloalkyne bicyclo-[6.1.0]-nonyne (BCN). The photophysical properties of both iminosydnones and pyrazoles were measured and compared to determine the turn-on fluorescence values (Fig. 1b and Table S1, ESI†). As expected, we observed a significant enhancement of the fluorescence signal upon formation of the pyrazole products **P3–6** with a bathochromic effect when electron-withdrawing substituents are connected to the pyrazole **P4** and **P6**. On the contrary, electron-donating OMe group linked to the iminosydnone **5** provoked reversed Internal Charge Transfer effect with an iminosydnone emitting at 591 nm while the corresponding pyrazole **P5** emitted at shorter wavelength.

We then investigated the possibility to switch on fluorophores linked at position N6 of the iminosydnone core. If successful, this would allow tracking by fluorescence the "release product" of the transformation. The synthetic strategy relied on the reaction between **7**, a cheap and easily accessible starting material, and different fluorescent electrophiles. Amongst the large variety of fluorophores motifs, we focused on the coumarin, naphthalimide and dansyl scaffolds.

The iminosydnones derivatized with those profluorophores were obtained by reacting **7** with the *in situ* generated isocyanates for ureas **8** and **9** and with commercially available dansyl chloride for the sulphonamide **10** (Fig. 2a).

The optical properties of both iminosydnones **8–10** and the fluorophores released during the click and release reaction were studied (Fig. 2b and Table S2, ESI†). In all cases, we observed a strong quenching of the tested fluorophores when linked to the N6 position of the iminosydnones leading to important fluorescence enhancements upon reaction with **BCN** and complete release of products **R8–10**. Iminosydnones bearing fluorescein and rhodamine fluorophores at position N6 were also synthesized and studied but no turn on effect after addition of **BCN** was observed in these cases (Table S4, ESI†).

Encouraged by these results, we investigated the possibility to combine those two quenching effects to generate double turn-on clickable molecules. Four probes bearing two different profluorophores were thus synthesized from compounds **4–6** using similar chemistry as the one described in Fig. 2 (see ESI† for details) and studied in the click and release reaction with **BCN** (Table 1 and Table S4, ESI†). Pleasantly, these studies revealed a significant double turn-on effect for all tested iminosydnones **11–14**. Because of its good solubility and stability[18] in pure water, and biological media, as well as its ability to release two distinguishable fluorophores, probe **13** was selected for further applications.

The simultaneous release of the fluorescent naphthalimide-urea **R13** and pyrazolo-pyrydinium **P13** was first studied *in vitro*, using the more reactive cycloalkyne **DBCO** (Fig. 3a–c). This reaction was performed at pH 7.4 in phosphate buffer (PBS, 0.1 M), human plasma or in PBS containing 1 mM of Glutathion (GSH). The formation of both products were independently monitored over time using appropriate excitation wavelengths. Kinetic constants were between 0.18 and 0.82 M^{-1} s^{-1}, depending on the medium.

