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Sydnone Reporters for Highly Fluorogenic Copper-Free Click Ligations

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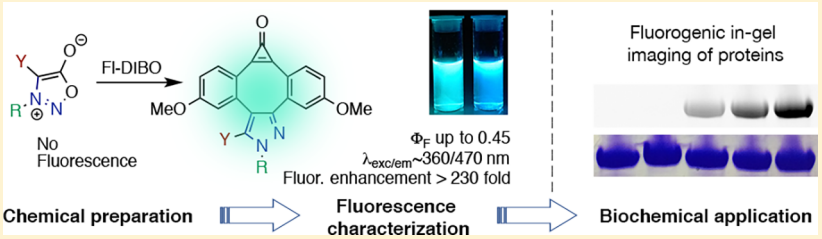
Supporting Information



ABSTRACT: Bioorthogonal fluorescent turn-on reactions are attractive for the sensitive real-time detection of a variety of phenomena including bioconjugation, chemical reactivity, and material assembly. Herein we describe the use of 3,4-disubstituted sydnones, a singular class of mesoionic dipoles, for highly fluorescent turn-on copper-free click cycloadditions with the fluorogenic dibenzocyclooctyne Fl-DIBO. Coherent with time-dependent density functional theory calculations, the pyrazole cycloadducts were found to be highly fluorescent with compelling photophysical properties including excellent fluorescence enhancement (up to 240-fold), high quantum yields (over 45%), and large Stokes shift (over 100 nm). Furthermore, the good stability and reactivity of 4-chlorosydnones with Fl-DIBO allowed us to employ them as chemical reporters for the challenging detection of modified-proteins in complex cellular extracts, with exquisite specificity in no-wash conditions. This novel fluorogenic system significantly expands our chemical biology toolbox and should be beneficial in countless applications.

INTRODUCTION

The recent advances in fluorescence microscopy, in coordination with the development of fluorescent probes, have revolutionized our understanding of cell biology and physiology.[1] Biomolecules such as proteins, lipids, and complex glycans can now be tracked effortlessly through the incorporation of fluorescent reporters, in a spatial and temporal manner, within their native environment.[2−4] Of particular importance, the bioorthogonal chemical reporter strategy has emerged as a key technology for the challenging labeling of post-translational modifications.[5−7] In this two-step approach, a uniquely reactive chemical functionality (the reporter) is first introduced into the biomolecule of interest and is subsequently utilized for the chemical ligation of a diverse set of probes (e.g., fluorescent tags), using strictly selective bioorthogonal reactions. While this technology can ensure the visualization of a specific target in complex biological settings, the fact that fluorescent labels are always "on" precludes their direct application for real-time bioimaging.

To overcome this limitation, fluorogenic molecules, that increase in fluorescence after chemical reactions or target-binding, have recently been developed for maximizing signal over background, expanding significantly our chemical biology toolbox.[8] Popular fluorogenic metal-free bioorthogonal chemistries include the Staudinger ligation,[9] the inverse electron-demand Diels−Alder reaction,[10−13] and the strain-promoted alkyne−azide cycloaddition (SPAAC). Although numerous fluorogenic azido-probes have been developed, notably for copper-click chemistry,[14,15] their utilization under metal-free conditions is consequently limited by the necessity of employing relatively bulky cyclooctynes as chemical reporters,[16] that may not be well tolerated by the cell's metabolic machinery.

To address this major drawback, fluorogenic cyclooctynes have recently been synthesized that can be activated upon SPAAC with inconspicuous azido-reporters (Figure 1A). Inspired by the coumarin quenching ability of terminal alkyne through the stabilization of a nonemissive excited triplet $^3(n,\pi^*)$ state,[17] Bertozzi and co-workers have developed a fluorogenic copper-free click reagent CoumBARAC (**1**) by incorporating a cyclooctyne ring into the coumarin dye.[18] Although the formation of the triazole moiety upon SPAAC with 2-azidoethanol led to a 10-fold fluorescence enhancement (FE), the requirement of relatively high energy of excitation (λ_{exc} ~ 300 nm) combined with a rather low fluorescence quantum yield (Φ_F = 0.04) made the reagent ill-suited for bioimaging. Following a similar design, the Wong group recently reported

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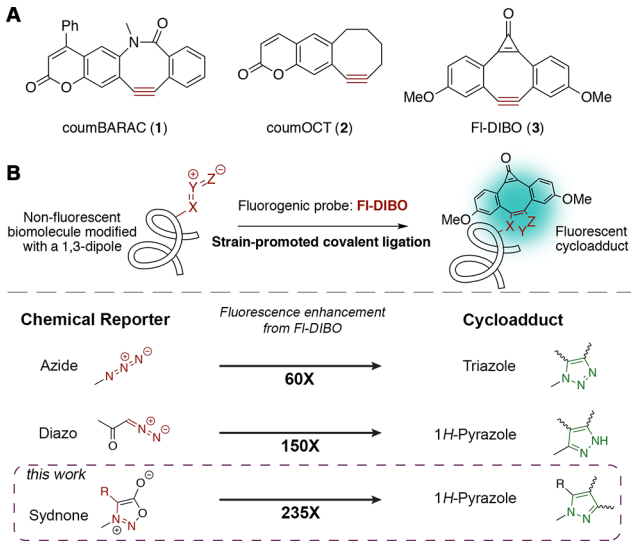


Figure 1. Fluorogenic strain-promoted reagents. A. Turn-on cyclooctynes. B. Fluorescence enhancement of Fl-DIBO (**3**) upon bioorthogonal reactions with various chemical reporters.

the fluorogenic probe CoumOCT (**2**) that exhibited improved photophysical properties (λ_{exc} = 330 nm, Φ_F = 0.23, FE = 20) and was successfully employed for the visualization of metabolically labeled azido-glycoconjugates in living cells.[19]

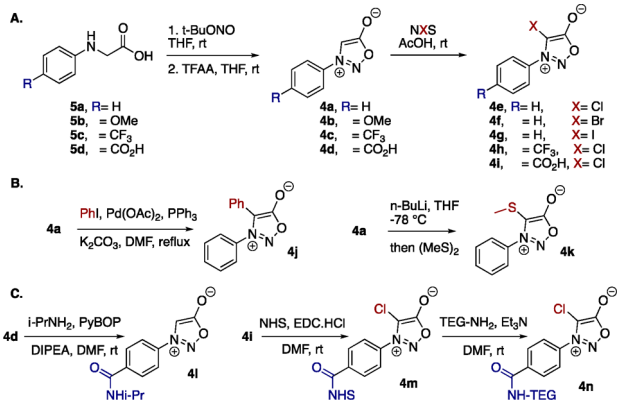
While turning off known fluorescent dyes with bioorthogonal functionalities clearly simplifies the design of novel fluorogenic probes, potential degradation or unwanted side-reactions of the quenching moiety[20,21] may potentially activate the probe leading to unspecific fluorescence. On the other hand, fluorogenic probes that chemically assemble the fluorophore during the bioorthogonal conjugation step are consequently more reliable constructs for fluorescent bioimaging. In this context, we recently developed a fluorogenic dibenzocyclooctyne (Fl-DIBO (**3**)), which upon SPAAC with azido-biomolecules generates ligated products with a 60-fold fluorescence enhancement (Figure 1B). The fluorescent triazoles exhibited good photostability and could be excited above 350 nm, the typical cutoff wavelength of standard fluorescence microscopes.[22] The fluorogenic behavior of Fl-DIBO was later shown to be highly dependent on the nature of the 1,3-dipole used. For instance, while the reaction with nitrile oxides, nitrones, and disubstituted diazo compounds gave cycloadducts with low quantum yield, monosubstituted diazo reagents produced 1*H*-pyrazole derivatives that displayed an improved 160-fold fluorescence enhancement.[23] However, the high reactivity of diazo compounds toward metals and their stability being strongly dependent on the electronic character of their substituents[24,25] make their synthesis and biological utilization greatly challenging.

