## Supplementary Information

## Fluorogenic sydnonimine probes for orthogonal labeling

Wenyuan $\mathrm{Xu}^{\mathrm{a} \ddagger}$, Zhuzhou Shao ${ }^{\text {aq }}$, Cheng Tang ${ }^{\text {a }}$, Chun Zhang ${ }^{\mathrm{b}^{*}}$, Yinghan Chen ${ }^{\mathrm{a}^{*}}$ and Yong Liang ${ }^{\mathrm{a}^{*}}$<br>${ }^{a}$ State Key Laboratory of Coordination Chemistry, Jiangsu Key Laboratory of Advanced Organic Materials, School of Chemistry and Chemical Engineering, Chemistry and Biomedicine Innovation Center, Nanjing University, Nanjing 210023, China<br>${ }^{b}$ School of Pharmaceutical Sciences, Jiangnan University, Wuxi, 214122, China<br>${ }^{7}$ These authors contributed equally.<br>*E-mails: zhangchun@jiangnan.edu.cn; qd_yinghan@163.com; yongliang@nju.edu.cn.

## CONTENTS

Abbreviations ..... S1
Computational details ..... S2
General information ..... S12
Organic synthesis ..... S13
Kinetics measurement ..... S23
Mutually orthogonal labeling of two fluorogenic probes ..... S24
Fluorescence spectra ..... S25
Confocal fluorescent microscope imaging ..... S27
References ..... S28
NMR spectra ..... S29


#### Abstract

Abbreviations The following abbreviations are used throughout the text of the SI file: BHQ, black hole quencher; Boc, tert-butoxycarbonyl; calcd., calculated; $\mathrm{CDCl}_{3}$, chloroform- $d$; DMSO, dimethylsulfoxide; DIPEA, $N, N$-diisopropylethylamine; DMF, $N, N-$ dimethylformamide; DCM, dichloromethane; equiv., equivalent; ESI, electrospray ionization; $\mathrm{Et}_{2} \mathrm{O}$, diethyl ether; EtOAc , ethyl acetate; M, mol/L; MeCN, acetonitrile; MeOH , methanol; MS, Mass Spectrometry; NaOAc, sodium acetate; PBS, phosphate buffered saline; PE, petroleum ether; RP-HPLC, reversed-phase high performance liquid chromatography; r.t., room temperature; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin layer chromatography; HATU, 2-(7-Aza-1 H -benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; TSTU, $N, N, N^{\prime}, N^{\prime}$-tetramethyl- $O$-( $N$-succinimidyl)uronium tetrafluoroborate; h, hour; HRMS, high resolution mass spectrum; 3RAX, 3-amino-7-(diethylamino)-5phenylphenazinium chloride; TAMRA, 2-(3,6-bis(dimethylamino)xanthylium-9-yl)-5carboxybenzoate; Cy5, 1-(5-carboxy-pentyl)-3,3-dimethyl-2-((1E,3E)-5-((E)-1,3,3-trimethyl-5-sulfoindolin-2-ylidene) penta-1,3-dien-1-yl)-3H-indol-1-ium-5-sulfonate.


## Computational details

All calculations were performed with Gaussian $09^{[1]}$. Geometry optimizations of all the minima and transition structures were carried out at the M06-2X level of theory ${ }^{[2,3]}$ with the $6-31 \mathrm{G}(\mathrm{d})$ basis set. Vibrational frequencies were evaluated at the same level to verify that optimized structure is an energy minimum or a transition state and to compute zero-point vibrational energies (ZPVE) and thermal corrections at 298 K. Solvent effects in water were calculated at the M06-2X/6-311+G(d,p) level using the gas-phase optimized structures with the CPCM model ${ }^{[4-6]}$, where UFF radii were used. The predicted second-order rate constants shown in Figure S2 were calculated by using the corrected activation free energies $\left[\Delta \mathrm{G}_{-}^{\ddagger} \text { corr }=\left(\Delta \mathrm{G}_{-}^{\ddagger} \mathrm{compt}+8.4\right) / 1.6\right]^{[7]}$, according to Eyring equation at 298 K .



Figure S1. The optimized structure of sydnonimine 4'.


Ph-SIN
$2 \pi$ cycloaddends

TS: $\Delta G_{\text {water }}(\mathrm{kcal} / \mathrm{mol})$
$\left[\boldsymbol{k}_{\mathbf{2}}\left(\mathrm{M}^{-1} \mathrm{~s}^{-1}\right)\right]$


1,3-Cp
TS3: 25.3
$2.3 \times 10^{-3}$


3,3-Cp
TS1: 29.5
$2.7 \times 10^{-5}$

cyclooctyne
TS4: 23.3
$1.8 \times 10^{-2}$

norbornene
TS2: 29.5
$2.7 \times 10^{-5}$

Figure S2. DFT-computed activation free energies for cycloadditions of Ph-SIN with $2 \pi$ cycloaddends at the CPCM(water)-M06-2X/6-311+G(d,p)//M06-2X/6-31G(d) level of theory and the predicted rate constants in water at $25^{\circ} \mathrm{C}$.

## Coordinates and Energies of Computed Stationary Points

| 4, | C -9.900140 2.795531-0.790937 |
| :---: | :---: |
| $G($ Water $)=-1882.027398$ Hartree | C-8.781162 $2.081810-0.364117$ |
|  | O 8.170762-1.462029-1.303154 |
| N 1.938298-0.881444-0.092767 | N 8.2670660 .3846240 .010479 |
| N 1.432042-1.954363 0.428846 | C 9.721218 0.435263-0.013142 |
| O 0.098723-1.737066 0.411301 | C 10.2158081 .8079510 .420041 |
| C -0.211788-0.502241-0.119786 | C 11.7375501 .8937560 .429148 |
| C 1.041639 0.052303-0.451323 | H $1.3000360 .970575-0.946505$ |
| C 3.365353-0.797659-0.224517 | H 3.555713-2.878584-0.724865 |
| N-1.404611-0.029111-0.258450 | H 6.055722-2.707677-0.965136 |
| C 4.079141-1.943463-0.560802 | H 5.854489 1.467207-0.017117 |
| C 5.457666-1.847035-0.685446 | H 3.3988251 .3013170 .255404 |
| C 6.107230-0.630585-0.468333 | H-3.786651 1.0754560 .531142 |
| C 5.365209 0.506881-0.146998 | H -3.961670 0.431023-1.121808 |
| C 3.983303 0.429169-0.017935 | H -5.533264-1.320090-0.310265 |
| C 7.606004-0.619045-0.628171 | H -5.248210-0.821984 1.364080 |
| S -2.671710-0.968002 0.257201 | H-6.304231 1.369275 0.942176 |
| C -4.005649 0.180361-0.058532 | H -6.423991 1.009514-0.791505 |
| O -2.846901-2.123839-0.617294 | H-7.602922-0.172029 2.475556 |
| O-2.590995-1.186025 1.699515 | H-8.961088-1.063543 1.764584 |
| C -5.332342-0.458374 0.334712 | H -7.320818-1.697802 1.599873 |
| C-6.465555 0.5575140 .205833 | H-10.231034-0.956016 0.038770 |
| N -7.773385-0.053583 0.358872 | H-12.208612 0.323938-0.662806 |
| C -8.882406 $0.722372-0.041029$ | H -9.792188 3.849075-1.032650 |
| C -7.919300-0.783639 1.613860 | H-7.833208 2.599258-0.270593 |
| C-10.140404 0.107121-0.162614 | H 7.7986130 .8704020 .763176 |
| C-11.250727 $0.828190-0.573526$ | H 10.140942-0.348475 0.633233 |
| C-11.141464 2.181911-0.892802 | H 10.034858 0.204664-1.034950 |

H 9.805882 2.567945-0.258144 H 9.8561222 .0485961 .428397 H 12.1330681 .1528951 .133461 H $12.1217291 .620418-0.568614$ H-12.010356 2.743362-1.220229 N 12.1530613 .2154730 .890622 H 13.1650373 .2491790 .983098 H 11.9094653 .9120930 .189349

