SUPPLEMENTARY INFORMATION

Strain-promoted sydnone bicyclo-[*6.1.0*]-nonyne cycloaddition

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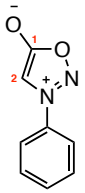
Synthetic Chemistry

General Methods:

NMR spectra were recorded on a Bruker Ultrashield^{TM} 400 Plus spectrometer (^{1}H: 400 MHz, ^{13}C: 101 MHz, ^{31}P: 162 MHz). Chemical shifts (δ) are reported in ppm and are referenced to the residual non-deuterated solvent peak: $CDCl_3$ (7.26 ppm), d_6-DMSO (2.50 ppm), MeOD (3.31 ppm) for ^{1}H-NMR spectra, $CDCl_3$ (77.0 ppm), d_6-DMSO (39.5 ppm), MeOD (49.0 ppm) for ^{13}C-NMR spectra. ^{13}C-NMR resonances are proton decoupled. Coupling constants (J) are measured to the nearest 0.1 Hz and are presented as observed. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; quin, quintet; sext, sextet; m, multiplet. Analytical thin-layer chromatography (TLC) was carried out on silica 60F-254 plates. The spots were visualized by UV light (254 nm) and by potassium permanganate or vanillin staining. Flash column chromatography was carried out on silica gel 60 (230-400 mesh or 70-230 mesh). ESI-MS was carried out using an Agilent 1200 LC-MS system with a 6130 Quadrupole spectrometer. The solvent system consisted of 0.2 % formic acid in H_2O as buffer A, and 0.2 % formic acid in acetonitrile (MeCN) as buffer B. Small molecule LC-MS was carried out using a Phenomenex Jupiter C18 column (150 x 2 mm, 5 μm). Variable wavelengths were used and MS acquisitions were carried out in positive and negative ion modes. Preparative HPLC purification was carried out using a Varian PrepStar/ProStar HPLC system, with automated fraction collection from a Phenomenex C18 column (250 x 30 mm, 5 μm). Compounds were identified by UV absorbance at 191 nm. All solvents and chemical reagents were purchased from commercial suppliers and used without further purification. Non-aqueous reactions were carried out in oven-dried glassware under an inert atmosphere of argon unless stated otherwise. All water used experimentally was distilled. Brine refers to a saturated solution of sodium chloride in water.

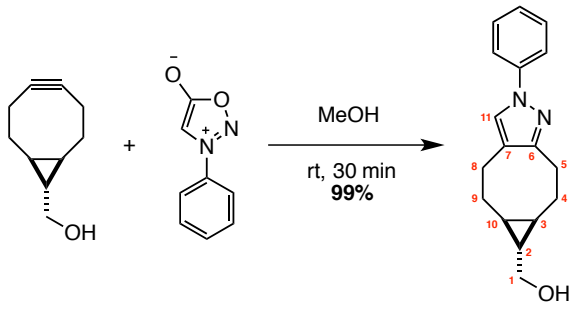
3-phenyl-1,2,3-oxadiazol-3-ium-5-olate (1)



Phenyl sydnone 1 was prepared in 2 steps from *N*-phenyl glycine according to a literature procedure.[15] δ_H(400 MHZ, d_6-DMSO) 7.96-7.93 (2H, m, Ar*H*), 7.79 (1H, s, *H*2), 7.77-7.69 (3H, m, Ar*H*); δ_C (101 MHz, d_6-DMSO) 168.5 (*C*1), 134.5 (Ar, 4˚ carbon), 132.4 (Ar), 130.2 (Ar), 121.6 (Ar), 94.9 (*C*2); υ_{max} (film)/cm^{-1} 3126, 1756, 1470, 1437, 1359, 1291, 1226, 1174, 1086, 1013, 946, 853, 757, 724; LRMS (ESI^+): m/z 163 (100% $[M+H]^+$).

***Exo*-((1*R*,8*S*)-bicyclo[6.1.0]non-4-yn-9-yl)methanol (2) was synthesized in 4 steps from 1,5-cyclooctadiene as reported previously.[4e]**

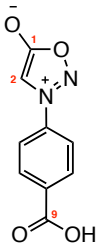
((5a*S*,6a*R*)-2-phenyl-2,4,5,5a,6,6a,7,8-octahydrocyclopropa[5,6]cycloocta[1,2-*c*]pyrazol-6-yl)methanol (3)



Phenyl sydnone **1** (25.5 mg, 0.16 mmol) was added to a stirring solution of *exo*-BCN-OH **2** (23.6 mg, 0.16 mmol) in methanol (1 mL) at room temperature. The mixture was stirred at room temperature for 30 min and concentrated under reduced pressure. The crude reaction mixture was purified by silica gel chromatography (10-50% EtOAc in hexane) to yield pyrazole **3** as a colorless oil (41.6 mg, 99%). δ_H(400 MHZ, CDCl3) 7.61-7.58 (3H, m, *H*14, *H*11), 7.41-7.37 (2H, m, *H*13), 7.21-7.17 (1H, m, *H*15), 3.54-3.44 (2H, m, *H*1), 3.07 (1H, ddd, J 15.0, 8.4, 2.4, $H5_A$), 2.80 (1H, ddd, J 15.0, 8.4, 2.5, $H8_A$), 2.70 (1H, ddd, J 15.0, 9.1, 2.5, $H5_B$), 2.54-2.40 (3H, m, $H4_A$, $H8_B$, $H9_A$), 1.34-1.31 (1H, br t, O*H*), 1.30-1.14 (2H, m, $H4_B$, $H9_B$), 0.99-0.88 (2H, m, *H*3, *H*10), 0.76-0.70 (1H, m, *H*2); δ_C (101 MHz, $CDCl_3$) 154.5 (*C*6), 140.0 (Ar, 4˚ carbon), 129.3 (Ar), 126.3 (Ar), 125.4 (*C*11), 122.4 (*C*7), 118.2 (Ar), 66.8 (*C*1), 29.8 (*C*9), 28.6 (*C*2), 28.4 (*C*4), 27.3 (*C*5), 23.62 (*C*10), 23.55 (*C*3), 23.5 (*C*8); υ_{max}