Sydnones are stable mesoionic 1,3-dipoles that undergo thermal [3 + 2] cycloadditions in the presence of various dipolarophiles.[26] For instance, 1,4-pyrazoles can be generated under mild conditions via regioselective copper(I)-catalyzed sydnone cycloadditions with terminal alkynes.[27] Similar to azides, sydnones can also undergo metal-free strain-promoted cycloadditions with various cyclooctyne-reporters.[28−30] Therefore, we envisaged that sydnones, which also generate 1*H*-pyrazoles upon [3 + 2] cycloadditions with alkynes, could be viable replacements of diazo reagents for highly fluorogenic copper-free click ligations with Fl-DIBO (**3**). Herein we report that the fluorogenic probe Fl-DIBO (**3**) can indeed be efficiently turned on upon reaction with sydnones and that the generated cycloadducts exhibit compelling photophysical properties including excellent fluorescence enhancement (up to 240-fold), high quantum yields (over 45%), and large Stokes shift (over 100 nm). Time-dependent density functional theory (TD-DFT) calculations indicate that the particular high fluorescence quantum yields observed for the 1,5-disubstituted pyrazoles are due to the large oscillator strengths of their $S_0 \leftrightarrow S_1$ transitions. Ultimately, the optimal bioorthogonal fluorogenic system was employed in a complex biological setting for the selective visualization of sydnone-modified proteins in cell lysate.

RESULTS AND DISCUSSION

Photophysical Properties of the Cycloadducts Generated from Fl-DIBO and Modified Sydnones. Previous studies on the fluorogenic probe Fl-DIBO (**3**) revealed that the nature of the heterocycle formed after cycloaddition is key for the cycloadduct fluorescence emission or quenching.[23] To attest whether Fl-DIBO (**3**) can be activated upon reactions with sydnones and investigate the fluorescence properties of the resulting click products, we prepared an array of 3,4-disubstituted sydnones (Scheme 1). Briefly, 3-modified

Scheme 1. Chemical Synthesis of Modified Sydnones 4a−n



sydnones **4a−d**, bearing aryl groups with various electron density, were synthesized in an efficient two-step protocol by nitrosation of *N*-substituted glycines **5a−d** with *tert*-butyl nitrite followed by trifluoroacetic anhydride-induced cyclization (Scheme 1A). Further C-4 chemical elaboration of the mesoionic rings could easily be achieved by either halogenation, Heck-coupling, or lithiation reactions generating the desired 3,4-disubstituted sydnones **4e−k** in good yields (Scheme 1A,B). Finally, amide coupling protocols on the benzoic acid derivatives allowed for additional modifications leading to sydnones **4l−n** (Scheme 1C).

Next we reacted 3-phenylsydnone **4a** with Fl-DIBO (**3**), and to our delight, the resulting 1-phenyl-5*H*-pyrazole **6a** was found to be strongly fluorescent when excited at 363 nm with a maximum emission at 468 nm and a quantum yield of 0.35 (Table 1). Motivated by this result, we investigated the influence of the substitution pattern of modified sydnones on the photophysical properties of the click adducts (Table 1, Figure 2, and Supporting Information, Figures S1−S13). While switching substituents at the *N*-position of the pyrazole ring had very little effect on the excitation wavelengths of the new

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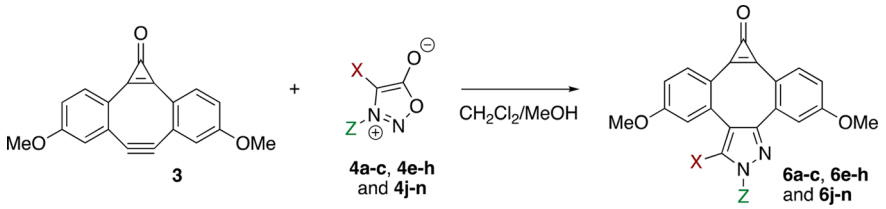
Table 1. Photophysical Properties of Pyrazoles 6a–c, 6e–h, and 6j–n in Methanol



pyrazole	Z	X	λ_{exc}[a] [nm]	λ_{em}[b] [nm]	Φ_F[c]	FE[d]
			Pyrazoles Obtained from 3-Modified Sydnones			
6a	Ph	H	363	468	0.35	175
6b	MeO-Ph	H	361	480	0.15	75
6c	F_3C-Ph	H	361	460	0.45	225
6l	*i*-PrNHCOPh	H	360	468	0.31	155
			Pyrazoles Obtained from 3,4-Modified Sydnones			
6e	Ph	Cl	354	451	0.47	235
6f	Ph	Br	355	450	0.12	60
6g	Ph	I	359	455	0.014	7
6h	F_3C-Ph	Cl	353	449	0.41	205
6j	Ph	Ph	361	456	0.48	240
6k	Ph	SMe	354	456	0.40	200
6n	TEGNHCOPh[e]	Cl	352	452	0.40	200
			353[f]	452[f]	0.47[f]	235[f]

[a]Determined at λ_{em} = 460 nm. [b]Determined at λ_{exc} = 360 nm. [c]Fluorescence quantum yield, quinine sulfate in aqueous 1.0 N H_2SO_4 as standard. [d]Fluorescence enhancement compared to Fl-DIBO (sydnones having negligible fluorescence emission at λ_{exc} = 360 nm, Figure S13 in SI). [e]TEGN = triethylene glycol monoamine. [f]Determined in 40% MeOH in PBS (pH 7.4).

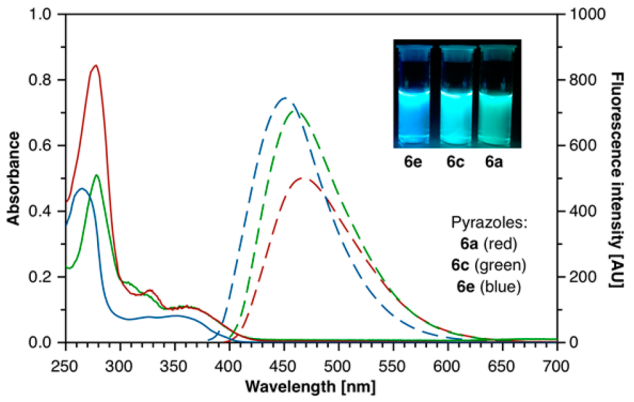


Figure 2. Absorption (solid traces; 30 μM solutions) and fluorescence emission (dashed traces; λ_{exc} = 360 nm; OD_{360} = 0.15) spectra of pyrazoles **6a**, **6c**, and **6e** in MeOH. Inset: Visual comparison of the fluorescence intensity of cycloadducts in MeOH with excitation at 365 nm.

fluorophores, both the emission maxima and fluorescence quantum yields were clearly impacted by the electron density of the modifications. For example, pyrazole **6b**, having an electron-donating group, found its fluorescence emission to be red-shifted (λ_{em} = 480 nm), accompanied by a decrease in quantum yield (Φ_F = 0.15). Conversely, pyrazole **6c**, containing an electron-withdrawing substituent, exhibited a clear hypsochromic shift (λ_{em} = 460 nm) with an exciting increase in fluorescence quantum yield (Φ_F = 0.45). In general, 3-modified sydnones, having substituents of moderate electron density, generate pyrazoles (**6a** and **6l**) with similar spectral identity ($\lambda_{exc/em} \approx$ 360/470 nm) and good fluorescence quantum yield (Φ_F = 0.30).

One of the main advantages of sydnones, compared to other 1,3-dipoles such as azides, is the possibility to modulate their structures at two different positions (3,4-substitutions of the mesoionic ring). Consequently, we prepared 1,5-substituted pyrazoles by reacting Fl-DIBO (**3**) with 3,4-modified sydnones **4e–h**, **4j–k**, and **4n** and evaluated the effect of these modifications on their fluorescence. While introducing a substituent on C-5 of the pyrazole moiety significantly affected the fluorescence quantum yield of the chromophores, only a marginal impact on their emission maxima ($\lambda_{exc/em} \approx$ 355/455 nm) was observed (Table 1). For instance, C5-modification of the pyrazole ring with a phenyl (**6j**) or an *S*-methyl (**6k**) functionality noticeably enhanced the fluorescence quantum yield to 0.48 and 0.40, respectively. Following a similar pattern, 4-chlorosydnones **4e**, **4h**, and **4n** also efficiently increased the quantum yield of their respective click products **6e**, **6h**, and **6n**, with a remarkable 235-fold fluorescence enhancement. On the other hand, substitution of the chlorine atom with other halogens such as bromine or iodine suppressed the fluorescence emission, probably through the heavy atom effect via intersystem crossing ($S_1 \rightarrow T_1$).[31] Because the polarity of solvent is known to affect the photophysical properties of chromophores, we measured the fluorescence emission of 5-chloropyrazole **6n** in pure methanol and in 40% methanol in phosphate-buffered saline (PBS, pH 7.4) and were pleased to observe similar spectral identification and quantum yield in both conditions ($\Phi_{F/MeOH}$ = 0.40 vs $\Phi_{F/40\%MeOH\text{-}PBS}$ = 0.47), a valuable result for bioimaging. Altogether, fluorogenic bioorthogonal strain-promoted cycloadditions between 3,4-modified sydnones and Fl-DIBO generate fluorescent pyrazoles with emissions ranging from 450 to 480 nm, large Stokes shift (~100 nm), and considerable high fluorescence enhancement (up to 240-fold).