## Ph-SIN

$G($ Water $)=-1136.105866$ Hartree

N-1.080131-0.615939 0.143371
N - 0.931911 -1.872886 0.417504
O $0.405910-2.0532380 .444881$
C $1.083323-0.8846340 .196308$
C $0.0645110 .072739-0.007642$
N 2.377886 - 0.9355560 .190529
S $3.1581940 .499805-0.022014$
O $2.3621111 .415382-0.848632$
O 3.6559680 .9933721 .254885
H 0.124642 1.104622-0.303001
C 4.549095-0.047485-0.985845
H 5.078015-0.814061-0.419713
H 4.184726-0.441375-1.934464
H $5.1851450 .824777-1.145457$
C - $2.413496-0.0924520 .032274$
C - $3.413187-0.907157-0.486863$
C - 2.6559171 .2101640 .450637

C -4.699911-0.390379-0.586148
H -3.176740-1.916745-0.803410
C - 3.9477071 .7125690 .335746
H-1.857252 1.8091630 .875772
C -4.966711 $0.915475-0.180068$
H -5.493602 -1.010150 -0.989412
H -4.156424 2.7268840 .658459
H -5.972447 1.313516-0.265848

## 3,3-Cp

$G($ Water $)=-195.1257316$ Hartree

C $1.2621720 .000000-0.647569$
C $1.262611-0.0000000 .647379$
C - 0.0944640 .0000000 .000147
H $1.809548-0.0000001 .578518$
C - $0.937167-1.2671780 .000026$
H-1.586180 - 1.3052430 .883394
H -1.586035-1.305322 -0.883478
H - 0.305240-2.159455 0.000062
C - 0.9371671 .2671780 .000026
H - $1.5860351 .305322-0.883478$
H-1.586180 1.3052440 .883394
H -0.305240 2.1594550 .000062
H $1.8094490 .000000-1.578528$
----------------------------------------
TS1
$G($ Water $)=-1331.184636$ Hartree ----------------------------------------

C $0.0612241 .378754-0.858453$ C 0.8045612 .0008710 .087044 C - 0.492768 2.659619-0.287192 H $0.1836261 .010641-1.868851$

H 1.8254622 .3318260 .229154
C - 0.398375 3.889218-1.187790 H -0.297361 $4.800471-0.586715$ H 0.458658 3.827466-1.864942 H-1.307337 3.991167-1.792167 C-1.704639 2.7599370 .628464 H-1.660766 3.7086541 .175730 H-2.619850 2.7737250 .024507 H-1.792296 1.9595151 .357802 N 1.183486-0.469047 0.649970 N 0.9683120 .4580051 .582205 O -0.371929 0.3083381 .876852 C -1.001717-0.368978 0.883951 C 0.047487-0.739256-0.026168 N -2.279243-0.513076 0.957725 S -2.994588-1.381472-0.258964 O -2.234892-1.251103-1.507484 O -3.322289-2.719032 0.213334 H - $0.020033-1.460734-0.823323$ C - $4.502105-0.454433-0.432654$ H -5.023576-0.464247 0.524335

H -4.250684 0.562727-0.733502
H -5.091995-0.954223-1.202749
C $2.520535-0.6443320 .193877$
C $3.556696-0.5394091 .119728$

C $2.768697-0.915675-1.149047$
C $4.865035-0.7096120 .683758$
H 3.325875 -0.330750 2.158287
C 4.083402-1.097496-1.565161
H $1.953034-0.970811-1.862101$
C 5.131316-0.993435-0.654316
H $5.678530-0.6292111 .397198$
H 4.286404-1.311344-2.609274
H 6.154603-1.132755-0.987132

norbornene
$G($ Water $)=-272.5457539$ Hartree
--------------------------------------
C - $1.275344-0.668267-0.500391$
C -0.084771-1.124602 0.325215
C 1.179937-0.777633-0.521120
C $1.1799600 .777613-0.521143$
C - $1.2753210 .668278-0.500390$
C - 0.0847421 .1246060 .325239
C -0.0307790 .0000051 .376394
H-1.915633-1.325220 -1.079425
H -0.114261-2.151779 0.692510
H 2.080314-1.172541-0.039936
H 1.123410-1.201886-1.526509
H 1.123255 1.201826-1.526539
H 2.080396 1.172565-0.040118
H-1.915564 1.325267-1.079429
H-0.114276 2.1517790 .692547
H - 0.8972910 .0000132 .042551

| H $0.896013-0.0000191 .961528$ | O -2.269336-1.292680-1.512166 |
| :---: | :---: |
|  | O -3.042678-3.075016 0.073286 |
| TS2 | H 0.020892-1.661107-0.845338 |
| $G($ Water $)=-1408.604663$ Hartree | C -4.523060-0.912619-0.203292 |
| -------------------------------------- | H -4.966500-1.101931 0.774105 |
| C 0.7815121 .7719430 .266431 | H -4.415716 0.157380-0.383687 |
| C -0.067371 2.9107750 .802239 | H -5.107407-1.385411-0.994331 |
| C-1.296020 1.906000-0.804003 | C 2.576003-0.869482 0.167619 |
| C $0.0286981 .162279-0.742586$ | C 3.639884-0.690762 1.052089 |
| H 1.8633231 .8679200 .226054 | C 2.796107-1.159451-1.177507 |
| H $0.4674510 .775550-1.658569$ | C 4.939266-0.803760 0.573853 |
| C-1.496749 2.3616240 .653679 | H 3.435439-0.471023 2.093741 |
| H-2.242730 3.1607270 .725219 | C 4.103941-1.282770-1.635755 |
| H-1.762405 1.5763001 .357596 | H 1.964365-1.281712-1.862928 |
| C -0.067172 3.962520-0.350865 | C 5.175883-1.103685-0.766480 |
| H-0.533723 $4.891920-0.009380$ | H 5.770629-0.665617 1.257376 |
| H $0.9451144 .202992-0.687908$ | H 4.281208-1.511624-2.681437 |
| C -0.924399 3.274052-1.454721 | H 6.193039-1.198031-1.132046 |
| H-1.831515 3.848714-1.667057 |  |
| H -0.382748 3.147955-2.396268 | 1,3-Cp |
| H 0.2218743 .3036321 .778358 | $G($ Water $)=-403.09152$ Hartree |
| H-2.111588 1.367321-1.289845 |  |
| N 1.251889-0.751136 0.664695 | C 2.075599-1.340850 0.012149 |
| N 1.0361700 .1425631 .640320 | C 1.179573-0.220732 0.465535 |
| O -0.294695-0.062275 1.960363 | C 2.636057-0.176795 0.099311 |
| C -0.928544-0.691129 0.941934 | H 2.147467-2.397607-0.195902 |
| C 0.100860-0.979166-0.014639 | H $0.897003-0.1653791 .522917$ |
| N-2.208098-0.845984 1.017461 | C $3.8039170 .725252-0.003608$ |
| S -2.909256-1.658781-0.242553 | H $4.6993180 .184751-0.318783$ |


| H 3.602976 1.526128-0.722534 | N -2.980533-1.675748 0.128895 |
| :---: | :---: |
| H 3.9977321 .2002960 .963537 | H -2.891429-0.685096 0.334386 |
| C $0.1203220 .364743-0.450773$ | C -4.123576-2.344076 0.417824 |
| H $0.4888090 .355860-1.485625$ | О -4.259035-3.536009 0.184632 |
| H-0.117259 1.397517-0.182545 | C -5.219375-1.507458 1.051554 |
| N-1.132117-0.364483-0.357339 | H -5.473780-1.942024 2.021369 |
| H-1.099803-1.369377-0.449124 | H -4.933924-0.461202 1.184735 |
| C-2.311529 0.229449-0.018235 | H -6.110255-1.564999 0.421439 |
| O-2.425244 1.4287180 .166352 | N $2.1021330 .659997-0.516487$ |
| C-3.488557-0.716488 0.138989 | N $1.7524180 .088063-1.669958$ |
| H -4.363135-0.258682-0.325626 | O $0.6146780 .780587-2.038716$ |
| H-3.316392-1.702051-0.300716 | C $0.0806441 .406143-0.965534$ |
| H-3.702233-0.837288 1.204745 | C 1.0231671 .1934050 .094984 |
| ----------------------------------- | N -1.080578 1.958631-1.102993 |
| TS3 | S -1.837340 2.4010520 .288550 |
| $G($ Water $)=-1539.157085$ Hartree | O -2.757679 1.330345 0.682357 |
| ---------------------------------- | O-0.897621 2.8850891 .302889 |
| C 0.063075-0.822882 0.319228 | H 1.0619671 .7304731 .029013 |
| C -0.804351-1.316670-0.799087 | C-2.808529 3.770620-0.288086 |
| C 0.659644-1.564361-0.641228 | H-2.133210 $4.561337-0.614982$ |
| H-0.031990-0.774858 1.396066 | H -3.435660 3.424050-1.109403 |
| H-1.132734-0.599031-1.557386 | H -3.417367 4.102108 0.554469 |
| C 1.613182-2.691189-0.835175 | C 3.2901280 .1888760 .108209 |
| H 2.504350-2.571165-0.211674 | C 4.404570-0.062446-0.689691 |
| H 1.119243-3.629101-0.553816 | C 3.311215-0.033253 1.482185 |
| H 1.921855-2.772016-1.880049 | C 5.559406-0.550173-0.091466 |
| C -1.860372-2.358324-0.490637 | H $4.3493390 .120558-1.757017$ |
| H-1.466751-3.129917 0.185321 | C 4.481477-0.507461 2.067427 |
| H-2.200311-2.870325-1.398391 | H 2.4242760 .1423682 .081954 |