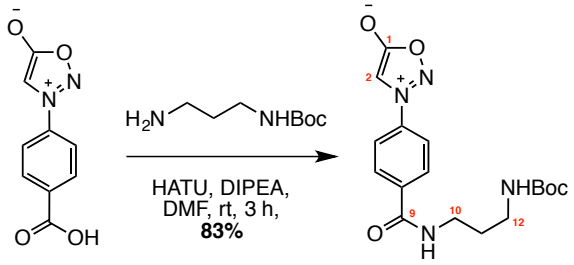
(film)/cm^{-1} 3347, 2929, 2856, 1599, 1503, 1409, 1381, 1027, 755; LRMS (ESI$^+$): m/z 269 (100% [M+H]$^+$).

3-(4-carboxyphenyl)-1,2,3-oxadiazol-3-ium-5-olate (7)



4-carboxyphenyl sydnone 7 was prepared in 3 steps from 4-aminobenzoic acid according to a literature procedure.[15] δ_H(400 MHZ, d_6-DMSO) 13.53 (1H, br s, COO*H*), 8.22 (2H, d, J 7.9, Ar*H*), 8.08 (2H, d, J 7.9, Ar*H*), 7.89 (1H, s, *H*2); δ_C (101 MHz, d_6-DMSO) 168.4 (*C*1), 165.9 (*C*9), 137.3 (Ar, 4˚ carbon), 134.2 (Ar, 4˚ carbon), 131.0 (Ar), 121.8 (Ar), 95.2 (*C*2); υ_{max} (film)/cm^{-1} 3002, 1729, 1697, 1605, 1454, 1233, 1187, 1114, 1082, 1014, 954, 881, 788, 768, 727; LRMS (ESI$^+$): m/z 207 (100% [M+H]$^+$).

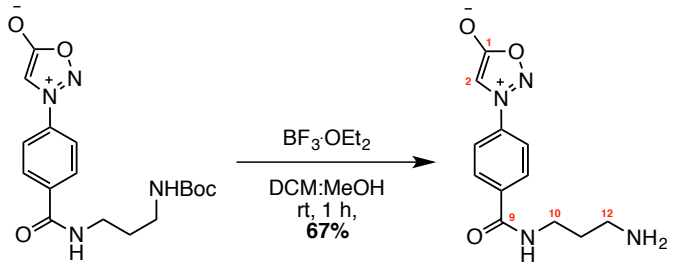
3-(4-((3-((*tert*-butoxycarbonyl)amino)propyl)carbamoyl)phenyl)-1,2,3-oxadiazol-3-ium-5-olate (8)



HATU (59 mg, 0.15 mmol) was added to a stirring solution of carboxylic acid **7** (32 mg, 0.15 mmol) and DIPEA (30 µL, 0.17 mmol) in DMF (2 mL) at 0 ˚C. The resulting mixture was stirred for 5 min at 0 ˚C and then *N*-Boc-1,3-propanediamine (30 µL, 0.17 mmol) was added dropwise. The resulting mixture was warmed to room temperature, stirred for 3 h, diluted with EtOAc (50 mL) and citric acid (20% aqueous, 50 mL) was added. The aqueous phase was extracted with EtOAc (3 X 20 mL), the combined organics were washed with water (50 mL), brine (50 mL), dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude reaction mixture was purified by silica gel chromatography (100% EtOAc) to yield sydnone propanamide **8** as a pale yellow oil (47 mg, 84%). δ_H(400 MHZ, d_6-DMSO) 8.71 (1H, t, J 5.3, N*H* amide), 8.11 (2H, d, J 8.8, Ar*H*), 8.06 (2H, d, J 8.8, Ar*H*), 7.87 (1H, s, *H*2), 6.84 (1H, t, J 5.3, N*H* carbamate), 3.29 (2H, td, J 6.4, 6.6, *H*10), 2.99 (2H, td, J

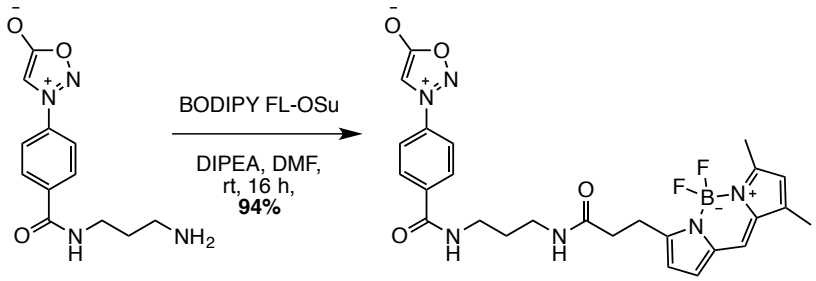
6.4, 6.4, *H*2), 1.66 (2H, tt, J 6.8, 6.8, *H*11), 1.38 (9H, s, ^{t}Bu); δ_C (101 MHz, d_6-DMSO) 168.4 (*C*1), 164.6 (*C*9), 155.6 (*C*13), 137.8 (Ar, 4° carbon), 136.1 (Ar, 4° carbon), 128.9 (Ar), 121.5 (Ar), 95.1 (*C*2), 77.5 (^{t}Bu, 4° carbon), 37.7 (*C*12), 37.2 (*C*10), 29.4 (*C*11), 28.2 (^{t}Bu); υ_{max} (film)/cm^{-1} 3332, 2977, 1746, 1689, 1652, 1538, 1508, 1451, 1365, 1275, 1251, 1168, 1009, 949, 858, 728; LRMS (ESI$^+$): m/z 307 (100% [(M–Boc)+formate]$^+$; m/z 747 (70% [2M+Na]$^+$).