Time-Dependent Density Functional Theory Calculations. To elucidate the observed influence of the C5-substitution of the pyrazole framework on their fluorescence quantum yield and emission wavelength, we decided to

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investigate the electronic properties of three representative model pyrazoles: 1-Me-5-H-pyrazole **6o**, 1-Me-5-Me-pyrazole **6p**, and 1-Me-5-Cl-pyrazole **6q**, by TD-DFT quantum chemical calculations at the B3LYP/6-31+G(d) level (Figure 3). We

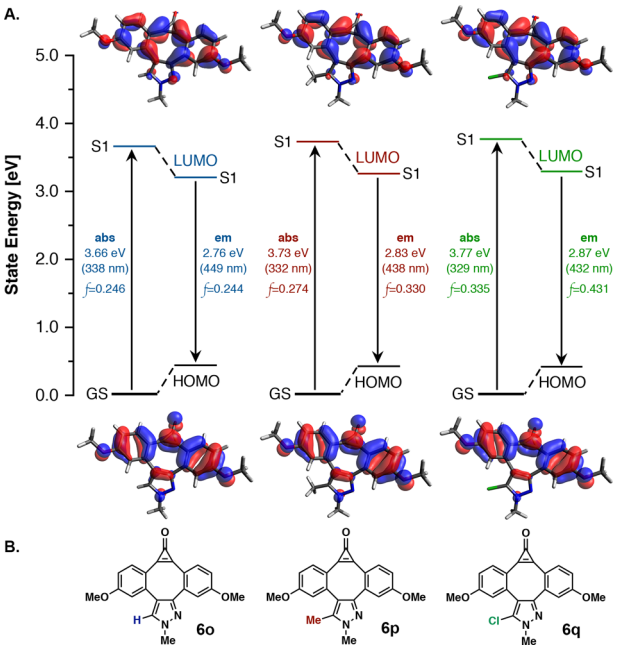

Figure 3. A. Energy diagrams and contour plots of the HOMO and LUMO of pyrazoles **6o**–**q** with their transitions oscillator strength values (*f*). B. Chemical structures of pyrazoles **6o**–**q**.

have previously shown that the absorption and emission data of pyrazole structures, generated from Fl-DIBO, were well predicted with this method when the long-range corrected hybrid functional CAM-B3LYP and a polarized continuum model (PCM) for solvent were used.[22] As depicted in Figure 3, the calculated first excited singlet state S_1 of all pyrazoles (**6o**–**q**) correspond to a HOMO–LUMO transition having a π,π^* character, with coefficients mainly located on the dibenzocyclooctene moiety and only to a small extent on the pyrazole ring, explaining for the overall very similar experimental absorption and emission profiles of these molecules. Nevertheless, introduction of either a methyl group or a chlorine atom on C5 of the pyrazole ring generated a slight but consistent increase in energy values of their $S_0 \leftrightarrow S_1$ transitions with the chloro-substitution having the most pronounced influence (over 0.1 eV difference), reflecting well the experimental hypsochromic shift observed for the chloropyrazole **6e**, **6h**, and **6n** absorption and emission wavelengths (Table 1). More remarkably, the calculated emission oscillator strength (*f*), which is a good representation of the radiative rate constant of chromophores,[31] significantly increased with the C5-substitution pattern, going from 0.24 for 1-Me-5-H pyrazole **6o** to 0.33 for 1-Me-5-Me pyrazole **4p** and 0.43 for 1-Me-5-Cl pyrazole **4q** (Figure 3). Taken together, these TD-DFT computed results suggest that the significantly high fluorescence quantum yields observed for the 1,5-disubstituted pyrazoles **6e**, **6h**–**k**, and **6n** is due to the large oscillator strengths of their $S_0 \leftrightarrow S_1$ transitions compared to their monosubstituted analogues **6a** and **6l**.

Kinetic and Stability Studies of Sydnones and Their Application for the Fluorogenic Labeling of Proteins. High reactivity and biological stability are critical features of bioorthogonal reagents. Therefore, we examined the effect of sydnone substitutions on their reaction kinetics with Fl-DIBO (**3**) (Figure 4). The second-order rate constants of cyclo-

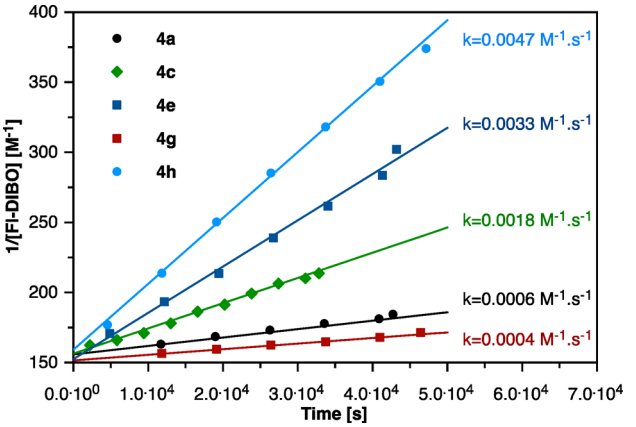

Figure 4. Kinetics data of cycloaddition reactions between Fl-DIBO (**3**) (6.6 mM in a mixture of $CDCl_3$ and MeOD (4:1) at 25 °C) in the presence of various sydnones (**4a**, **4c**, **4e**, **4g**, and **4h** (6.6 mM)). The lines shown were drawn using parameters obtained by linear fitting with *k* representing the second-order rate constant of the reaction.

addition between Fl-DIBO (**3**) and sydnones **4a**, **4c**, **4e**–**h**, and **4j**–**k** were determined by monitoring product formation by ^{1}H NMR spectroscopy in a mixture of $CDCl_3$ and CD_3OD (Supporting Information, Figures S14–S17). While 3-phenyl sydnone **4a** reacted with Fl-DIBO (**3**) at a rate of 6.0×10^{-4} M^{-1} s^{-1}, adding an electron-withdrawing substituent was clearly beneficial with *p*-CF_3-phenyl sydnone **4c** having a second-order rate constant of 1.8×10^{-3} M^{-1} s^{-1}. Regarding 3,4-disubstituted sydnones, introduction of bulky substituents, such as phenyl, *S*-methyl, and iodine, on C-4 of the mesoionic ring drastically decreased their reactivity with kinetics below 4.0×10^{-4} M^{-1} s^{-1}, probably due to steric hindrance. On the other hand, 4-substitution of the sydnone scaffold with the smaller chlorine atom significantly enhanced the 1,3-dipole reactivity with second-order rate constants up to 4.7×10^{-3} M^{-1} s^{-1}, a rate twice as fast as the previously reported reaction between Fl-DIBO and ethyl diazoacetate[23] and 30-fold faster than the Staudinger ligation, a bioconjugation reaction often used for the *in vitro* and *in vivo* labeling of biological macromolecules.[32]

Next we investigated the stability of sydnones in aqueous solution. 4-Chloro-3-benzoic acid sydnone **4i** was incubated in a mixture of DMSO-d_6 and deuterated water (1:1), and its stability was monitored by ^{1}H NMR spectroscopy over time (Supporting Information, Figure S18). The 4-chlorosydnone was found highly stable, as no decomposition was observed for over 25 h. Overall, 4-chlorosydnones are stable 1,3-dipoles in aqueous solution that react fast with the Fl-DIBO probe to give highly fluorescent pyrazoles.