| C 5.602464-0.769215 1.284742 | TS4 |
| :---: | :---: |
| H 6.432316-0.754008-0.702795 | $G($ Water $)=-1447.855654$ Hartree |
| H 4.508329-0.684011 3.137517 |  |
| H 6.509726-1.145893 1.745453 | C -0.034920 1.024580 0.325790 |
| ----------------------------------- | C -0.764613 1.316672-0.639356 |
| cyclooctyne | C 0.7839421 .4383891 .486242 |
| $G($ Water $)=-311.7869537$ Hartree | H 0.1649101 .3860582 .393058 |
| ----------------------------------- | H 1.6179060 .7465271 .643629 |
| C 0.6811291 .3414240 .376746 | C -1.498781 2.267996-1.487823 |
| C -0.682343 1.341190-0.376880 | H-2.501859 1.884639-1.707269 |
| C 0.603634-1.453056 0.028187 | H-0.979814 $2.341288-2.453281$ |
| C-1.857400 0.5818250 .279100 | C 1.3032752 .8714191 .302399 |
| C -0.602258-1.452377-0.028549 | H 1.8825093 .1268922 .197792 |
| C -1.957951-0.910097-0.119865 | H 2.0062692 .8864110 .459522 |
| C 1.856814 0.582765-0.278986 | C 0.2219053 .9448711 .082667 |
| C 1.958707-0.909006 0.120049 | H-0.656672 3.7029861 .696915 |
| H-0.539204 $0.960586-1.396591$ | H 0.6166274 .8816401 .490519 |
| H-1.762279 0.6468401 .369540 | C -1.580923 3.647761-0.821139 |
| H-0.998167 $2.383992-0.489899$ | H-2.053818 $4.332813-1.534334$ |
| H-2.803074 1.070506 0.016803 | H -2.253212 3.5857560 .043366 |
| H-2.672084-1.434049 0.523759 | C -0.226857 4.230223-0.373362 |
| H -2.326611-0.999077-1.149093 | H -0.290642 5.317612-0.487132 |
| H $2.8020351 .072273-0.016545$ | H 0.556411 3.904187-1.071192 |
| H $1.7619370 .647589-1.369447$ | N-1.022683-1.267662-0.603129 |
| H $2.673865-1.432067-0.523167$ | N -0.971273-0.505365-1.697552 |
| H $2.326957-0.9976341 .149457$ | O 0.367432-0.554549-2.058400 |
| H 0.9965252 .3843550 .489920 | C 1.114289-0.896688-0.982651 |
| H 0.5381040 .9606781 .396452 | C 0.155743-1.204883 0.052952 |
| -------------------- | N 2.398540-0.858560-1.097003 |


| S 3.299026-1.462024 0.149250 | H $2.8932381 .799067-0.849368$ |
| :---: | :---: |
| O 2.584930-1.351645 1.429603 | H 5.070937 0.626362-0.693658 |
| O 3.848290-2.758721-0.216950 | H 5.151013-1.768365-0.039702 |
| H $0.357062-1.7322660 .971345$ | H 3.040084-2.982878 0.456690 |
| C 4.621283-0.2712170.163364 | C $0.511670-1.8544560 .359067$ |
| H 5.095915-0.273774-0.817828 | C -0.700455-1.811226 0.393815 |
| H 4.2060210 .7103740 .394894 | C -1.973158-1.207373 0.158655 |
| H 5.323574-0.588991 0.935888 | C -3.202887-1.651505 0.647399 |
| C -2.302502-1.452271-0.011922 | C -1.881186-0.014442-0.597546 |
| C -3.368441-1.812024-0.833132 | C -4.349338-0.905905 0.399387 |
| C -2.467427-1.262540 1.356846 | H-3.247866-2.569310 1.224231 |
| C -4.622807-1.991630-0.262957 | C -3.038413 $0.726491-0.809560$ |
| H-3.199333-1.949603-1.895621 | C -4.265882 0.284912-0.319439 |
| C -3.726379-1.462260 1.914858 | H -5.307407-1.247608 0.778171 |
| H-1.629869 -0.945238 1.968973 | H-2.967716 1.665686-1.350525 |
| C -4.802476-1.824238 1.109294 | H-5.159573 0.875285-0.496177 |
| H-5.460776-2.274221-0.891588 | C -0.559765 0.402912-1.220638 |
| H-3.866094-1.321116 2.981604 | N $0.5026040 .794068-0.268845$ |
| H -5.782692-1.974124 1.550091 | H -0.159080-0.422280-1.814735 |
| ------------------------------------ | H-0.741841 1.253657-1.883558 |
| DIBAC | C 0.2619421 .9628410 .427708 |
| $G($ Water $)=-785.236177$ Hartree | O -0.731419 2.6263760 .188166 |
| ------------------------------ | C 1.2395762 .3602561 .516372 |
| C 1.792874-1.272296 0.126711 | H 1.7001261 .4981142 .002256 |
| C 1.745302 0.101539-0.228318 | H 2.0356862 .9889621 .107396 |
| C $2.9341500 .759350-0.538324$ | H 0.6811262 .9510262 .242812 |
| C 4.154740 0.094635-0.457710 |  |
| C 4.199516-1.249526-0.094739 | TS5 |
| C 3.022046-1.932408 0.185954 | $G($ Water $)=-1921.308232$ Hartree |


|  | C 3.247798-1.735154 2.572751 |
| :---: | :---: |
| C -0.233042-1.504886 0.742980 | H 3.294629-2.705676 3.075221 |
| C 0.890835-2.332146 0.982926 | H 4.124505-1.158880 2.869810 |
| C 0.764332-3.460456 1.791961 | H 2.334393 -1.228548 2.890036 |
| C -0.449529-3.772702 2.393931 | N -0.896078 $2.147807-0.043811$ |
| C -1.558950-2.964122 2.163983 | N -0.339238 $2.059391-1.245237$ |
| C -1.450375-1.845004 1.346774 | O -1.307841 1.431784-2.010398 |
| H 1.631852-4.098688 1.932966 | C -2.202123 0.804044-1.208353 |
| H -0.528156-4.651223 3.026389 | C-1.775929 1.140215 0.134788 |
| H-2.514894-3.198511 2.621534 | N -3.123545 0.107654-1.773313 |
| H-2.325177-1.224751 1.179579 | S -4.385661-0.460117-0.869964 |
| C - $0.052750-0.353498-0.110431$ | O -4.165273-0.246021 0.567875 |
| C $0.7113990 .280998-0.878874$ | O -5.624989 0.019848-1.452831 |
| C 1.924460 0.354802-1.663413 | H-2.335249 1.001894 1.044945 |
| C 2.340718 1.509683-2.339804 | C -4.249152-2.206896-1.184455 |
| C 2.723728-0.807641-1.722677 | Н -5.091443-2.685427-0.681438 |
| C $3.5424171 .521691-3.037350$ | H -4.301871-2.364219-2.261722 |
| H 1.710542 2.390715-2.324968 | H -3.301072-2.565401-0.779127 |
| C 3.932631-0.769439-2.409036 | C -0.188582 2.8104080 .999886 |
| C 4.345841 0.385319-3.066361 | C 1.1818033 .0264220 .886665 |
| H 3.850573 2.422792-3.558339 | C -0.903294 3.2193232 .123279 |
| H 4.558445-1.656867-2.409979 | C 1.8425363 .6648541 .932015 |
| H $5.2914020 .397058-3.599261$ | H 1.7211182 .6909790 .007746 |
| C 2.250017-2.093799-1.090763 | C -0.224958 3.8468633 .160861 |
| N 2.135271-2.031901 0.375101 | H-1.976821 3.0663282 .173705 |
| H 1.258566-2.351061-1.473464 | C 1.146968 4.0715353 .067125 |
| H 2.944383-2.899133-1.349993 | H 2.9113593 .8354731 .856340 |
| C 3.322950-1.911978 1.068801 | H -0.774444 4.1691034 .039112 |
| O 4.385952-1.910829 0.474134 | H 1.6717614 .5664143 .877662 |