3-(4-((3-aminopropyl)carbamoyl)phenyl)-1,2,3-oxadiazol-3-ium-5-olate (9)



Boron trifluoride diethyl etherate (225 µL, 1.82 mmol) was added drop-wise to a stirring solution of *tert*-butyl carbamate **8** (33 mg, 91 µmol) in DCM:MeOH (9:1, 3 mL) at room temperature. The mixture was stirred at room temperature for 1 h and concentrated under reduced pressure. The crude reaction mixture was purified by silica gel chromatography (90:10:1 $CHCl_3$:MeOH:NH_4OH) to yield primary amine **9** as a pale yellow oil (16 mg, 67%). δ_H(400 MHZ, MeOD) 8.12-8.09 (2H, m, Ar*H*), 8.03-7.99 (2H, m, Ar*H*), 7.49 (1H, s, *H*2), 3.50 (2H, t, J6.7, *H*10), 2.87 (1H, t, J 7.2, *H*12), 1.89 (2H, tt, J 6.9, 6.7, *H*11); δ_C (101 MHz, d_6-DMSO) 171.5 (*C*1), 168.4 (*C*9), 139.3 (Ar, 4° carbon), 138.3 (Ar, 4° carbon), 96.2 (*C*2), 39.1 (*C*12), 38.2 (*C*10), 31.1 (*C*11); υ_{max} (film)/cm^{-1} 3358, 2926, 2360, 1743, 1644, 1607, 1590, 1507, 1449, 1318, 1010, 859, 727; LRMS (ESI$^+$): m/z 263 100% [M+H]$^+$.

3-(4-((3-(3-(5,5-difluoro-7,9-dimethyl-5*H*-5λ^4,6λ^4-dipyrrolo[1,2-*c*:2',1'-*f*][1,3,2]diazaborinin-3-yl)propanamido)propyl)carbamoyl)phenyl)-1,2,3-oxadiazol-3-ium-5-olate (6)



BODIPY-FL-OSu (5mg, 10 µmol) was added to a stirring solution of primary amine **9** (4 mg, 15 µmol) and DIPEA (3.5 µL, 20 µmol) in DMF (1 mL) at 0 °C. The mixture was warmed to

room temperature, stirred for 16 h and concentrated under reduced pressure. Residual DMF was removed by azeotropic evaporation with toluene. The crude reaction mixture was dissolved in 25 mL of 70:30:0.7 water:acetonitrile:formic acid and purified by reversed-phase HPLC. Appropriate fractions were concentrated under reduced pressure and freeze-dried to afford BODIPY-derivative **6** as a red fluorescent solid (5.0 mg, 96%). LRMS (ESI^+): m/z 535 100% $[M–H]^–$, expected 535.

***N*^{6}-((((1*R*,8*S*)-bicyclo[6.1.0]non-4-yn-9-yl)methoxy)carbonyl)-*L*-lysine (BCNK, 5) was synthesized as reported previously[10e] and was purified by reverse-phase chromatography before use.** δ_H (400 MHz, d_6-DMSO/D_2O (1:1)) 4.14-3.76 (m, 3H), 3.56-3.29 (m, 2H), 3.18-2.81 (m, 3H), 2.31-1.98 (m, 5H), 1.71-1.52 (m, 4H), 1.51-1.29 (m, 4H), 1.29-1.08 (m, 3H), 0.95-0.66 (m, 2H); δ_C (101 MHz, d_6-DMSO/D_2O (1:1)) 169.4, 165.9, 101.3, 76.0, 55.8, 31.8, 30.1, 29.9, 25.2, 23.2, 22.1, 21.0, 18.7; LRMS (ESI^+): m/z 323 100% $[M+H]^+$, expected 323.

Small Molecule Kinetics

Rate constant k for the reaction of phenyl sydnone with BCN was measured under *pseudo*-first order reaction conditions by using a 10- to 80-fold excess of *exo*-((1*R*,8*S*)-bicyclo[6.1.0]non-4-yn-9-yl)methanol (BCN) in methanol/water mixtures by following the exponential decay in UV absorbance of phenyl sydnone at 310 nm over time. Stock solutions were prepared of phenyl sydnone (0.1 mM in 9/1 water/methanol) and BCN (1, 3, 5 and 8 mM in methanol). Mixing equal volumes of the stock solutions resulted in final concentrations of 0.05 mM phenyl sydnone and 0.5, 1.5, 2.5 and 4 mM BCN, corresponding to 10 to 80 equivalents. Spectra were recorded using the following instrument parameters: wavelength = 310 nm; spectral-band-width (SBW) = 1.0 nm; path length = 1.0 cm; increment of data point collection = 2.0 s. All data was recorded at 21 ˚C. Each data set was fit to a single-exponential equation. Measurements were performed in triplicate with new solutions of each reactant each time. The mean of the observed reaction rate constants **k'** were plotted against the concentration of BCN to afford a linear plot with gradient **k**. All data processing was performed using the Kaleidagraph software program.