Finally, to evaluate the performance of 4-chlorosydnones as reporters for the fluorogenic labeling of biomolecules, we functionalized the exposed lysines of bovine serum albumin (BSA), as a model protein, with the mesoionic dipoles. Accordingly, NHS-activated benzoic ester chlorosydnone **4m** was reacted with BSA in PBS (pH 7.4) (Figure 5A). The modified protein (BSA-PhSydCl) was then incubated with Fl-DIBO (**3**) in PBS (containing 4% of DMF) at 37 °C and visualized by in-gel fluorescence imaging. As expected, while no

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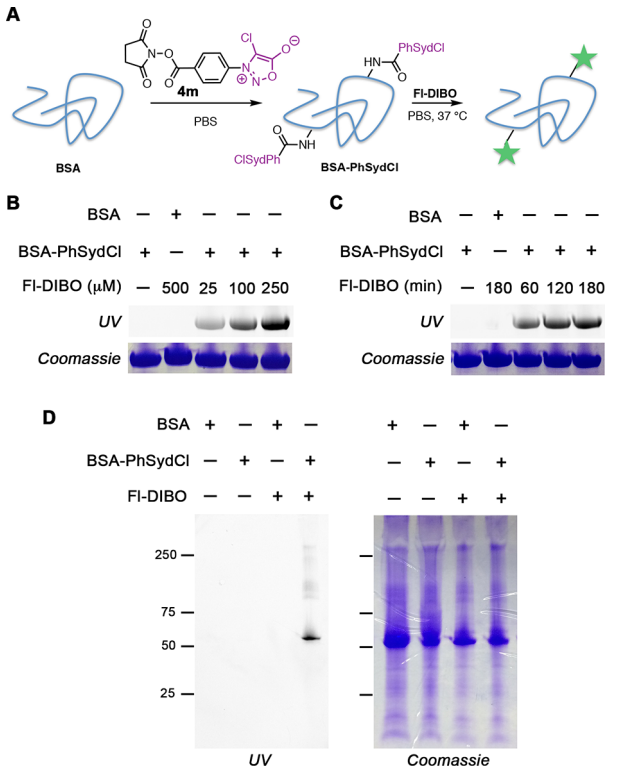

Figure 5. Fluorogenic labeling of chlorosydnone-modifed BSA conjugate (BSA-PhSydCl) with Fl-DIBO (**3**). A. Incorporation of 4-chloro-3-arylsydnones **4m** onto BSA using NHS-chemistry and subsequent reaction with Fl-DIBO in PBS at 37 °C. B. In-gel visualization of BSA-PhSydCl or native BSA incubated with Fl-DIBO (25−500 μM) or no reagent (−) for 3 h. C. In-gel visualization of BSA-PhSydCl or native BSA incubated with Fl-DIBO (100 μM) or no reagent (−) for 60−180 min. D. In-gel visualization of BSA-PhSydCl or native BSA incubated with Fl-DIBO (100 μM) or no reagent (−) for 3 h in U2OS cell extracts.

labeling was detected when native BSA was used, BSA displaying chlorosydnone moieties showed a clear dose- and time-dependent increase in fluorescence intensity (Figure 5B,C). Optimal fluorescent labeling was achieved at a concentration of 100 μM of Fl-DIBO after 3 h of incubation. Cyclooctynes have recently been reported to react with biological entities such as thiols[20] and sulfenic acids,[21] which could potentially generate background labeling. To challenge the signal specificity of our fluorogenic system in a more stringent biological environment, we performed the turn-on protein ligation in cell lysate. Human bone osteosarcoma (U2OS) cell lysate was spiked with 10% of BSA-PhSydCl, or regular BSA for control, and incubated for 3 h at 37 °C in the presence of Fl-DIBO (Figure 5D). Gratifyingly, only BSA-PhSydCl conjugate displayed a robust fluorescent signal, whereas all controls showed no detectable labeling.

CONCLUSION

In summary, strain-promoted cycloaddition reactions between 3,4-disubstituted sydnones and Fl-DIBO generated highly fluorescent pyrazoles, exhibiting emissions ranging from 450 to 480 nm, large Stokes shift (~100 nm), and high fluorescence quantum yields (up to 47%), leading to remarkable fluorescence enhancements (up to 240-fold). In addition, 4-chlorosydnones proved to be stable reporters in aqueous solution that react noticeably fast with the Fl-DIBO probe, making them suitable for biochemical applications. Accordingly, these new reporters were appended to protein surface and the modified protein could be detected by Fl-DIBO in complex cellular extracts with exquisite specificity via in-gel fluorescence imaging. Interestingly, Taran and co-workers recently reported the synthesis of 4-fluorosydnones that was found to react exceptionally fast toward alkynes,[33] highlighting the promising future of this new class of reagents. However, the challenging isolation of the fluorosydnones preclude their current use as bioorthogonal chemical reporters. While highly fluorogenic reactions between sydnone reporters and Fl-DIBO clearly offer advantages in cell biology, where background labeling is often an issue, we look forward to using this exciting system for other applications including the screening of chemical reactivity, the sensitive detection of material assembly, and the sensing of various analytes.

EXPERIMENTAL SECTION

Materials and Methods. All reactions were carried out under a dry argon environment. All solvents were of reagent grade and used as received. Dichloromethane and tetrahydrofuran were dried over alumina columns under nitrogen through a solvent purification system. Chemicals and reagents were used as commercially supplied without any further purification unless otherwise stated. Room temperature refers to ambient temperature (20−22 °C). Column chromatography was carried out using silica gel G60 (Fluka analytical, 230−400 mesh, 40−63 μm particle size, 60 Å) as the stationary phase. ^{1}H, ^{13}C, and ^{19}F NMR spectra were recorded on a Brüker 300 MHz spectrometer. Chemical shifts are reported in δ units, parts per million (ppm) downfield from TMS. Coupling constants (J) are reported in hertz (Hz) without adjustments. Splitting patterns are designed as follows: s, singlet; d, doublet; t, triplet; dd, doublet of doublets; dt, doublet of triplets; td, triplet of doublets; ddd, doublet of doublet of doublets; tt, triplet of triplets; m, multiplet; br, broad. All NMR signals were assigned on the basis of ^{1}H NMR, COSY, HSQC, and ^{13}C experiments. High-resolution mass spectra were obtained with an electrospray ionization Thermo Exactive orbitrap mass spectrometer.

General Procedure for the Formation of 3-Modified Sydnones 4a−d. *tert*-Butyl nitrite (0.27 mL, 2.2 mmol) was added dropwise to a solution of *N*-aryl glycine (2 mmol) in THF (5 mL). The reaction mixture was stirred at room temperature for 30 min. Trifluoroacetic anhydride (0.31 mL, 2.2 mmol) was then added to the previous solution, and the reaction mixture was stirred for an additional 1 h. The mixture was then quenched with a saturated aqueous solution of $NaHCO_3$ (10 mL). The organic layer was extracted with ethyl acetate (3 × 15 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel (20 g) using a mixture of 1% of methanol in dichloromethane to afford the respective pure 3-substituted sydnone **4a−d**.

3-Phenyl-1,2,3-oxadiazol-3-ium-5-olate ***4a.*** White solid (289 mg, 89%): ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.69−7.73 (m, 3H), 7.77 (s, 1H), 7.93 (dd, J = 8.0, 1.9 Hz, 2H); ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 95.0 (CH), 121.6 (2 × CH), 130.2 (2 × CH), 132.4 (CH), 134.6 (C), 168.5 (C−O$^-$) in agreement with the literature data.[28]

3-(4′-Methoxyphenyl)-1,2,3-oxadiazol-3-ium-5-olate ***4b.*** Brown solid (227 mg, 59%): ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.90 (s, 3H), 6.65 (s, 1H), 7.07 (d, J = 9.1 Hz, 2H), 7.64 (d, J = 9.1 Hz, 2H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 56.0 (CH_3), 93.5 (CH), 115.4 (2 × CH), 122.8 (2 × CH), 134.5 (C), 162.6 (C), 169.2 (C−O$^-$) in agreement with the literature data.[28]

3-[4′-(Trifluoromethyl)phenyl]-1,2,3-oxadiazol-3-ium-5-olate ***4c.*** White solid (298 mg, 65%): ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.84 (s, 1H), 7.92 (s, 4H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 94.0 (CH), 122.1 (2 × CH), 123.0 (q, J = 272.9 Hz, CF_3), 127.8 (q, J = 3.7 Hz, 2 × CH), 134.7 (q, J = 33.7 Hz, C), 137.3 (C), 168.7 (C−O$^-$); ^{19}F NMR (282 MHz, $CDCl_3$) δ −63.08 (s) in agreement with the literature data.[28]

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3-(4′-Carboxyphenyl)-1,2,3-oxadiazol-3-ium-5-olate **4d**. Orange solid (240 mg, 58%): ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.88 (s, 1H), 8.07 (d, J = 8.8 Hz, 2H), 8.21 (d, J = 8.8 Hz, 2H); ^{13}C NMR (75.5 MHz, DMSO-d_6): δ 95.3 (CH), 121.9 (2 × CH), 131.0 (2 × CH), 134.3 (C), 137.3 (C), 166.0 (C═O), 168.4 (C−O$^-$) in agreement with the literature data.[28]

General Procedure for the Formation of 4-Chlorosydnone 4e and 4h−i. *N*-Chlorosuccinimide (134 mg, 1.0 mmol) was added to a solution of 3-aryl sydnone (0.5 mmol) in acetic acid (1.2 mL). The reaction mixture was stirred at room temperature for 8 h and then diluted with ethyl acetate, and all the volatiles were removed under reduced pressure. The residue was then purified by flash chromatography on silica gel (15 g) using an appropriate mixture of solvents to afford pure 4-chloro-3-arylsydnone.