## General information

All chemical reagents were obtained from commercial sources and used without further purification. TLC analysis was performed with silica gel-coated plates with 0.2 mm silica gel-coated HSGF 254 plates. Compounds were purified by column chromatography on silica gel (200-300 mesh) or neutrality $\mathrm{Al}_{2} \mathrm{O}_{3}$ (200-300 mesh).
${ }^{1} \mathrm{H}$ NMR and ${ }^{13} \mathrm{C}$ NMR spectra were recorded on a Bruker Avance III HD 500 spectrometer. Chemical shifts for ${ }^{1} \mathrm{H}$ NMR spectra are reported as $\delta$ in units of parts per million (ppm) downfield from $\mathrm{SiMe}_{4}(\delta 0.0)$ and relative to the signal of chloroform- $d$ ( $\delta 7.26$, singlet) or dimethyl sulfoxide- $d_{6}$ (DMSO- $d_{6}$ ) ( $\delta 2.50$, quintet). Multiplicities were given as: s (singlet); d (doublet); t (triplet); q (quartet); dd (doublets of doublet) or $m$ (multiplets). The number of protons ( n ) for a given resonance is indicated by n H . Coupling constants are reported as a $J$ value in Hertz. Chemical shifts for ${ }^{13} \mathrm{C}$ NMR spectra are reported as $\delta$ in units of parts per million (ppm) downfield from $\mathrm{SiMe}_{4}(\delta$ 0.0 ) and relative to the signal of chloroform- $d$ ( $\delta 77.16$, triplet), or dimethyl sulfoxide$d_{6}$ (DMSO- $d_{6}$ ) ( $\delta 39.52$, heptet). High-resolution mass spectra (HRMS) analyses were performed on an Agilent quadrupole time flight high resolution mass spectrometer mass spectrometer ( 6540 Q-TOF LC/MS). Liquid chromatogram was detected by Shimadzu HPLC (LC-20AD, SPD-M20A detector). Analyses were performed using an ACE Excel 5 AQ column ( $4.6 \times 250 \mathrm{~mm}, 5 \mu \mathrm{~m}$ ). Samples were prepared by semi-preparative RP-HPLC (LC-20A, SIL-10AP, SPD-20A detector, Shimadzu) with ACE Excel 5 AQ column ( $10 \times 250 \mathrm{~mm}, 5 \mu \mathrm{~m}$ ). Fluorescence spectra were recorded at room temperature by LS55 (PE, America) spectrophotometer from molecular devices using a cuvette with 1 nm path length. Fluorescence microscopy images were taken on a: Zeiss LSM 710 laser scanning confocal microscope.

## Organic synthesis



Ethyl 4-((cyanomethyl)amino)benzoate (6). To a solution of ethyl 4-aminobenzoate (5) $(2.0 \mathrm{~g}, 12.1 \mathrm{mmol}, 1$ equiv.) in toluene ( 25 mL ) was added $\mathrm{KI}(200 \mathrm{mg}, 1.21 \mathrm{mmol}$, 0.1 equiv.), TEA ( $4.9 \mathrm{~g}, 48.4 \mathrm{mmol}, 4.0$ equiv.) and chloroacetonitrile ( $1.37 \mathrm{~g}, 18.2$ mmol, 1.5 equiv.) successively. The mixture was refluxed for 48 hours. After the starting material was consumed completely which was detected by TLC, the reaction mixture was concentrated under reduced pressure and partitioned between ethyl acetate and water. The organic layer was separated and washed with brine solution, dried over sodium sulfate, concentrated under reduced pressure and recrystallization to give the desired product $6(2.27 \mathrm{~g}, 92 \%)$.
${ }^{1} \mathbf{H}$ NMR ( $500 \mathrm{MHz}, \mathrm{CDCl}_{3}$ ) $\delta 7.96(\mathrm{~d}, J=8.8 \mathrm{~Hz}, 2 \mathrm{H}), 6.69(\mathrm{~d}, J=8.8 \mathrm{~Hz}, 2 \mathrm{H}), 4.33$ $(\mathrm{q}, J=7.1 \mathrm{~Hz}, 2 \mathrm{H}), 4.15(\mathrm{~s}, 2 \mathrm{H}), 1.37(\mathrm{t}, J=7.1 \mathrm{~Hz}, 3 \mathrm{H}) ;{ }^{13} \mathbf{C}$ NMR ( $125 \mathrm{MHz}, \mathrm{CDCl}_{3}$ ) $\delta 166.7,149.3,131.4,120.8,116.7,112.2,60.5,31.7,14.2 ;$ HRMS (ESI ${ }^{+}$): calcd. for $\mathrm{C}_{11} \mathrm{H}_{12} \mathrm{~N}_{2} \mathrm{NaO}_{2}{ }^{+}[\mathrm{M}+\mathrm{Na}]^{+}: 227.0791$, found: 227.0789.


5-amino-3-(4-(ethoxycarbonyl)phenyl)-1,2,3-oxadiazol-3-ium chloride (7). To a solution of ethyl 4-((cyanomethyl)amino)benzoate (6) ( $2.27 \mathrm{~g}, 11.1 \mathrm{mmol}, 1.0$ equiv.) in dry THF ( 30 mL ) was added isoamyl nitrite ( $1.95 \mathrm{~g}, 30 \mathrm{mmol}, 1.5$ equiv.). The mixture was stirred for 2 hours at room temperature. After the starting material was consumed completely which was detected by TLC, the reaction mixture was concentrated under reduced pressure. Then a solution of 4 M HCl in dioxane ( 5 mL ) was added to the mixture and the reaction was stirred 24 hours at room temperature. The precipitate was collected by filtration and washed with dry diethyl ether. The desired product 7 is obtained ( $808 \mathrm{mg}, 27 \%$ ).
${ }^{1} \mathbf{H}$ NMR ( 500 MHz, DMSO- $d_{6}$ ) $\delta 9.93(\mathrm{~s}, 2 \mathrm{H}), 8.75(\mathrm{~s}, 1 \mathrm{H}), 8.29(\mathrm{~d}, J=8.7 \mathrm{~Hz}, 2 \mathrm{H})$, $8.20(\mathrm{~d}, J=8.7 \mathrm{~Hz}, 2 \mathrm{H}), 4.39(\mathrm{q}, J=7.1 \mathrm{~Hz}, 2 \mathrm{H}), 1.36(\mathrm{t}, J=7.1 \mathrm{~Hz}, 3 \mathrm{H}) ;{ }^{13} \mathbf{C}$ NMR ( 125 MHz, DMSO- $d_{6}$ ) $\delta$ 169.6, 164.3, 135.8, 134.1, 131.0, 123.3, 102.8, 61.7, 14.1; HRMS ( $\mathrm{ESI}^{+}$): calcd. for $\mathrm{C}_{11} \mathrm{H}_{12} \mathrm{~N}_{3} \mathrm{O}_{3}{ }^{+}\left[\mathrm{M}-\mathrm{Cl}^{-}\right]^{+}: 234.0873$, found: 234.0877.