Protein Expression and Purification

Chemically competent *E.coli* DH10B cells were transformed with *p*BKBCNRS (which encodes *Mb*PylRS with three amino acid substitutions Y271M, L274G and C313A in the enzyme active site) and *psf*GFP_{150TAG}*Pyl*T-His_6 (which encodes *Mb*$tRNA_{CUA}$ and a *C*-terminally hexahistidine-tagged *sf*GFP gene with an AMBER codon at position 150). Cells were recovered in 1mL of S.O.B medium (supplemented with 0.2% glucose) for 1 h at 37 ˚C,

before incubation (16 h, 37 ˚C, 220 rpm) in 50 mL of LB containing ampicillin (100 µg/mL) and tetracycline (25 µg/mL). 5mL of this overnight culture was used to inoculate 100 mL of LB supplemented with ampicillin (50 µg/mL) and tetracycline (12 µg/mL) and incubated at 37 ˚C (220 rpm). At OD_{600} = 0.4-0.5 a solution of **5** in H_2O (+10% 0.1 M $NaOH_{(aq)}$) was added to a final concentration of 2 mM. After 30 min of incubation at 37 ˚C (220 rpm), protein expression was induced by the addition of arabinose to a final concentration of 0.2%. After 4 hours of induction cells were harvested by centrifugation and frozen at –80 ˚C until required. Cells were thawed on ice and lysed by suspension in 20 mL BugBuster® supplemented with 10 mM Tris-HCl, 20 mM imidazole, 100 µg/mL DNaseA 1 x Roche protease tablet and rotated at room temperature for 20 min. The soluble extract was clarified by centrifugation (25 min, 21000 g, 4 ˚C). 300 µL of pre-washed Ni^{2+}-NTA beads (Qiagen) were added to the extract and the mixture was incubated with agitation for 1 h at 4 ˚C. Beads were collected by centrifugation (10 min, 1000 g). The beads were resuspended in wash buffer (3 X 30 mL, 20 mM Tris-HCl, 30 mM imidazole, 300 mM NaCl, pH 8.0) and spun down at 100 g for 10 min. The beads were suspended in 10 mL of wash buffer and transferred to a column. The protein was eluted with 3 mL of wash buffer supplemented with 200 mM imidazole. Purified protein was analyzed by 4-12% SDS-PAGE and its mass confirmed by mass spectrometry. *sf*GFP with incorporated Boc-lysine (**4**) was prepared in a similar fashion, except that *E.coli* DH10B cells were transformed with *p*BK*Pyl*RS (which encodes wild-type pyrrolysine *t*RNA-synthetase) and *psf*GFP_{150TAG}*Pyl*T-His_6 (which encodes *Mb*$tRNA_{CUA}$ and a *C*-terminally hexahistidine-tagged *sf*GFP gene with an AMBER codon at position 150). Western blots were performed with an antibody against the hexahistidine tag (Cell Signaling Technology, His Tag 27E8 mouse mAb (HRP conjugate) #9991).

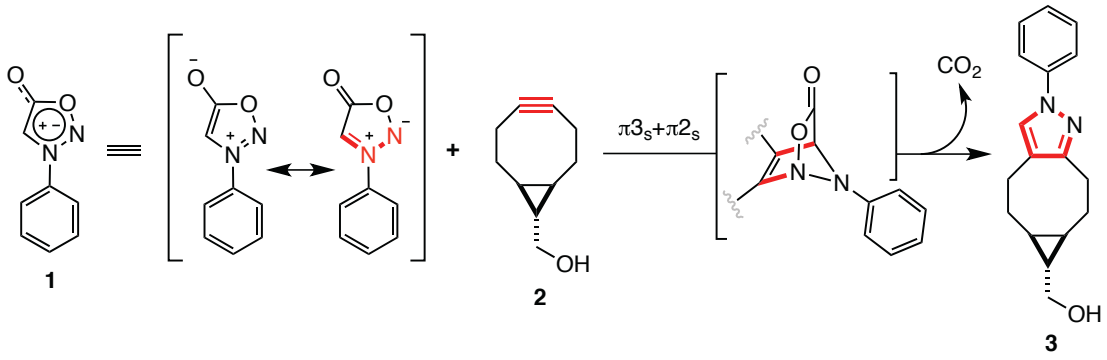
Protein Mass Spectrometry

Protein LC-MS (ESI) was carried out using a Phenomenex Jupiter C4 column (150 x 2 mm, 5 µm) and samples were analyzed in the positive mode, following protein UV absorbance at 214 and 280 nm. Total protein masses were calculated by deconvolution within the MS ChemStation software (Agilent Technologies).

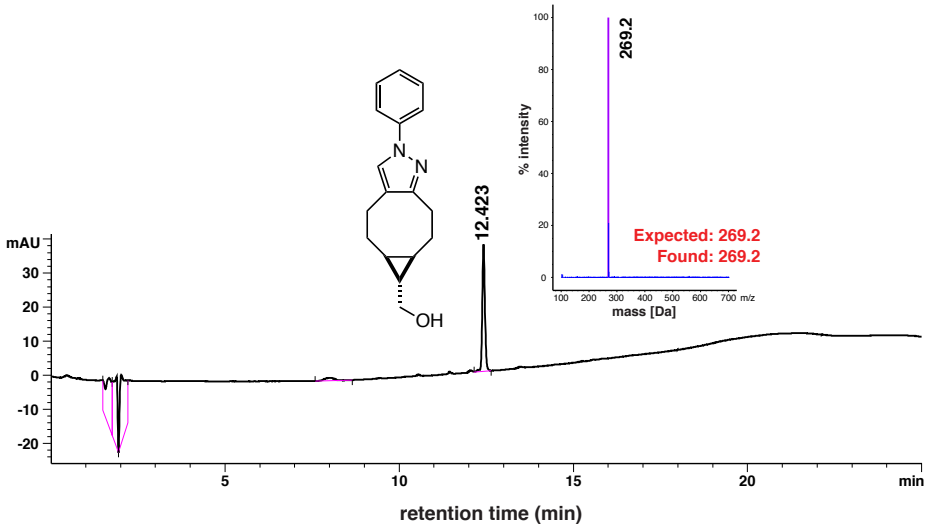
***E.coli* Lysate Labeling with Sydnone-BODIPY-FL**