4-Chloro-3-phenyl-1,2,3-oxadiazol-3-ium-5-olate **4e**. Purified with a mixture of petroleum ether and ethyl acetate (7:3); green solid (16 mg, 16%): ^{1}H NMR (300 MHz, methanol-d_4): δ 7.70−7.78 (m, 5H); ^{13}C NMR (200 MHz, methanol-d_4): δ 100.3 (C−Cl), 126.1 (2 × CH), 131.3 (2 × CH), 134.0 (CH), 134.5 (C), 166.1 (C−O$^-$) in agreement with literature data.[28]

4-Chloro-3-[4′-(trifluoromethyl)phenyl]-1,2,3-oxadiazol-3-ium-5-olate **4h**. Purified with a mixture of petroleum ether and ethyl acetate (4:1); orange solid (127 mg, 96%): ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.84 (d, J = 8.4 Hz, 2H), 7.97 (d, J = 8.4 Hz, 2H); δ ^{13}C NMR (75.5 MHz, $CDCl_3$): 123.0 (q, J = 273.1, CF_3), 125.3 (2 × CH), 127.6 (q, J = 3.6 Hz, 2 × CH), 135.0 (q, J = 33.7 Hz, C), 135.7 (C), 163.7 (C−O$^-$), missing C−Cl signal due to relaxation issue; ^{19}F NMR (282 MHz, $CDCl_3$): δ − 63.11 (s); HRMS (m/z): [M + H$^+$] calcd for $C_9H_5ClF_3N_2O_2$, 264.9986; found, 264.9987.

3-(4′-Carboxyphenyl)-4-chloro-1,2,3-oxadiazol-3-ium-5-olate **4i**. On 2.4 mmol scale; purified with a mixture of 1% of methanol in dichloromethane with 0.5% acetic acid; white solid (64 mg, 20%): ^{1}H NMR (300 MHz, methanol-d_4): δ 7.90 (d, J = 8.8 Hz, 2H), 8.30 (d, J = 8.8 Hz, 2H); ^{13}C NMR (75 MHz, methanol-d_4): δ 126.4 (2 × CH), 132.4 (2 × CH), 136.3 (C), 137.5 (C), 166.0 (C−O$^-$), 167.6 (C═O), missing C−Cl signal due to relaxation issue in agreement with literature data.[28]

4-Bromo-3-phenyl-1,2,3-oxadiazol-3-ium-5-olate **4f**. *N*-Bromosuccinimide (178 mg, 1.6 mmol) was added to a solution of 3-phenylsydnone **4a** (172 mg, 1.06 mmol) in acetic acid (4 mL). The reaction mixture was stirred at room temperature for 2 h. Water (30 mL) was added, and the resulting precipitate was washed with cold ethanol (2 mL) and dried under reduced pressure to afford the pure brominated sydnone **4f** as an orange solid (174 mg, 68%): ^{1}H NMR (300 MHz, methanol-d_4): δ 7.67−7.79 (m, 5H); ^{13}C NMR (75.5 MHz, methanol-d_4): δ 86.3 (C−Br), 126.3 (2 × CH), 131.2 (2 × CH), 133.9 (CH), 135.4 (C), 168.0 (C−O$^-$) in agreement with literature data.[28]

4-Iodo-3-phenyl-1,2,3-oxadiazol-3-ium-5-olate **4g**. *N*-Iodosuccinimide (169 mg, 0.75 mmol) was added to a solution of 3-phenylsydnone **4a** (81 mg, 0.5 mmol) in acetic acid (1.3 mL). The reaction mixture was stirred at room temperature for 1 h 30 min. Water (20 mL) was added, and the resulting precipitate was washed with cold ethanol (3 mL) and dried under reduced pressure to afford the iodinated sydnone **4g** as a light orange solid (95 mg, 66%): ^{1}H NMR (300 MHz, methanol-d_4): δ 7.58−7.73 (m, 5H); ^{13}C NMR (75.5 MHz, methanol-d_4): δ 50.6 (C−I), 125.3 (2 × CH), 130.2 (2 × CH), 132.7 (CH), 135.3 (C) 168.9 (C−O$^-$) in agreement with literature data.[28]

3,4-Diphenyl-1,2,3-oxadiazol-3-ium-5-olate **4j**. Iodobenzene (0.08 mL, 0.75 mmol) was added to a solution of 3-phenylsydnone **4a** (81 mg, 0.5 mmol), palladium(II) acetate (5.6 mg, 0.025 mmol), potassium carbonate (138 mg, 1 mmol), and triphenylphosphine (13 mg, 0.05 mmol) in wet DMF (2 mL). The reaction mixture was refluxed for 12 h. After being cooled to room temperature, water was added to the mixture and the aqueous layer was extracted with ethyl acetate (3 × 15 mL). The combined organic layers were then dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel (10 g) using a mixture of petroleum ether and ethyl acetate (5:1) to afford the pure desired sydnone **4j** as a brown solid (95 mg, 80%): ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.27−7.29 (br s, 5H), 7.46−7.50 (m, 2H), 7.54−7.60 (m, 2H), 7.63−7.69 (m, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 108.0 (C), 124.5 (C), 124.9 (2 × CH), 127.5 (2 × CH), 128.8 (CH), 128.8 (2 × CH), 130.3 (2 × CH), 132.2 (CH), 134.8 (C), 167.3 (C−O$^-$) in agreement with literature data.[34]

4-(Methylsulfanyl)-3-phenyl-1,2,3-oxadiazol-3-ium-5-olate **4k**. A solution of *n*-butyllithium in THF (1.6 M, 0.34 mL, 0.55 mmol) was added dropwise to a cold (−78 °C) solution of 3-phenylsydnone **4a** (81 mg, 0.5 mmol) in THF (2 mL). After 1 h, dimethyl disulfide (0.04 mL, 0.5 mmol) was then added dropwise and the reaction mixture was stirred for 1 h (the temperature did not rise above −50 °C). The reaction was then quenched by addition of water (20 mL), and the aqueous layer was extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel (10 g) using a mixture of petroleum ether and ethyl acetate (5:1) to afford the pure desired sydnone **4k** as a brown solid (49 mg, 73%): ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.27 (s, 3H), 7.58−7.71 (m, 5H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 18.0 (CH_3), 125.0 (2 × CH), 129.0 (C), 129.9 (2 × CH), 131.1 (C), 132.5 (CH), 168.1 (C−O$^-$) in agreement with literature data.[35]

3-{4′-[Isopropylamino)carbonyl]phenyl}-1,2,3-oxadiazol-3-ium-5-olate **4l**. Isopropylamine (0.05 mL, 0.55 mmol) was added to a solution of PyBop (312 mg, 0.6 mmol), 3-carboxyphenylsydnone **4d** (103 mg, 0.5 mmol), and DIPEA (0.17 mL, 1 mmol) in DMF (5 mL). The reaction mixture was stirred overnight and then diluted with ethyl acetate. The organic layer was washed with a saturated aqueous solution of NH_4Cl (3 × 30 mL) and brine (30 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel (10 g) using a mixture of dichloromethane and acetone (95:5) to afford the pure sydnone **4l** as an off-white solid (50 mg, 40%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.30 (d, J = 6.6 Hz, 6H), 4.31 (hept, J = 6.6 Hz, 1H), 6.05 (br d, J = 6.6 Hz, 1H), 6.76 (s, 1H), 7.80 (d, J = 8.8 Hz, 2H), 8.01 (d, J = 8.8 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 22.9 (2 × CH_3), 42.6 (CH), 93.8 (CH), 121.6 (2 × CH), 129.1 (2 × CH), 136.7 (C), 139.0 (C), 164.5 (C═O), 168.8 (C−O$^-$); HRMS (m/z): [M + H$^+$] calcd for $C_{12}H_{14}N_3O_3$, 248.1030; found, 248.1031.