((3-chloropropyl)sulfonyl)(3-(4-(ethoxycarbonyl)phenyl)-1,2,3-oxadiazol-3-ium-
5-yl)amide (8). To a solution of 5-amino-3-(4-(ethoxycarbonyl)phenyl)-1,2,3-oxadiazol-3-ium chloride (7) ( $500 \mathrm{mg}, 1.85 \mathrm{mmol}, 1.0$ equiv.) in dry DCM ( 10 mL ) was added 3-Chloropropanesulfonyl chloride ( $393 \mathrm{mg}, 2.22 \mathrm{mmol}, 1.2$ equiv.) and cooled to $-30^{\circ} \mathrm{C}$. The mixture was stirred and added a solution of TEA ( $468 \mathrm{mg}, 4.63$ mmol, 2.5 equiv.) in dry DCM ( 5 mL ) slowly. Then kept the temperature for 5 hours. After the starting material was consumed completely which was detected by TLC, the reaction mixture was concentrated under reduced pressure. The crude product was purified by flash chromatography on neutrality $\mathrm{Al}_{2} \mathrm{O}_{3}(\mathrm{PE}: \mathrm{EtOAc}=1: 1)$ to give the desired product 8 ( $622 \mathrm{mg}, 90 \%$ ).
${ }^{1} \mathbf{H}$ NMR ( $500 \mathrm{MHz}, \mathrm{CDCl}_{3}$ ) $\delta 8.33(\mathrm{~d}, J=8.5 \mathrm{~Hz}, 2 \mathrm{H}), 7.90(\mathrm{~d}, J=8.6 \mathrm{~Hz}, 2 \mathrm{H}), 7.86$ $(\mathrm{s}, 1 \mathrm{H}), 4.45(\mathrm{q}, J=7.1 \mathrm{~Hz}, 2 \mathrm{H}), 3.72(\mathrm{t}, J=6.3 \mathrm{~Hz}, 2 \mathrm{H}), 3.33-3.26(\mathrm{~m}, 2 \mathrm{H}), 2.42-$ $2.31(\mathrm{~m}, 2 \mathrm{H}), 1.44(\mathrm{t}, J=7.1 \mathrm{~Hz}, 3 \mathrm{H}) ;{ }^{13} \mathbf{C}$ NMR ( $125 \mathrm{MHz}, \mathrm{CDCl}_{3}$ ) $\delta 170.5,164.4$, 136.4, 135.3, 132.0, 121.8, 100.8, 62.3, 51.7, 43.2, 27.3, 14.4; HRMS (ESI ${ }^{+}$): calcd. for $\mathrm{C}_{14} \mathrm{H}_{17} \mathrm{ClN}_{3} \mathrm{O}_{5} \mathrm{~S}^{+}[\mathrm{M}+\mathrm{H}]^{+}: 374.0572$, found: 374.0567.

(3-(4-(ethoxycarbonyl)phenyl)-1,2,3-oxadiazol-3-ium-5-yl)((3-(methyl(phenyl)
amino)propyl)sulfonyl)amide (9) To a mixture of ((3-chloropropyl)sulfonyl)(3-(4-(ethoxycarbonyl)phenyl)-1,2,3-oxadiazol-3-ium-5-yl)amide (8) (530 mg, 1.41 mmol , 1.0 equiv.) in toluene ( 20 mL ) was added successively $\mathrm{KI}(23.2 \mathrm{mg}, 0.14 \mathrm{mmol}, 0.1$ equiv.), TEA ( $712 \mathrm{mg}, 7.05 \mathrm{mmol}, 5.0$ equiv.) and $N$-methylaniline ( $302 \mathrm{mg}, 2.82 \mathrm{mmol}$,
2.0 equiv.). The mixture was stirred at $120^{\circ} \mathrm{C}$ for 24 hours. After the starting material was consumed completely which was detected by TLC, the reaction mixture was filtrated and concentrated under reduced pressure. The crude product was purified by flash chromatography on neutrality $\mathrm{Al}_{2} \mathrm{O}_{3}(\mathrm{MeOH}: \mathrm{DCM}=1: 100)$ to give the desired product 9 ( $250 \mathrm{mg}, 40 \%$ ).
${ }^{1} \mathbf{H} \operatorname{NMR}\left(500 \mathrm{MHz}, \mathrm{CDCl}_{3}\right) \delta 8.33(\mathrm{~d}, J=8.8 \mathrm{~Hz}, 2 \mathrm{H}), 7.86(\mathrm{~d}, J=8.8 \mathrm{~Hz}, 2 \mathrm{H}), 7.80$ (s, 1H), $7.21(\mathrm{dd}, J=8.8,7.3 \mathrm{~Hz}, 2 \mathrm{H}), 6.72(\mathrm{~d}, J=8.1 \mathrm{~Hz}, 2 \mathrm{H}), 6.69(\mathrm{t}, J=7.3 \mathrm{~Hz}, 1 \mathrm{H})$, 4.46 (q, $J=7.1 \mathrm{~Hz}, 2 \mathrm{H}), 3.49$ (t, $J=7.2 \mathrm{~Hz}, 2 \mathrm{H}$ ), $3.24-3.14$ (m, 2H), 2.94 ( $\mathrm{s}, 3 \mathrm{H}$ ), $2.23-2.13(\mathrm{~m}, 2 \mathrm{H}), 1.44(\mathrm{t}, J=7.1 \mathrm{~Hz}, 3 \mathrm{H}) ;{ }^{13} \mathbf{C}$ NMR ( $125 \mathrm{MHz}, \mathrm{CDCl}_{3}$ ) $\delta 170.4$, $164.4,149.2,136.4,135.2,131.9,129.4,121.8,116.7,112.5,100.7,62.3,52.1,51.2$, 38.4, 21.7, 14.4; HRMS (ESI $)$ : calcd. for $\mathrm{C}_{21} \mathrm{H}_{25} \mathrm{~N}_{4} \mathrm{O}_{5} \mathrm{~S}^{+}[\mathrm{M}+\mathrm{H}]^{+}$: 445.1540, found: 445.1541 .

(3-(4-((3-((tert-butoxycarbonyl)amino)propyl)carbamoyl)phenyl)-1,2,3-oxadiazol
-3-ium-5-yl)((3-(methyl(phenyl)amino)propyl)sulfonyl)amide (11). To a solution of (3-(4-(ethoxycarbonyl)phenyl)-1,2,3-oxadiazol-3-ium-5-yl)((3-(methyl(phenyl)amino) propyl)sulfonyl)amide (9) ( $200 \mathrm{mg}, 0.45 \mathrm{mmol}, 1.0$ equiv.) in THF ( 10 mL )was added a solution of $\mathrm{LiOH}(58.9 \mathrm{mg}, 2.25 \mathrm{mmol}, 5.0$ equiv. $)$ in water $(2 \mathrm{~mL})$ at room temperature and stirred for 3 hours. After the starting material was consumed completely which was detected by TLC, the reaction mixture was concentrated under reduced pressure and poured into water ( 5 mL ), adjusted the pH to $1-2$ with 1 M HCl aqueous solution, then extracted with dichloromethane. The organic layer was separated and washed with brine solution, dried over sodium sulfate and concentrated under reduced pressure to give desired product $\mathbf{1 0}$. The crude product $\mathbf{1 0}$ was used directly for the next step without any purification process.