E.coli DH10B cells containing either *psf*GFP_{150TAG}*Pyl*T-His_6 and *p*BKBCNRS or *psf*GFP_{150TAG}*Pyl*T-His_6 and *p*BK*Pyl*RS were inoculated into LB containing ampicillin (for *p*BKBCNRS, 100 µg/mL) or kanamycin (for *p*BK*Pyl*RS, 50 µg/mL) and tetracycline (25 µg/mL). The cells were incubated for 16 h at 37 ˚C (220 rpm). 5 mL of overnight culture was use to inoculate into 100 mL of LB supplemented with ampicillin (50 µg/mL) and tetracycline (12 µg/mL) and incubated at 37 ˚C (220 rpm). At OD_{600} = 0.4-0.5, 3 mL culture

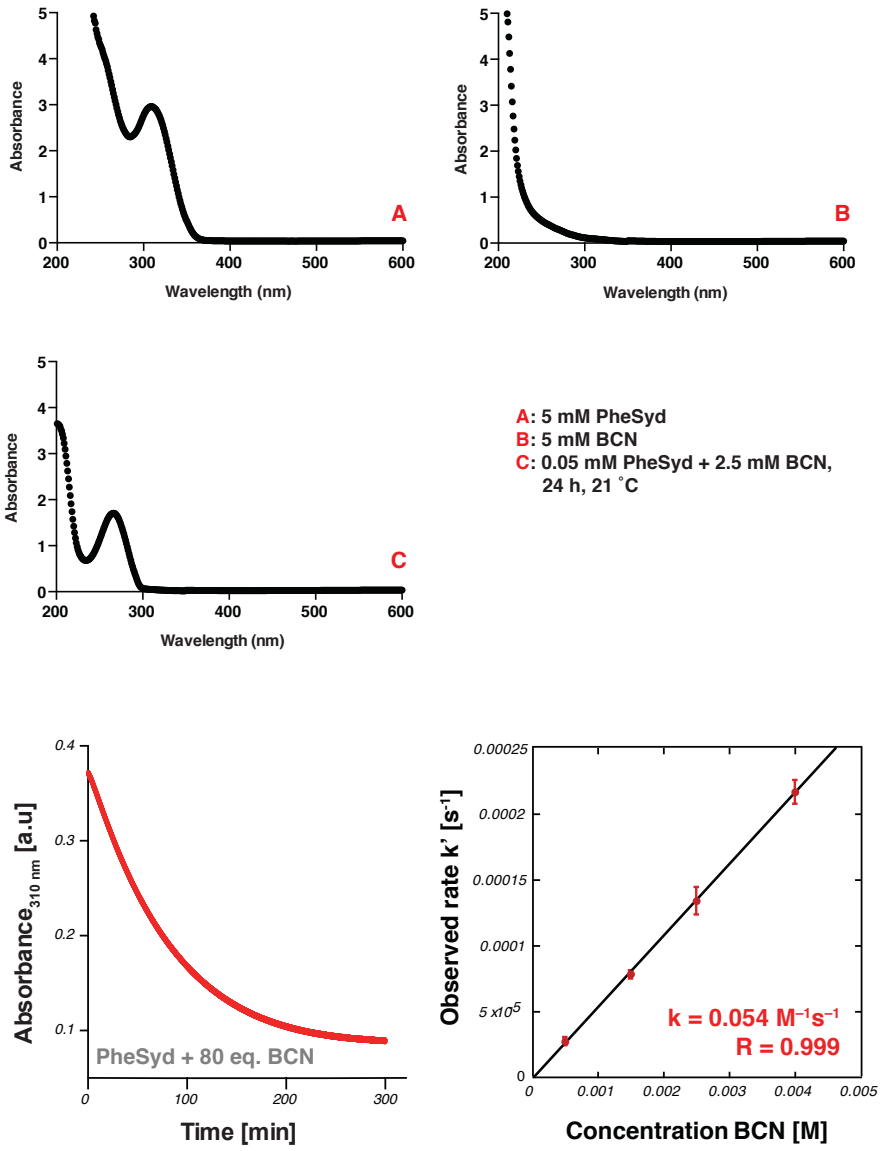
aliquots were removed and supplemented with a solution of either BCNK (**5**) in H_2O (+10% 0.1 M $NaOH_{(aq)}$) or BocK (**4**) in H_2O (+10% 0.1 M $NaOH_{(aq)}$) to a final concentration of 2 mM. After 30 min of incubation at 37 ˚C (220 rpm), protein expression was induced by the addition of arabinose to a final concentration of 0.2%. After 2 h of expression, 1 mL cell suspensions were harvested by centrifugation (16000 g, 10 min) and frozen at –80 ˚C until required. Cell pellets were thawed on ice, suspended in in 500 µL of PBS buffer, centrifuged (16000 g, 10 min) and the supernatant discarded. This process was repeated two times. Finally, the washed cell pellet was suspended in 100 µL of PBS buffer and incubated with 1 µL of PheSyd-BODIPY-FL (**6**, 5 mM in DMSO) at 37 ˚C for 6 h. The cell suspension was centrifuged (16000 g, 10 min) and the supernatant discarded. The cell pellet was suspended in 100 µL of 1X NuPAGE LDS sample buffer supplemented with 5% β-mercaptoethanol and BCN (**2**, final concentration of 1 mM), incubated at 37 ˚C for 2 h, heated at 93 ˚C for 10 min and centrifuged at 16000 g for 10 min. The crude cell lysates were analyzed by 4-12% SDS-PAGE to assess protein levels. Gels were either Coomassie stained or scanned with a Typhoon imager in order to visualize fluorescent bands.