NHS-Activated 3-(4′-Carboxyphenyl)-4-chloro-1,2,3-oxadiazol-3-ium-5-olate **4m**. *N*-(3-(Dimethylamino)propyl)-*N*′-ethylcarbodiimide hydrochloride (39 mg, 0.2 mmol) was added to a solution of 3-(4′-carboxyphenyl)-4-chlorosydnone **4i** (43 mg, 0.17 mmol) and *N*-hydroxysuccinimide (23 mg, 0.2 mmol) in DMF (3 mL). The reaction mixture was stirred at room temperature overnight. The reaction was then quenched with a saturated aqueous solution of NH_4Cl. The aqueous layer was extracted with ethyl acetate (2 × 20 mL). The combined organic layers were washed with brine (2 × 30 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel (5 g) using a mixture of dichloromethane and methanol (99:1) to afford the pure compound as an off-white solid (37 mg, 65%): ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.95 (s, 4H), 7.87 (d, J = 8.6 Hz, 2H), 8.45 (d, J = 8.6 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 25.8 (2 × CH_2), 125.2 (2 × CH), 129.6 (C), 132.5 (2 × CH), 137.6 (C), 160.3 (C), 168.8 (3 × C), missing C−Cl signal due to relaxation issue; HRMS (m/z): [M + H$^+$] calcd for $C_{13}H_9ClN_3O_6$, 338.0174; found, 338.0175.

4-Chloro-3-{4-[({2-[2-(2-hydroxyethoxy)ethoxy]ethyl}amino)-carbonyl]phenyl}-1,2,3-oxadiazol-3-ium-5-olate **4n**. A solution of NHS-chlorosydnone **4m** (0.24 mmol, 81 mg) in DMF (2 mL) was added to a solution of 2-[2-(2-aminoethoxy)ethoxy]ethanol (0.2 mmol, 30 mg) and triethylamine (0.8 mmol, 0.1 mL) in DMF (2 mL). The reaction mixture was stirred at room temperature during 48 h. The reaction was quenched with a saturated aqueous solution of NH_4Cl. The aqueous layer was extracted with dichloromethane (2 × 20 mL), and the organic layer was successively washed with water (3 × 30 mL) and brine (30 mL), dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel (5 g) using a mixture of dichloromethane and methanol (95:5) to afford the pure chlorosydnone **4n** (26 mg,

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35%) as an orange solid: ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.62−3.65 (m, 2H), 3.66−3.75 (m, 11H), 7.23 (br s, 1H), 7.73 (d, J = 8.8 Hz, 2H), 8.15 (d, J = 8.7 Hz, 2H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 40.2 (CH_2), 61.8 (CH_2), 69.9 (CH_2), 70.4 (CH_2), 70.5 (CH_2), 72.6 (CH_2), 124.7 (2 × CH), 129.3 (2 × CH), 135.0 (C), 138.8 (C), 161.9 (C−O^-), 165.4 (C═O), missing C−Cl signal due to relaxation issue; HRMS (m/z): [M+Na^+] calcd for $C_{15}H_{18}ClN_3O_6Na$, 394.0776; found, 394.0775.

General Procedure for the Cycloaddition of Fl-DIBO with Various Sydnones. A solution of Fl-DIBO (**3**) (14.4 mg, 0.05 mmol) and the respective sydnone (0.1 mmol) in a mixture of dichloromethane and methanol (4:1, 5 mL, solvent system A) or a mixture of 1,2-dichloroethane and methanol (4:1, 5 mL, solvent system B) was stirred at 50 °C (system A) or 70 °C (system B). All volatiles were then removed under reduced pressure, and the residue was purified by flash chromatography on silica gel (5 g) using a mixture of 1% of methanol in dichloromethane, affording the desired pure cycloadducts, respectively.

5,11-Dimethoxy-2-phenyldibenzo[3,4:7,8]cyclopropa[5,6]-cycloocta[1,2-c]pyrazol-8(2H)-one ***6a***. Obtained with system A overnight. Light yellow solid (18 mg, 89%): ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.89 (s, 3H), 3.91 (s, 3H), 6.89 (dd, J = 8.5, 2.5 Hz, 1H), 6.95 (dd, J = 8.5, 2.7 Hz, 1H), 7.00 (d, J = 2.5 Hz, 1H), 7.34 (tt, J = 7.4, 1.5 Hz, 1H), 7.43 (d, J = 2.7 Hz, 1H), 7.48 (t, J = 7.4 Hz, 2H), 7.63 (d, J = 8.5 Hz, 1H), 7.65 (d, J = 8.5 Hz, 1H), 7.74 (dd, J = 7.4, 1.5 Hz, 2H), 7.86 (s, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 55.8 (2 × CH_3), 112.6 (CH), 114.5 (CH), 117.4 (C), 117.7 (CH), 117.7 (C), 118.3 (CH), 119.1 (2 × CH), 122.3 (C), 127.4 (CH), 129.7 (2 × CH), 130.7 (CH), 133.7 (CH), 134.0 (CH), 137.0 (C), 138.3 (C), 139.3 (C), 148.4 (C), 149.8 (C), 150.2 (C), 152.8 (C═O), 162.6 (C), 162.9 (C); HRMS (m/z): [M + H^+] calcd for $C_{26}H_{19}N_2O_3$, 407.1390; found, 407.1393.

5,11-Dimethoxy-2-(4-methoxyphenyl)dibenzo[3,4:7,8]-cyclopropa[5,6]cycloocta[1,2-c]pyrazol-8(2H)-one ***6b***. Obtained with system B during 48 h. Light yellow solid (14 mg, 65%): ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.85 (s, 3H), 3.88 (s, 3H), 3.90 (s, 3H), 6.87 (dd, J = 8.5, 2.6 Hz, 1H), 6.93 (dd, J = 8.5, 2.7 Hz, 1H), 6.06−6.99 (m, 3H), 7.41 (d, J = 2.7 Hz, 1H), 7.61 (d, J = 8.5 Hz, 1H), 7.62 (d, J = 8.5 Hz, 1H), 7.63 (d, J = 9.1 Hz, 2H), 7.76 (s, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 55.7 (3 × CH_3), 112.5 (CH), 114.3 (CH), 114.7 (2 × CH), 117.4 (C), 117.6 (CH), 117.7 (C), 118.2 (CH), 120.8 (2 × CH), 121.8 (C), 130.8 (CH), 133.0 (C), 133.7 (CH), 133.9 (CH), 137.1 (C), 138.4 (C), 147.8 (C), 149.9 (C), 150.2 (C), 152.8 (C═O), 158.9 (C), 162.6 (C), 162.8 (C); HRMS (m/z): [M + H^+] calcd for $C_{27}H_{21}N_2O_4$, 437.1496; found, 437.1500.

5,11-Dimethoxy-2-(4-trimethylfluorophenyl)dibenzo[3,4:7,8]-cyclopropa[5,6]-cycloocta[1,2-c]pyrazol-8(2H)-one ***6c***. Obained with system A overnight. Light yellow solid (23 mg, 97%): ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.89 (s, 3H), 3.91 (s, 3H), 6.90 (dd, J = 8.5, 2.6 Hz, 1H), 6.97 (dd, J = 8.5, 2.7 Hz, 1H), 7.01 (d, J = 2.6 Hz, 1H), 7.41 (d, J = 2.7 Hz, 1H), 7.64 (d, J = 8.5 Hz, 1H), 7.65 (d, J = 8.5 Hz, 1H), 7.74 (d, J = 8.6 Hz, 2H), 7.87 (d, J = 8.6 Hz, 2H), 7.93 (s, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 55.8 (CH_3), 55.8 (CH_3), 112.8 (CH), 114.5 (CH), 117.4 (C), 117.6 (C), 117.8 (CH), 118.4 (CH), 118.7 (2 × CH), 123.2 (C), 123.9 (q, J = 272.0 Hz, CF_3), 127.0 (q, J = 3.7 Hz, 2 × CH), 129.2 (q, J = 33.0 Hz, C), 130.6 (CH), 133.8 (CH), 134.0 (CH), 136.5 (C), 137.8 (C), 141.6 (C), 149.4 (C), 149.7 (C), 150.1 (C), 152.7 (C═O), 162.6 (C), 162.9 (C); ^{19}F NMR (282 MHz, $CDCl_3$): δ − 62.35 (s); HRMS (m/z): [M + H^+] calcd for $C_{27}H_{18}F_3N_2O_3$, 475.1264; found, 475.1269.