A solution of $\mathbf{1 0}$ ( $100 \mathrm{mg}, 0.24 \mathrm{mmol}, 1$ equiv.) in DMF ( 5 mL ) was added HATU (137 $\mathrm{mg}, 0.36 \mathrm{mmol}, 1.5$ equiv.) and DIPEA ( $124 \mathrm{mg}, 0.96 \mathrm{mmol}, 4.0$ equiv.) and stirred for

15 minutes at room temperature. Then $N$-Boc-1,3-diaminopropane ( $84 \mathrm{mg}, 0.48 \mathrm{mmol}$, 2.0 equiv.) was added and stirred for 5 hours. After the starting material was consumed completely which was detected by TLC, water was added to quench the reaction and extracted with EtOAc. The organic layer was separated and washed with brine solution, dried over sodium sulfate and concentrated under reduced pressure. The crude product was purified by flash chromatography on neutrality $\mathrm{Al}_{2} \mathrm{O}_{3}(\mathrm{MeOH}: \mathrm{DCM}=1: 50)$ to give the desired product $11(120 \mathrm{mg}, 87 \%)$.
${ }^{1} \mathbf{H}$ NMR ( $500 \mathrm{MHz}, \mathrm{CDCl}_{3}$ ) $\delta 8.19(\mathrm{~d}, J=8.4 \mathrm{~Hz}, 2 \mathrm{H}), 8.02(\mathrm{~s}, 1 \mathrm{H}), 7.85(\mathrm{~d}, J=8.6$ $\mathrm{Hz}, 2 \mathrm{H}), 7.79(\mathrm{~s}, 1 \mathrm{H}), 7.21(\mathrm{dd}, J=8.5,7.4 \mathrm{~Hz}, 2 \mathrm{H}), 6.72(\mathrm{~d}, J=8.2 \mathrm{~Hz}, 2 \mathrm{H}), 6.68(\mathrm{t}$, $J=7.3 \mathrm{~Hz}, 1 \mathrm{H}), 4.87(\mathrm{~s}, 1 \mathrm{H}), 3.53(\mathrm{dd}, J=11.9,6.1 \mathrm{~Hz}, 2 \mathrm{H}), 3.49(\mathrm{t}, J=7.2 \mathrm{~Hz}, 2 \mathrm{H})$, $3.29(\mathrm{~d}, J=5.6 \mathrm{~Hz}, 2 \mathrm{H}), 3.22-3.14(\mathrm{~m}, 2 \mathrm{H}), 2.93(\mathrm{~s}, 3 \mathrm{H}), 2.22-2.12(\mathrm{~m}, 2 \mathrm{H}), 1.77-$ $1.71(\mathrm{~m}, 2 \mathrm{H}), 1.47(\mathrm{~s}, 9 \mathrm{H}) ;{ }^{13} \mathbf{C}$ NMR ( $125 \mathrm{MHz}, \mathrm{CDCl}_{3}$ ) $\delta 170.5,164.6,157.7,149.2$, $139.5,135.2,129.6,129.4,121.8,116.7,112.5,100.6,80.3,52.1,51.2,38.4,37.0,36.1$, 30.0, 28.5, 21.8; HRMS (ESI ${ }^{+}$): calcd. for $\mathrm{C}_{27} \mathrm{H}_{37} \mathrm{~N}_{6} \mathrm{O}_{6} \mathrm{~S}^{+}[\mathrm{M}+\mathrm{H}]^{+}$: 573.2490, found: 573.2491.

(3-(4-((3-ammoniopropyl)carbamoyl)phenyl)-1,2,3-oxadiazol-3-ium-5-yl)((3(methyl(phenyl)amino)propyl)sulfonyl)amide 2,2,2-trifluoroacetate (4) A solution of $\mathbf{1 1}$ in DCM was added $\mathrm{CF}_{3} \mathrm{COOH}$, the mixture was stirred for 3 hours at room temperature. After the starting material was consumed completely which was detected by TLC, the reaction mixture was concentrated under reduced pressure to give desired product 4. The crude product 4 was used directly for the next step without any purification process.

HRMS ( $\mathrm{ESI}^{+}$): calcd. for $\mathrm{C}_{22} \mathrm{H}_{29} \mathrm{~N}_{6} \mathrm{O}_{4} \mathrm{~S}^{+}\left[\mathrm{M}-\mathrm{CF}_{3} \mathrm{COO}^{-}\right]^{+}$: 473.1966 , found: 473.1981.

(3-(4-((3-aminopropyl)carbamoyl)phenyl)-1,2,3-oxadiazol-3-ium-5-yl)((3-((4-((E)-(2,5-dimethoxy-4-((E)-(4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methyl) amino)propyl)sulfonyl)amide (13) Fast black K salt hemi (zinc chloride) salt (dye content $\sim 30 \%) \mathbf{1 2}(24 \mathrm{mg}, 28.6 \mu \mathrm{~mol}, 1.2$ equiv.) was dissolved in a mixture of $130 \mu \mathrm{~L}$ NaOAc buffer ( $0.1 \mathrm{M}, \mathrm{pH} 4.0$ ) and $130 \mu \mathrm{~L} \mathrm{MeCN}$. The resulting mixture was stirred at r.t. for 10 min , and then sonicated to get near complete solubilization. In another roundbottom flask, the compound 4 ( $14 \mathrm{mg}, 23.9 \mu \mathrm{~mol}, 1.0$ equiv.) was dissolved in MeCN $(60 \mu \mathrm{~L})$ and cooled to $0^{\circ} \mathrm{C}$. The diazonium salt solution was directly filtrated and added to the solution of compound 4 and the resulting mixture was stirred at r.t. for 2 h . The fine precipitate newly formed was recovered by filtration and washed twice with a mixture of deionized water and $\operatorname{MeCN}(1: 1, \mathrm{v} / \mathrm{v})$. The resulting solid was then dried by lyophilization to afford the desired product $13(11.7 \mathrm{mg}, 52 \%)$.
${ }^{1} \mathbf{H}$ NMR ( 400 MHz, DMSO- $d_{6}$ ) $\delta 8.95(\mathrm{t}, J=5.5 \mathrm{~Hz}, 1 \mathrm{H}), 8.44(\mathrm{dd}, J=10.8,5.7 \mathrm{~Hz}$, 2H), 8.17 (q, $J=9.2 \mathrm{~Hz}, 4 \mathrm{H}), 8.07$ (d, $J=9.0 \mathrm{~Hz}, 1 \mathrm{H}), 7.85-7.73$ (m, 4H), 7.45 (s, $1 \mathrm{H}), 7.37(\mathrm{~s}, 1 \mathrm{H}), 6.91(\mathrm{~d}, J=9.1 \mathrm{~Hz}, 1 \mathrm{H}), 4.00(\mathrm{~s}, 3 \mathrm{H}), 3.95(\mathrm{~s}, 3 \mathrm{H}), 3.64(\mathrm{dd}, J=16.6$, $9.0 \mathrm{~Hz}, 4 \mathrm{H}), 3.18(\mathrm{dd}, J=14.8,6.8 \mathrm{~Hz}, 2 \mathrm{H}), 3.09(\mathrm{~s}, 3 \mathrm{H}), 2.88(\mathrm{~d}, J=11.1 \mathrm{~Hz}, 2 \mathrm{H})$, $2.08-1.96(\mathrm{~m}, 2 \mathrm{H}), 1.89-1.80(\mathrm{~m}, 2 \mathrm{H})$. HRMS (ESI $\left.{ }^{+}\right)$: calcd. for $\mathrm{C}_{36} \mathrm{H}_{40} \mathrm{~N}_{11} \mathrm{O}_{8} \mathrm{~S}^{+}$ $[\mathrm{M}+\mathrm{H}]^{+}: 786.2777$, found: 786.2774.

(3-(4-((3-(4-(3,6-bis(dimethylamino)xanthylium-9-yl)-3-carboxylatobenzamido) propyl)carbamoyl)phenyl)-1,2,3-oxadiazol-3-ium-5-yl)((3-((4-((E)-(2,5-dimethoxy -4-((E)-(4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methyl)amino)propyl)
sulfonyl)amide (1a) A solution of TAMRA ( $7.8 \mathrm{mg}, 17.4 \mu \mathrm{~mol}, 1.1$ equiv.) in DMF (4 mL ) was added DIPEA ( $20.7 \mathrm{mg}, 0.16 \mathrm{mmol}, 10.0$ equiv.) and TSTU ( $5.2 \mathrm{mg}, 17.4$ $\mu$ mol, 1.1 equiv.) in sequence and stirred at room temperature for an hour. After TAMRA was consumed completely which was detected by MS, 13 ( $12.4 \mathrm{mg}, 15.8$ $\mu \mathrm{mol}, 1.0$ equiv.) was added into the reaction mixture and stirred for another 5 hours in the dark. DMF was removed under reduced pressure and the dye-conjugate was purified by semi-preparative RP- HPLC with $80 \%$ to $100 \% \mathrm{MeOH}$ in water over 20 minutes at a flow rate of $5 \mathrm{~mL} / \mathrm{min}$. Visible detection was achieved at $\lambda=254 \mathrm{~nm}$. The productcontaining fractions were lyophilized to give $\mathbf{1 a}(6.6 \mathrm{mg}, 35 \%)$.