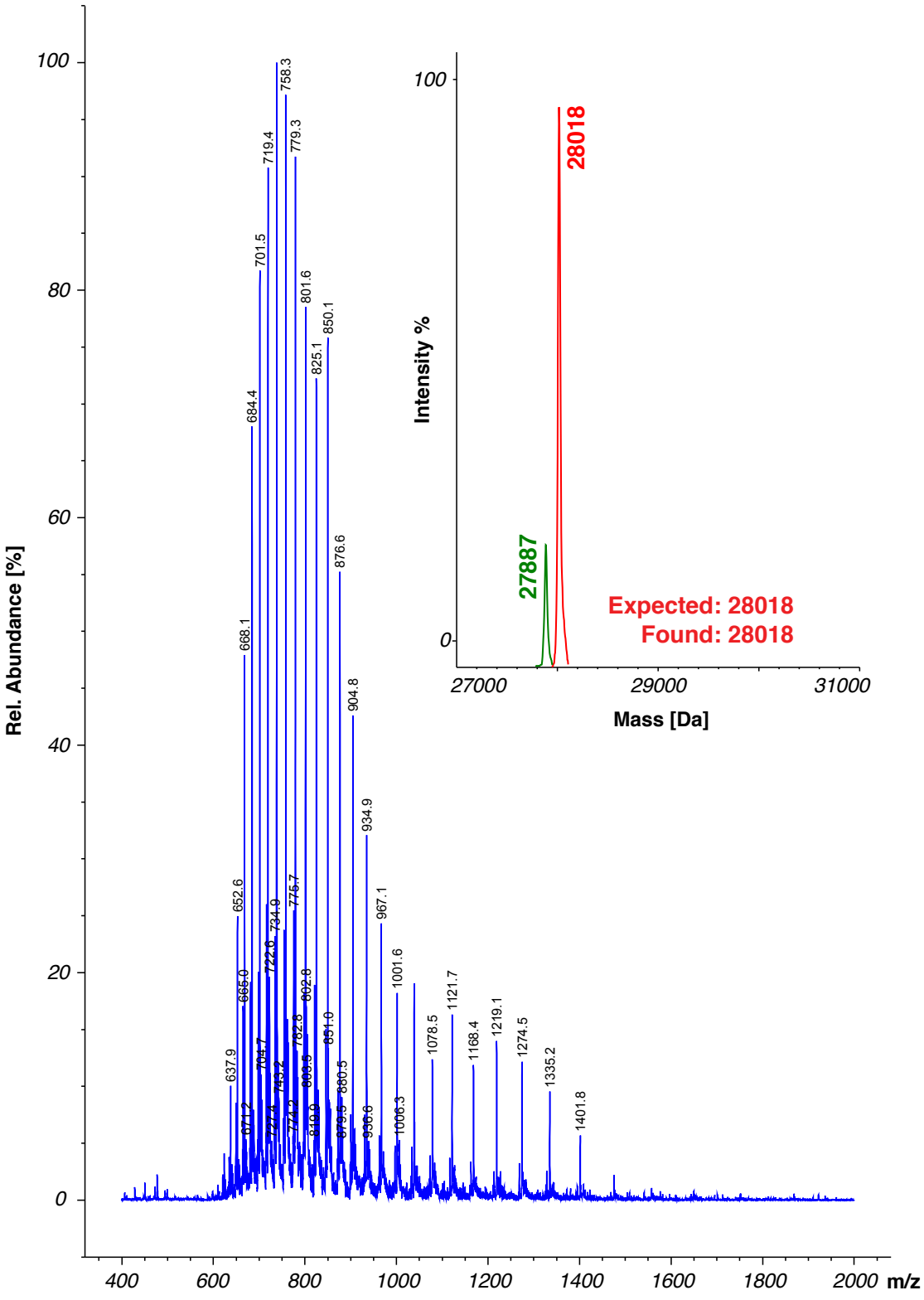
Supplementary Figure S1. The proposed mechanism for the 1,3-dipolar cycloaddition of *N*-phenyl sydnone (**1**) with BCN (**2**). We propose that the reaction proceeds *via* initial suprafacial [3+2] cycloaddition of a sydnone tautomer to afford an initial diaza-[*2.2.1*]-bicyclic lactone, which undergoes cycloreversion with the extrusion of carbon dioxide to afford a cyclooctane-fused *N*-phenyl pyrazole **3**.