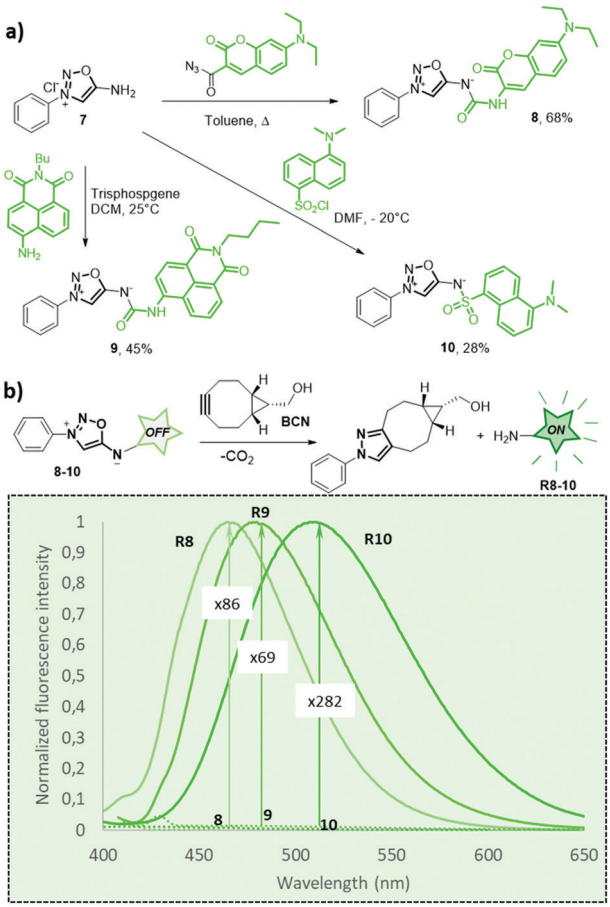

Fig. 2 (a) Synthetic routes to N6-iminosydnones profluorophores. (b) Fluorescence turn-on during the click and release reaction with BCN. Iminosydnones are dotted lines, released fluorophores are in plain lines. Solvent: DMSO.

With these results in hand, we investigated the use of **13** in cells and monitored the double turn-on click and release reaction by confocal microscopy imaging. The probe was first used on fixed and permeabilized CHO cells to prove the biocompatibility of the reaction. As shown in Fig. 3d, the turn on effect for both compounds **P13** and **R13** was clearly observed after overnight incubation of probe **13** and **DBCO**. Superposition of the fluorescence channels indicated only partial colocalisation of the two released products. The turn on effect due to the click and release reaction was also observed in living cells and in different cell lines (Fig. 3e and Fig. S2–S5, ESI†). Interestingly, while the starting iminosydnone **13** is water-soluble when used at 10 μM, both products formed during the bioorthogonal reaction precipitate in the cells as highlighted by the fluorescent blue and green spots in Fig. 3e. A zoom on cell distributions indicates different cytoplasmic localizations of the two release products confirming that the two fluorophores are no more connected and moved inside the cells before their precipitation. These experiments confirmed the compatibility of this reaction inside fixed or living cells and the possibility to monitor the release of the two products by means of a double turn-on fluorescence.

In conclusion, we developed a series of iminosydnone probes that can be efficiently cleaved to release and unquench two distinct fluorophores in biological media upon bioorthogonal reaction with cycloalkynes. These versatile iminosydnones can be considered as fluorogenic cleavable linkers allowing the cellular tracking of the two products generated from the click and release reaction. These new tools should therefore find interesting applications in drug delivery by allowing the tracking of both the released drug and its vector.

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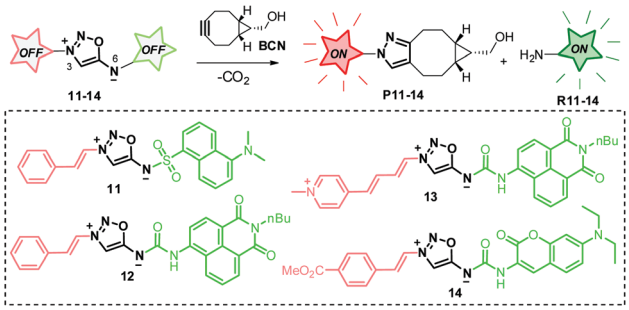
Table 1 Fluorescence properties of iminosydnone **11–14** functionalized with 2 profluorophores and the associated products **P** and **R**, after reaction with **BCN**. Solvent: DMSO



	Iminosydnones				Clicked pyrazole P				Released product R			
Entry	No	λ_{ex} (nm)	λ_{em} (nm)	Φ_F (%)	λ_{ex} (nm)	λ_{em} (nm)	Φ_F (%)	Turn-on[b]	λ_{ex} (nm)	λ_{em} (nm)	Φ_F (%)	Turn-on[b]
1	**11**	345	377	0.3	339	374	20	29	338	510	44	97
2	**12**	415	473	1.2	339	374	20	26	392	483	50	31
3[a]	**13**	396	482	1.5	403	582	11	18	392	483	50	22
4	**14**	349	467	0.7	354	455	23	33	387	465	51	22

[a] Water/DMSO, 80/20. [b] Ratio between fluorescence intensities of the pyrazole products and the iminosydnones substrates obtained after excitation at the λ_{max} of the pyrazole products.