HRMS (ESI ${ }^{+}$): calcd. for $\mathrm{C}_{61} \mathrm{H}_{60} \mathrm{~N}_{13} \mathrm{O}_{12} \mathrm{~S}^{+}[\mathrm{M}+\mathrm{H}]^{+}: 1198.4200$, found: 1198.4196.


Figure S3. HRMS of 1a.


Figure S4. HPLC trace of purified 1a using a gradient of $70 \%$ to $100 \% \mathrm{MeOH}$ in water over $30 \mathrm{~min}(548 \mathrm{~nm})$.

(E)-(3-(4-((3-aminopropyl)carbamoyl)phenyl)-1,2,3-oxadiazol-3-ium-5-yl)((3-((4-((7-(diethylamino)-5-phenylphenazin-5-ium-3-yl)diazenyl)phenyl)(methyl)amino) propyl)sulfonyl)amide chloride (15) Methylene Violet 3RAX 14 ( $24.3 \mathrm{mg}, 0.064$ mmol, 1.0 equiv.) was dissolved in $\mathrm{H}_{2} \mathrm{O}(1 \mathrm{~mL})$, then added $200 \mu \mathrm{~L}$ concentrated hydrochloric acid and cooled by ice-salt bath. The solution was added a solution of $\mathrm{NaNO}_{2}$ ( $4.8 \mathrm{mg}, 0.07 \mathrm{mmol}, 1.1$ equiv.) in $\mathrm{H}_{2} \mathrm{O}(1 \mathrm{~mL})$, and stirred for 30 min until the reaction mixture turned dark blue. Solid $\mathrm{NaOAc}(280 \mathrm{mg})$ was added and adjusted the pH to 7-8 to preform $N$-nitrosamine/diazonium salt intermediate. A solution of 4 (37.5 $\mathrm{mg}, 0.064 \mathrm{mmol}, 1.0$ equiv.) in THF ( 5 mL ) was slowly added into the preformed $N$ nitrosamine/diazonium salt intermediate and stirred for a further 5 hours. The reaction mixture was concentrated under reduced pressure and the crude product was purified by semi-preparative RP-HPLC with $70 \% \mathrm{MeOH}$ in $0.1 \%$ TFA distilled water as eluents at a flow rate of $5 \mathrm{~mL} / \mathrm{min}$. Visible detection was achieved at $\lambda=254 \mathrm{~nm}$. The productcontaining fractions were lyophilized to give $\mathbf{1 5}(17 \mathrm{mg}, 50 \%)$.
${ }^{1} \mathbf{H}$ NMR ( $\left.500 \mathrm{MHz}, \mathrm{MeOD}\right) \delta 8.28(\mathrm{~d}, J=9.0 \mathrm{~Hz}, 1 \mathrm{H}), 8.22(\mathrm{~s}, 1 \mathrm{H}), 8.15-8.03(\mathrm{~m}$, $6 \mathrm{H}), 7.96-7.92(\mathrm{~m}, 3 \mathrm{H}), 7.87(\mathrm{dd}, J=9.9,2.6 \mathrm{~Hz}, 1 \mathrm{H}), 7.70-7.64(\mathrm{~m}, 4 \mathrm{H}), 7.24(\mathrm{~d}$, $J=1.9 \mathrm{~Hz}, 1 \mathrm{H}), 6.82(\mathrm{~d}, J=9.3 \mathrm{~Hz}, 2 \mathrm{H}), 5.83(\mathrm{~d}, J=2.5 \mathrm{~Hz}, 1 \mathrm{H}), 3.81(\mathrm{~s}, 2 \mathrm{H}), 3.73-$ $3.66(\mathrm{~m}, 2 \mathrm{H}), 3.50(\mathrm{t}, J=6.7 \mathrm{~Hz}, 2 \mathrm{H}), 3.42(\mathrm{~s}, 2 \mathrm{H}), 3.23(\mathrm{t}, J=7.2 \mathrm{~Hz}, 2 \mathrm{H}), 3.13(\mathrm{~s}$, $3 \mathrm{H}), 3.02(\mathrm{t}, J=7.4 \mathrm{~Hz}, 2 \mathrm{H}), 2.22-2.13$ (m, 2H), $2.02-1.94(\mathrm{~m}, 2 \mathrm{H}), 1.34(\mathrm{~s}, 3 \mathrm{H})$, $1.08(\mathrm{~s}, 3 \mathrm{H})$. HRMS $\left(\mathrm{ESI}^{+}\right)$: calcd. for $\mathrm{C}_{44} \mathrm{H}_{48} \mathrm{~N}_{11} \mathrm{O}_{4} \mathrm{~S}^{+}\left[\mathrm{M}-\mathrm{Cl}^{-}\right]^{+}: 826.3606$, found: 826.3624.

((3-((4-((E)-(7-(diethylamino)-5-phenylphenazin-5-ium-3-yl)diazenyl)phenyl) (methyl)amino)propyl)sulfonyl)(3-(4-((3-(6-(3,3-dimethyl-5-sulfonato-2-(( $1 E, 3 E)$ -5-((E)-1,3,3-trimethyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-3H-indol-1-ium-1-yl)hexanamido)propyl)carbamoyl)phenyl)-1,2,3-oxadiazol-3-ium-5-yl)
amide chloride (1b) A solution of Cy5 ( $5 \mathrm{mg}, 7.5 \mu \mathrm{~mol}, 1.0$ equiv.) in DMF ( 2 mL ) was added DIPEA ( $9.7 \mathrm{mg}, 75 \mu \mathrm{~mol}, 10.0$ equiv.) and TSTU ( $2.5 \mathrm{mg}, 8.3 \mu \mathrm{~mol}, 1.1$ equiv.) in sequence and stirred at room temperature for an hour. After Cy5 was consumed completely which was detected by MS, $\mathbf{1 5}$ ( $7.78 \mathrm{mg}, 8.3 \mu \mathrm{~mol}, 1.1$ equiv.) was added into the reaction mixture and stirred for another 5 hours in the dark. DMF was removed under reduced pressure and the dye-conjugate was purified by semipreparative RP- HPLC with $45 \% \mathrm{MeCN}$ in $0.1 \%$ TFA distilled water as eluents at a flow rate of $5 \mathrm{~mL} / \mathrm{min}$. Visible detection was achieved at $\lambda=254 \mathrm{~nm}$. The productcontaining fractions were lyophilized to give $\mathbf{1 b}(9.8 \mathrm{mg}, 84 \%)$.

HRMS (ESI ${ }^{+}$): calcd. for $\mathrm{C}_{76} \mathrm{H}_{84} \mathrm{~N}_{13} \mathrm{O}_{11} \mathrm{~S}_{3}{ }^{+}\left[\mathrm{M}-\mathrm{Cl}^{-}\right]^{+}: 1450.5570$, found: 1450.5561 .


Figure S5. HRMS of 1b.


Figure S6. HPLC trace of purified 1b in $70 \% \mathrm{CH}_{3} \mathrm{OH} / \mathrm{H}_{2} \mathrm{O}$ over $20 \mathrm{~min}(254 \mathrm{~nm})$.


2,5-dioxopyrrolidin-1-yl
5-(((2-methylcycloprop-2-en-1-yl)methyl)amino)-5oxopentanoate (1,3-Cp-NHS) (16) was prepared from cyclopropene alcohol according to the literature procedure ${ }^{[8]}$.


Tetrazine-BODIPY 504 (Tz-BDP) (17) was prepared in two steps from 4(aminomethyl)benzonitrile and formamidine acetate salt according to the literature procedure ${ }^{[9]}$.


2,5-dioxopyrrolidin-1-yl
4-(didehydrodibenzo[b,f]azocin-5(6H)-yl)-4oxobutanoate (DIBAC-NHS) (19) was prepared from DIBAC 2 according to the literature procedure ${ }^{[10]}$.