3-Chloro-5,11-dimethoxy-2-phenyldibenzo[3,4:7,8]cyclopropa-[5,6]cycloocta[1,2-c]pyrazol-8(2H)-one ***6e***. Obtained with system A overnight. Yellowish solid (21 mg, 93%): ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.87 (s, 3H), 3.89 (s, 3H), 6.94 (dd, J = 8.6, 2.5 Hz, 1H), 6.96 (dd, J = 8.5, 2.6 Hz, 1H), 7.14 (d, J = 2.6 Hz, 1H), 7.33 (dd, J = 2.5 Hz, 1H), 7.49−7.56 (m, 3H), 7.60−7.66 (m, 4H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 55.8 (CH_3), 55.8 (CH_3), 113.4 (CH), 114.9 (CH), 117.3 (C−Cl), 117.7 (CH), 117.8 (C), 119.1 (C), 119.4 (CH), 125.6 (2 × CH), 128.3 (C), 129.2 (CH), 129.3 (2 × CH), 133.0 (CH), 133.2 (CH), 134.6 (C), 138.1 (C), 138.2 (C), 148.8 (C), 151.2 (C), 151.8 (C), 152.3 (C═O), 162.3 (C), 162.7 (C); HRMS (m/z): [M + H^+] calcd for $C_{26}H_{18}ClN_2O_3$, 441.1000; found, 441.1010.

3-Bromo-5,11-dimethoxy-2-phenyldibenzo[3,4:7,8]cyclopropa-[5,6]cycloocta[1,2-c]pyrazol-8(2H)-one ***6f***. Obtained with system A overnight. Off-white solid (21 mg, 88%): ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.86 (s, 3H), 3.89 (s, 3H), 6.93 (dd, J = 8.5, 2.7 Hz, 1H), 6.95 (dd, J = 8.5, 2.7 Hz, 1H), 7.16 (d, J = 2.6 Hz, 1H), 7.31 (d, J = 2.6 Hz, 1H), 7.47−7.64 (m, 7H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 55.7 (CH_3), 55.8 (CH_3), 113.5 (CH), 114.9 (CH), 116.8 (C−Br), 117.6 (CH), 118.0 (C), 119.2 (C), 119.9 (CH), 120.2 (C), 126.2 (2 × CH), 129.2 (2 × CH), 129.3 (CH), 132.8 (CH), 133.0 (CH), 135.3 (C), 138.1 (C), 139.0 (C), 149.3 (C), 151.4 (C), 152.1 (C), 152.2 (C═O), 162.2 (C), 162.6 (C); HRMS (m/z): [M + H^+] calcd for $C_{26}H_{18}BrN_2O_3$, 485.0495; found, 485.0494.

3-Iodo-5,11-dimethoxy-2-phenyldibenzo[3,4:7,8]cyclopropa[5,6]-cycloocta[1,2-c]pyrazol-8(2H)-one ***6g***. Obtained with system A during 24 h. Yellow solid (11 mg, 41%): ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.86 (s, 3H), 3.91 (s, 3H), 6.94 (dd, J = 8.5, 2.6 Hz, 1H), 6.95 (dd, J = 8.5, 2.7 Hz, 1H), 7.18 (d, J = 2.6 Hz, 1H), 7.30 (d, J = 2.6 Hz, 1H), 7.49−7.57 (m, 5H), 7.59 (d, J = 8.5 Hz, 1H), 7.61 (d, J = 8.5 Hz, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 55.7 (CH_3), 55.8 (CH_3), 91.1 (C−I), 113.7 (CH), 114.9 (CH), 117.5 (CH), 118.1 (C), 119.5 (C), 120.4 (CH), 125.5 (C), 127.0 (2 × CH), 129.2 (2 × CH), 129.5 (CH), 132.6 (CH), 133.0 (CH), 136.6 (C), 138.1 (C), 140.6 (C), 149.5 (C), 151.7 (C), 152.2 (C), 152.5 (C═O), 162.0 (C), 162.6 (C); HRMS (m/z): [M + H^+] calcd for $C_{26}H_{18}IN_2O_3$, 533.0357; found, 533.0357.

3-Chloro-5,11-dimethoxy-2-[4-(trifluoromethyl)phenyl]dibenzo-[3,4:7,8]cyclopropa[5,6]cycloocta[1,2-c]pyrazol-8(2H)-one ***6h***. Obtained with system A overnight. Off-white solid (25 mg, 98%): ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.88 (s, 3H), 3.90 (s, 3H), 6.95 (dd, J = 8.7, 2.6 Hz, 1H), 6.98 (dd, J = 8.7, 2.6 Hz, 1H), 7.13 (d, J = 2.6 Hz, 1H), 7.31 (d, J = 2.6 Hz, 1H), 7.62 (d, J = 8.6 Hz, 1H), 7.66 (d, J = 8.6 Hz, 1H), 7.80 (s, 4H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 55.7 (CH_3), 55.8 (CH_3), 113.5 (CH), 114.9 (CH), 117.8 (CH), 117.9 (C−Cl), 118.3 (C), 119.1 (C), 119.5 (CH), 123.7 (q, J = 271.7 Hz, C), 125.5 (2 × CH), 126.5 (q, J = 3.6 Hz, 2 × CH), 128.2 (C), 131.0 (q, J = 33.2 Hz, C), 133.0 (CH), 133.2 (CH), 134.1 (C), 137.7 (C), 140.8 (C), 149.8 (C), 151.3 (C), 151.9 (C), 152.1 (C═O), 162.4 (C), 162.6 (C); ^{19}F NMR (282 MHz, $CDCl_3$): δ − 62.62 (s); HRMS (m/z): [M + H^+] calcd for $C_{27}H_{17}ClF_3N_2O_3$, 509.0874; found, 509.0876.

5,11-Dimethoxy-2,3-diphephenyldibenzo[3,4:7,8]cyclopropa-[5,6]cycloocta[1,2-c]pyrazol-8(2H)-one ***6j***. Obtained with system B during 120 h. Yellow solid (10 mg, 42%): ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.30 (s, 3H), 3.93 (s, 3H), 6.29 (d, J = 2.6 Hz, 1H), 6.75 (dd, J = 8.5, 2.6 Hz, 1H), 6.81−7.06 (m, 3H), 7.16−7.35 (m, 8H), 7.50 (d, J = 2.6 Hz, 1H), 7.58 (d, J = 8.5 Hz, 1H), 7.62 (d, J = 8.5 Hz, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 55.2 (CH_3), 55.7 (CH_3), 114.4 (CH), 114.6 (CH), 117.9 (CH), 118.0 (C), 119.1 (CH), 119.4 (C), 125.3 (2 × CH), 127.9 (CH), 128.7 (CH), 128.8 (2 × CH), 129.0 (2 × CH), 130.0 (2 × C), 130.9 (2 × CH), 132.5 (CH), 133.3 (CH), 136.6 (C), 138.7 (C), 139.5 (C), 144.5 (C), 148.7 (C), 151.4 (C), 151.9 (C), 152.6 (C═O), 161.8 (C), 162.6 (C); HRMS (m/z): [M + H^+] calcd for $C_{32}H_{23}N_2O_3$, 483.1703; found, 483.1710.