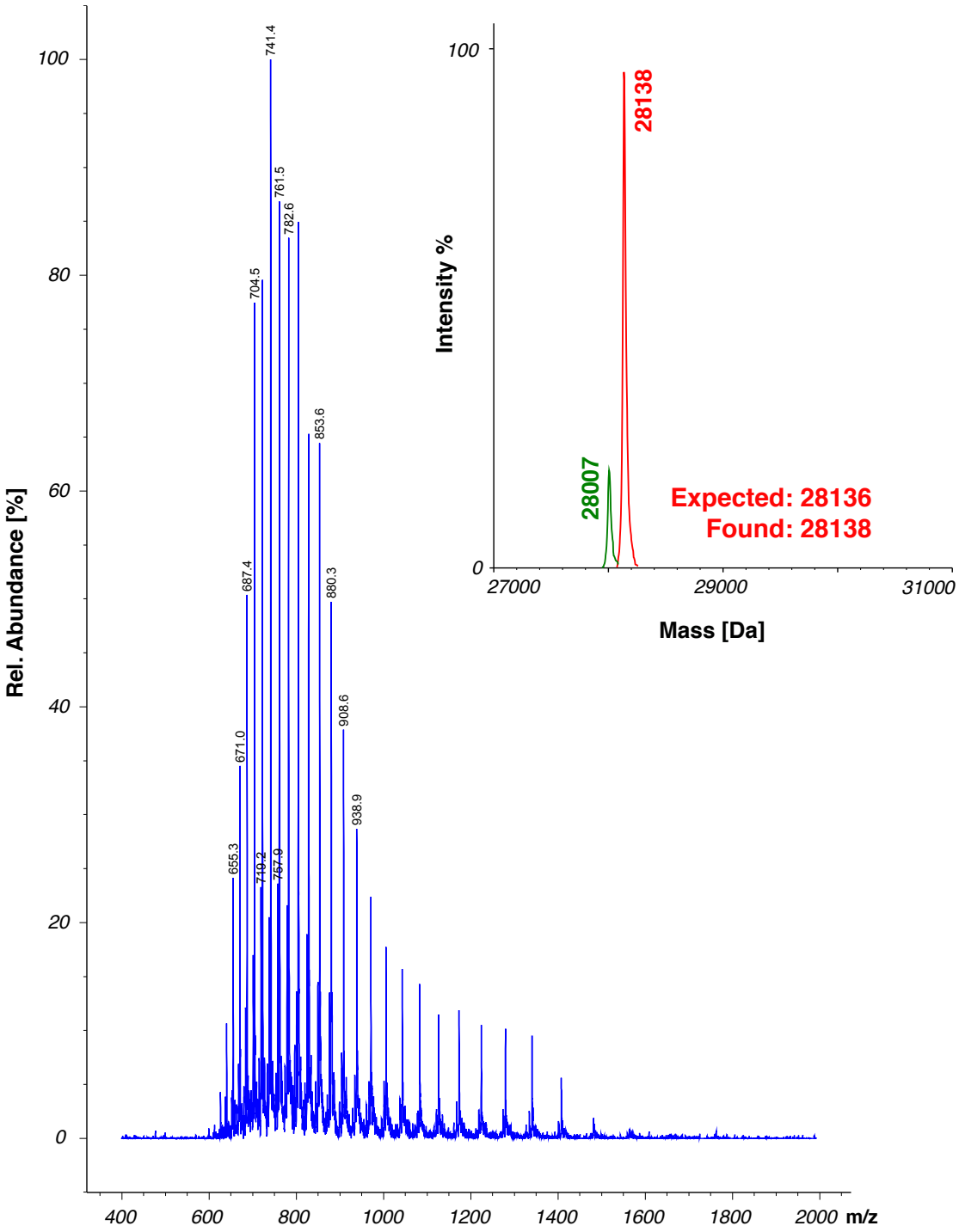
Supplementary Figure S2. LC/MS (254 nm) showing the traceless formation of *N*-phenyl pyrazole **3** from the reaction of *N*-phenyl sydnone **1** with 1 eq. of BCN **2** in MeOH (c = 80 mM, rt, 30 min). Molecular mass is quoted in Daltons (Da).



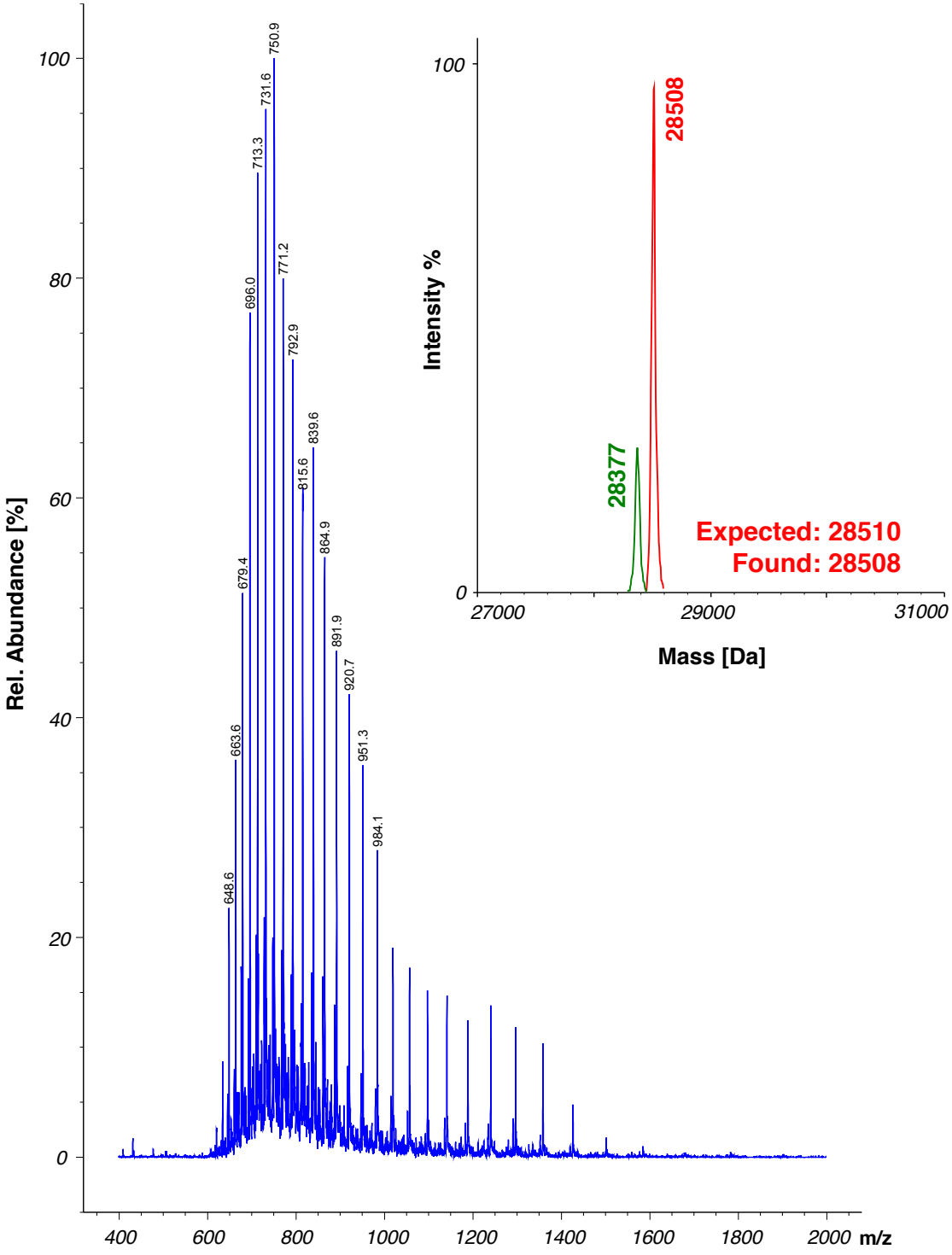
Supplementary Figure S3. UV-vis spectra of *N*-phenyl sydnone (**1**), BCN (**2**) and the product of the cycloaddition *N*-phenyl pyrazole (**3**) after reaction of 1 with 50 eq of 2 in 55:45 MeOH:H_2O after 24 h at room temperature. The rate constant for the reaction was determined under *pseudo*-first order conditions by following the exponential decay in phenyl sydnone absorbance at 310 nm over time upon reaction with a 10-80 fold excess of BCN in 55:45 MeOH:H_2O. The mean of the observed reaction rate constants k' were plotted against the concentration of BCN to afford a linear plot with gradient k. The calculated rate constant for the reaction was 0.054 $M^{-1}s^{-1}$ (±0.00067 $M^{-1}s^{-1}$) at 21 ˚C.