## Preparation of DIBAC/1,3-Cp-TG beads ${ }^{[11-13]}$



Scheme S1. Synthesis of DIBAC/1,3-Cp-TG beads.
TentaGel S NH $\mathrm{H}_{2}$ resin ( $29 \mathrm{mg}, 7.25 \mu \mathrm{~mol}, 130 \mu \mathrm{~m}$ beads) was swollen in double distilled water for 24 hours. Water was removed by centrifuged and the beads were rapidly washed with $\mathrm{DCM} / \mathrm{Et}_{2} \mathrm{O}(55: 45)(2 \times 2 \mathrm{~mL})$.

## DIBAC/1,3-Cp-TG beads

A solution of DIBAC-NHS 19 ( $2.8 \mathrm{mg}, 7.25 \mu \mathrm{~mol}$ ) in $2 \mathrm{~mL} \mathrm{DCM} / \mathrm{Et}_{2} \mathrm{O}$ (55:45) was
added to the beads, followed by DIPEA ( $2.5 \mu \mathrm{~L}, 15 \mu \mathrm{~mol}$ ) and rotated in a plastic tube for 30 min to modify the outer layer of the beads. The beads were then centrifuged and washed with $\mathrm{DCM} / \mathrm{Et}_{2} \mathrm{O}(55: 45)(1 \times 30 \mathrm{~s}), \mathrm{DCM}(3 \times 30 \mathrm{~s})$ and $\mathrm{DMF}(5 \times 30 \mathrm{~s})$. Then swollen in 2 mL DMF then added 1,3-Cp-NHS 16 ( $2.1 \mathrm{mg}, 7.25 \mu \mathrm{~mol}$ ) and DIPEA ( 2.5 $\mu \mathrm{L}, 15 \mu \mathrm{~mol}$ ) successively. The beads were rotated for 1.5 h then centrifuged and washed with DMF ( $5 \times 30 \mathrm{~s}$ ). The beads were dried under vacuum to give DIBAC/1,3-Cp-TG beads.

## Kinetics measurement

The kinetics of cycloadditions of sydnonimine $\mathbf{1 1}$ with DIBAC 2 were measured by HPLC in 1:1 DMSO/ $\mathrm{H}_{2} \mathrm{O}$ at $25^{\circ} \mathrm{C}$. Stock solutions of sydnonimine 11, DIBAC 2 and internal standard (2, 6-dichloropyridine) in DMSO were prepared. Prepared respective concentration solutions in $1: 1 \mathrm{DMSO} / \mathrm{H}_{2} \mathrm{O}$, leading to the final concentration of sydnonimine 11 was $50 \mu \mathrm{M}$, DIBAC 2 and internal standard was $500 \mu \mathrm{M}$. Reactions were monitored by the absorption peak area of sydnonimine $\mathbf{1 1}$ compared with the internal standard. Consumption of 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.


Figure S7. Kinetic measurement of cycloaddition of sydnonimine 11 with DIBAC 2, measured second-order rate constant in $\mathrm{DMSO} / \mathrm{H}_{2} \mathrm{O}$ is $0.71 \pm 0.007 \mathrm{M}^{-1} \cdot \mathrm{~s}^{-1}$. [A]-concentration of sydnonimine $\mathbf{1 1}(\mathrm{M})$

## Mutually orthogonal labeling of two fluorogenic probes

Sydnonimine $11(2 \mu \mathrm{~mol})$ and 1,3-Cp-NHS $16(2 \mu \mathrm{~mol})$ were dissolved in $500 \mu \mathrm{~L}$ DMSO- $d_{6} / \mathrm{D}_{2} \mathrm{O}(9: 1, \mathrm{~V} / \mathrm{V})$. The mixture was thoroughly mixed, incubated at room temperature and monitored by ${ }^{\mathbf{1}} \mathbf{H}$ NMR several time points. After 24 hours, the components signals stayed the same.


Figure S8. ${ }^{1}$ H NMR analysis of sydnonimine 11 and 1,3-Cp-NHS 16 in DMSO- $d_{6} / \mathrm{D}_{2} \mathrm{O}(9: 1, \mathrm{~V} / \mathrm{V})$.

## Fluorescence spectra



Figure S9. Fluorescence emission spectra of 1a. Measurement conditions: $20 \mu \mathrm{M}$ in $1 \% \mathrm{DMSO} / \mathrm{PBS}$, $25^{\circ} \mathrm{C}$ and $\lambda_{\mathrm{ex}}=559 \mathrm{~nm}$.


Figure S10. Fluorescence emission spectra of 1b. Measurement conditions: $20 \mu \mathrm{M}$ in $1 \%$ $\mathrm{DMSO} / \mathrm{PBS}, 25^{\circ} \mathrm{C}$ and $\lambda_{\mathrm{ex}}=622 \mathrm{~nm}$.


Figure S11. Fluorescence emission spectra of 3a. Measurement conditions: $20 \mu \mathrm{M}$ in $1 \% \mathrm{DMSO} / \mathrm{PBS}$, $25^{\circ} \mathrm{C}$ and $\lambda_{\mathrm{ex}}=559 \mathrm{~nm}$.


Figure S12. Fluorescence emission spectra of 3b. Measurement conditions: $20 \mu \mathrm{M}$ in $1 \%$ $\mathrm{DMSO} / \mathrm{PBS}, 25^{\circ} \mathrm{C}$ and $\lambda_{\mathrm{ex}}=622 \mathrm{~nm}$.

## Confocal fluorescent microscope imaging

A small portion of the beads ( 2 mg ) was added in $25 \mu \mathrm{LCH}_{3} \mathrm{CN} / \mathrm{H}_{2} \mathrm{O}$ (1:1) in a plastic tube, followed by $10 \mu \mathrm{~L} \mathbf{1 b}(1 \mathrm{mM})$ and $10 \mu \mathrm{~L} \mathbf{1 7}(1 \mathrm{mM})$ in DMSO. After 20 min , the beads were imaged under a Leica confocal microscope with corresponding filters without washing.

## References

[1] M.J. Frisch, G.W. Trucks, H.B. Schlegel, et al., Gaussian 09, revision D.01, ed., Gaussian Inc., Wallingford, CT, 2013.
[2] Y. Zhao, D.G. Truhlar, Theor. Chem. Acc. 120 (2008) 215-241.
[3] Y. Zhao, D.G. Truhlar, Acc. Chem. Res. 41 (2008) 157-167.
[4] V. Barone, M. Cossi, J. Phys. Chem. A 102 (1998) 1995-2001.
[5] M. Cossi, N. Rega, G. Scalmani, V. Barone, J. Comput. Chem. 24 (2003) 669-681.
[6] Y. Takano, K.N. Houk, J. Chem. Theory Comput. 1 (2005) 70-77.
[7] F. Liu, Y. Liang, K.N. Houk, Acc. Chem. Res. 50 (2017) 2297-2308.
[8] J. Yang, Y. Liang, J. Seckute, K.N. Houk, N.K. Devaraj, Chem. Eur. J. 20 (2014) 3365-3375.
[9] N.K. Devaraj, S. Hilderbrand, R. Upadhyay, R. Mazitschek, R. Weissleder, Angew. Chem., Int. Ed. 49 (2010) 2869-2872.
[10] L.S. Campbell-Verduyn, L. Mirfeizi, A.K. Schoonen, et al., Angew. Chem., Int. Ed. 50 (2011) 11117-11120.
[11] S.J. Siegl, J. Galeta, R. Dzijak, M. Dracinsky, M. Vrabel, Chempluschem 84 (2019) 493-497.
[12] F. Bédard, A. Girard, É. Biron, Int. J. Pept. Res. Ther. 19 (2012) 13-23.
[13] R. Liu, J. Marik, K.S. Lam, J. Am. Chem. Soc. 124 (2002) 7678-7680.

## NMR spectra





$\mathrm{EtO}_{2} \mathrm{C}$








| 190 | 180 | 170 | 160 | 150 | 140 | 130 | 120 | 110 | 100 | 90 | 80 | 70 | 60 | 50 | 40 | 30 | 20 | 10 | 0 |
| :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- |






|  | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| 30 | 180 | 170 | 160 | 150 | 140 | 130 | 120 | 110 | 100 | $\begin{gathered} 90 \\ \mathrm{fl}(\mathrm{ppm}) \end{gathered}$ | 80 | 70 | 60 | 50 | 40 | 30 | 20 | 10 | 0 | -10 |