5,11-Dimethoxy-3-(methylsulfanyl)-2-phenyldibenzo[3,4:7,8]-cyclopropa[5,6]-cycloocta[1,2-c]pyrazol-8(2H)-one ***6k***. Obtained with system B during 48 h. Yellow solid (21 mg, 91%): ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.79 (s, 3H), 3.85 (s, 3H), 3.90 (s, 3H), 6.94 (dd, J = 8.5 Hz, 2.6 Hz, 1H), 6.93 (dd, J = 8.5 Hz, 2.6 Hz, 1H), 7.26 (d, J = 2.6 Hz, 1H), 7.32 (d, J = 2.6 Hz, 1H), 7.45−7.54 (m, 3H), 7.57−7.64 (m, 4H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 18.6 (CH_3), 55.7 (CH_3), 55.8 (CH_3), 113.6 (CH), 114.7 (CH), 117.6 (CH), 117.9 (C), 119.2 (C), 119.8 (CH), 123.6 (C), 126.0 (2 × CH), 128.9 (CH), 129.1 (2 × CH), 132.5 (CH), 132.9 (CH), 136.2 (C), 137.7 (C), 138.5 (C), 139.5 (C), 148.7 (C), 151.6 (C), 152.3 (C), 152.4 (C═O), 162.2 (C), 162.6 (C); HRMS (m/z): [M + H^+] calcd for $C_{27}H_{21}N_2O_3S$, 453.1267; found, 453.1265.

5,11-Dimethoxy-2-(4-isopropylamidophenyl)dibenzo[3,4:7,8]-cyclopropa[5,6]-cycloocta[1,2-c]pyrazol-8(2H)-one ***6l***. Obtained with system B overnight. yellow solid (4 mg, 16%, low yield due to

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difficulties associated with the purification): ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.29 (d, J = 6.6 Hz, 6H), 3.89 (s, 3H), 3.92 (s, 3H), 4.31 (hept, J = 6.6 Hz, 1H), 5.97 (d, J = 7.8 Hz, 1H), 6.91 (dd, J = 8.5, 2.6 Hz, 1H), 6.97 (dd, J = 8.5, 2.7 Hz, 1H), 7.01 (d, J = 2.5 Hz, 1H), 7.43 (d, J = 2.6 Hz, 1H), 7.4 (d, J = 8.5 Hz, 1H), 7.65 (d, J = 8.5 Hz, 1H), 7.80 (d, J = 8.8 Hz, 2H), 7.88 (d, J = 8.8 Hz, 2H), 7.91 (s, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 23.0 (2 × CH_3), 42.3 (CH), 55.8 (2 × CH_3), 112.7 (CH), 114.5 (CH), 117.5 (C), 117.7 (C), 117.8 (CH), 118.4 (CH), 118.5 (2 × CH), 123.0 (C), 128.6 (2 × CH), 130.6 (CH), 133.5 (C), 133.8 (CH), 134.0 (CH), 136.6 (C), 137.9 (C), 141.2 (C), 149.1 (C), 149.9 (C), 150.2 (C), 152.7 (C), 162.6 (C), 162.9 (C), 165.7 (C); HRMS (m/z): [M + H$^+$] calcd for $C_{30}H_{26}N_3O_4$, 492.1918; found, 492.1923.

4-(3-Chloro-5,11-dimethoxy-8-oxodibenzo[3,4:7,8]cyclopropa-[5,6]cycloocta[1,2-c]pyrazol-2(8H)-yl)-N-{2-[2-(2-hydroxyethoxy)-ethoxy]ethyl}benzamide **6n**. Obtained with system A overnight; Purified with a mixture of 5% of methanol in dichloromethane; Yellow solid (18 mg, 60%): ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.59−3.62 (m, 2H), 3.66−3.73 (m, 11H), 3.87 (s, 3H), 3.88 (s, 3H), 6.94 (dd, J = 8.5, 2.6 Hz, 1H), 6.96 (dd, J = 8.5, 2.6 Hz, 1H), 7.12 (d, J = 2.5 Hz, 1H), 7.15 (br s, 1H), 7.31 (d, J = 2.6 Hz, 1H), 7.60 (d, J = 8.6 Hz, 1H), 7.64 (d, J = 8.6 Hz, 1H), 7.71 (d, J = 8.7 Hz, 2H), 7.99 (d, J = 8.7 Hz, 2H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 40.0 (CH_2), 55.8 (CH_3), 55.8 (CH_3), 61.8 (CH_2), 70.1 (CH_2), 70.4 (CH_2), 70.5 (CH_2), 72.7 (CH_2), 113.5 (CH), 114.9 (CH), 117.7 (CH), 118.0 (C−Cl), 119.0 (C), 119.4 (CH), 125.2 (2 × CH), 128.2 (C), 128.3 (2 × CH), 133.0 (CH), 133.2 (CH), 134.3 (C), 134.9 (C), 137.8 (C), 140.3 (C), 149.4 (C), 151.1 (C), 151.7 (C), 152.2 (C═O), 162.3 (C), 162.6 (C), 166.5 (C═O); HRMS (m/z): [M + H$^+$] calcd for $C_{33}H_{31}ClN_3O_7$, 616.1845; found, 616.1846.

Absorption and Fluorescence Measurements. UV−vis spectra were recorded at 25 ± 0.1 °C using a Varian Cary 300 UV−vis spectrophotometer. Fluorescence spectra were recorded with a SpectraMax M2E Multi-Mode Cuvette/Microplate Reader from molecular devices using a cuvette with 1 cm path length. For spectra, see Supporting Information Figures S1− S13.

Quantum Yield Determination. Quantum yields were determined from the slope of the integrated fluorescence emission between 380 and 700 nm (excitation at 360 nm) versus absorbance using quinine sulfate in 1.0 N H_2SO_4 (Φ_F = 0.54 ± 0.03) as fluorescence standard. For each compound, four data points were acquired with absorbances ranging between 0.1 and 0.5 (l = 1 cm).

Kinetics Measurements. The rate measurements of cycloaddition of Fl-DIBO (**3**) with modified sydnones **4a**, **4c**, **4e**, and **4h** were conducted by using ^{1}H NMR spectroscopy (Brüker 400 MHz) at 25 °C. A 20 mM solution of sydnones (0.2 mL) in $CDCl_3$:MeOD (4:1) was added to a thermally equilibrated solution of Fl-DIBO (**3**) (10 mM, 0.4 mL) in a mixture of $CDCl_3$:MeOD (4:1), leading to a mixture of both reactants in 1:1 ratio with a respective concentration of 6.66 mM. Reactions were monitored by following the decay of characteristic peaks of Fl-DIBO and sydnone as well as the formation of characteristic pyrazole peaks. Consumption of starting materials followed a second-order equation, and the second-order rate constants were obtained by least-squares fitting of the data to a linear equation. For graphs, see Supporting Information Figures S14− S17.

In-Gel Visualization. Following reaction with Fl-DIBO (**3**), some protein samples were analyzed via SDS-PAGE (10% gradient) and visualized by in-gel fluorescence scanning (BioRad, ChemiDoc MP imager, trans-UV excitation (302 nm)/standard emission filter). The gels were then stained with Coomassie Blue to reveal total protein content.

Strain-Promoted Click Ligation between Fl-DIBO and BSA-PhSydCl. Fl-DIBO (**3**) (2 μL, 2.5 mM in DMF) was incubated with BSA-PhSydCl (48 μL, 2 mg/mL) in PBS (0.01 M, pH 7.4) at 37 °C for 3 h and analyzed by in-gel fluorescence imaging.

Computational Studies. All quantum chemical studies were performed with the Gaussian 09 (Rev. A02) software package.[36] Molecular structures for compounds **6o**, **6p**, and **6q** were obtained by optimizing their gas-phase geometries by DFT with the B3LYP hybrid functional and Pople's 6-31G(d) split valence basis set. To account for bulk solvent effects, the gas-phase geometries were further geometry-optimized with inclusion of the polarizable continuum model (PCM), which creates a solute cavity based on a set of overlapping spheres.[37] Coordinates for the final geometries are listed in Supporting Information, Tables S1−S3. All structures were validated based on vibrational frequency analysis to ensure a stationary point on the ground-state potential surface. Vertical excitation energies were computed based on the solvent-equilibrated ground-state geometries (B3LYP/6-31G(d)) using TD-DFT with the CAM-B3LYP functional[38] and the 6-31+G(d) basis set with added diffuse functions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.7b03004.

^{1}H and ^{13}C NMR spectra of all synthesized compounds, absorbance and emission spectra of pyrazoles **6a−c**, **6e−h**, and **6j−n**, kinetics plots, ^{1}H NMR data for the stability study of sydnone **4e**, and TD-DFT calculations (PDF)

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Notes

The authors declare no competing financial interest.

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