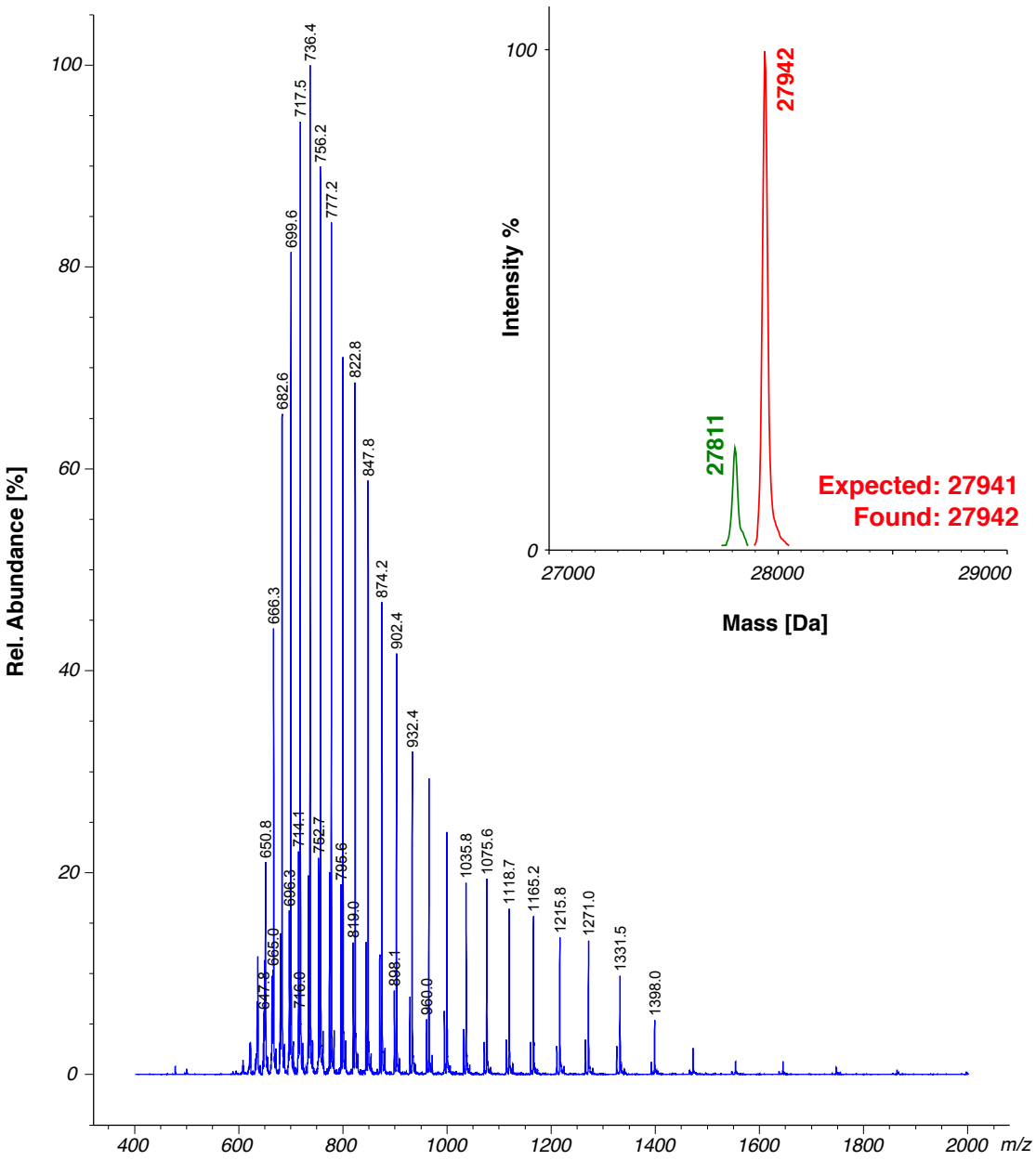
Supplementary Figure S4. Protein MS characterization of *sf*GFP-$\mathbf{5}_{150}$. All masses are given in Daltons (Da). The minor green peak represents proteolysis of the *N*-terminal methionine (–131 Da).



Supplementary Figure S5. Protein MS characterization of *sf*GFP-$\mathbf{5}_{150}$ quantitatively labeled with *N*-phenyl sydnone (**1**). All masses are given in Daltons (Da). The minor green peak represents proteolysis of the *N*-terminal methionine (–131 Da).



Supplementary Figure S6. Protein MS characterization of *sf*GFP-$\mathbf{5}_{150}$ quantitatively labeled with PheSyd-BODIPY-FL (**6**). All masses are given in Daltons (Da). The minor green peak represents proteolysis of the *N*-terminal methionine (–131 Da).



Supplementary Figure S6. Protein MS characterization of *sf*GFP-$\mathbf{4}_{150}$. All masses are given in Daltons (Da). The minor green peak represents proteolysis of the *N*-terminal methionine (–131 Da).