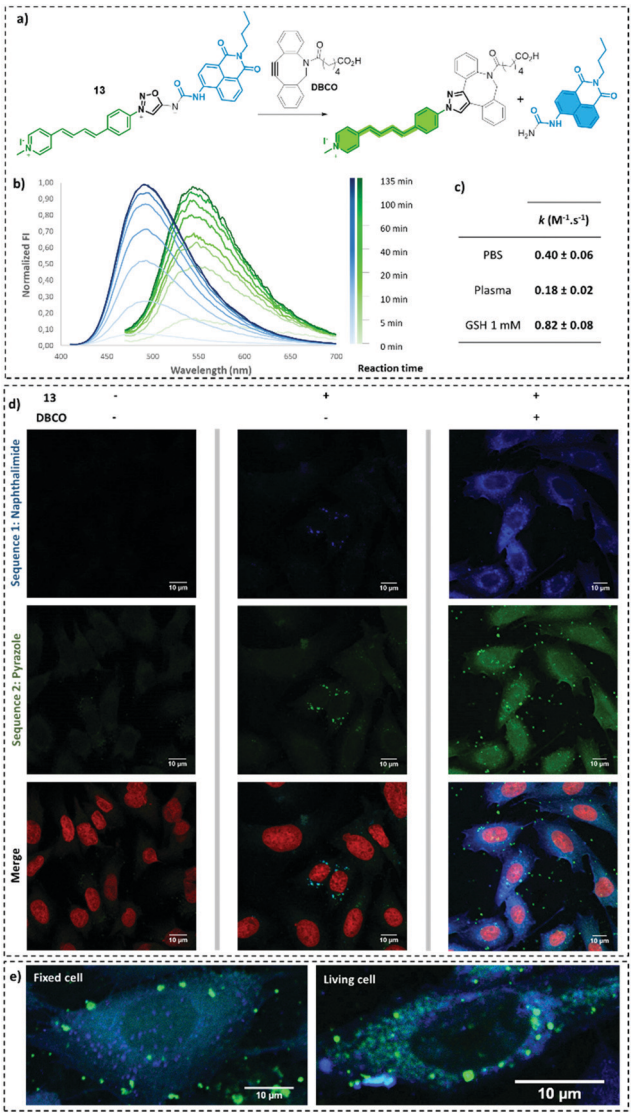


Fig. 3 (a) Reaction of probe **13** with **DBCO**. (b) Fluorescence analysis over time during click and release reaction between probe **13** and **DBCO** in PBS. [**13**] = 10 µM; [**DBCO**] = 1 mM. (λ_{ex} = 405 and 458 nm). (c) Second-order reaction rates obtained in different aqueous media. (d) Fluorescence was analyzed by confocal miscroscopy. CHO cells were fixed, permeabilized and treated with indicated compounds for 17 hours. [**13**] = 10 µM; [**DBCO**] = 80 µM. Cell nucleus were labeled using Draq5. Images were obtained on a SP8 LEICA confocal miscroscope with a ×63 zoom. (e) Comparison of the distribution of the two released products in fixed *vs.* living cells after reaction of **13** with **DBCO**. No cell nucleus labelling.

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Conflicts of interest

There are no conflicts to declare.

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18 Fluorescence monitoring in 0.1 M PBS Buffer, in a 0.1 M glutathione solution and in blood plasma indicated complete stability of probe **13** over 48 h.

