nature chemistry

SUPPLEMENTARY INFORMATION

DOI: 10.1038/NCHEM.1250

Genetically encoded norbornene directs site-specific cellular protein labelling via a rapid bioorthogonal reaction

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Supplementary Methods:

Protein Mass Spectrometry

Using an Agilent 1200 LC-MS system, ESI-MS was carried out 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. LC-ESI-MS on proteins 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). Protein mass spectrometry was additionally carried out with an LCT TOF mass spectrometer (Micromass, see below). Additionally, protein total mass was determined on an LCT time-of-flight mass spectrometer with electrospray ionization (ESI, Micromass). Proteins were rebuffered in 20 mM of ammonium bicarbonate and mixed 1:1 acetonitrile, containing 1 % formic acid. Alternatively samples were prepared with a C4 Ziptip (Millipore) and infused directly in 50% aqueous acetonitrile containing 1 % formic acid. Samples were injected at 10 μL min^{-1} and calibration was performed in positive ion mode using horse heart myoglobin. 30 scans were averaged and molecular masses obtained by maximum entropy deconvolution with MassLynx version 4.1 (Micromass). Theoretical masses of wild-type proteins were calculated using Protparam (http://us.expasy.org/tools/protparam.html), and theoretical masses for unnatural amino acid containing proteins were adjusted manually.

Determination of Kinetic Rate Constants for Small Molecules Cycloadditions

Rate constants k for different tetrazines were measured under pseudo first order conditions with a 10- to 100-fold excess of 5-norbornene-2-ol in methanol/water mixtures by following the exponential decay in UV absorbance of the tetrazine at 320 or 300 nm over time (**Supplementary Fig. 3** and **Supplementary Table1**).

Stock solutions were prepared for each tetrazine (0.1 mM in 9/1 water/methanol) and for 5-norbornene-2-ol (1 to 10 mM in either methanol or water). Mixing equal volumes of the prepared stock solutions resulted in a final concentration of 0.05 mM tetrazine and of 0.5 to 5 mM 5-norbornene-2-ol, corresponding to 10 to 100 equivalents. Spectra were recorded using the following instrumental parameters: wavelength, 320 nm for **6** and **8**; 300 nm for **5** and 3,6-dipyridyl-1,2,4,5-tetrazine, 280

nm for **7**; spectral band width (SBW), 1.0 nm; increment of data point collection, 0.5 s or 2.0 s. All data were recorded at 21 ºC. Data were fit to a single-exponential equation. Each measurement was carried out three times and the mean of the observed rates k' was plotted against the concentration of 5-norbornene-2-ol to obtain the rate constant k from the slope of the plot. All data processing was performed using Kaleidagraph software (Synergy Software, Reading, UK).

Cloning for Mammalian Cell Applications

An amber codon was introduced at position 128 of the EGFR-EGFP fusion protein with the following primers:

forward: ACCAGggtctcGATGCAtagAAAACCGGACTGAAGGAGCTGCCCATG,

reverse: TTGCAggtctcTGCATCATAGTTAGATAAGACTGCTAAGGCATAG.

After PCR the product was digested with BsaI and then ligated to circularize. The mutagenesis was verified by sequencing through the EGFR. The initial mutagenesis was carried out on an EGFR-EGFP fusion in the pEGFPN1 vector. The EGFR was then digested out of the pEGFPN1 vector using the enzymes NheI and MfeI (NEB). Similarly *pMmPylRS-mCherry-TAG-EGFP-HA*[46] was digested with the same enzymes to remove the mCherry-TAG-EGFP-HA reporter. The EGFR-EGFP was ligated into the *pMmPylRS-mCherry-TAG-EGFP-HA* vector in place of the mCherry-EGFP using T4 DNA ligase (NEB) to create *pMmPylRS-EGFR(128TAG)-GFP-HA*.

MS/MS Analysis

Cells were grown on 100 mm tissue culture dishes to ~ 90 % confluence. Cells were transfected with *pMmPylRS-mCherry-TAG-EGFP-HA* and *p4CMVE-U6-PylT* using lipofectamine 2000 (Invitrogen). After 16 – 24 hours in the presence of 1 mM **2** cells were lysed in RIPA buffer and mCherry-eGFP fusion protein was purified using the GFP_Trap_A system (Chromotek). MS/MS analysis was either performed by NextGen Sciences or by an in house facility. For the former, the eluate was added to 4X NuPage LDS Sample buffer and run out on an SDS-PAGE gel. The band corresponding to the full length mCherry-eGFP fusion was then excised. The gel plugs were digested overnight in trypsin. The digests were then analyzed by LC/MS/MS with a 30 minute gradient on an LTQ Orbitrap XL mass spectrometer. Product-ion data were searched against a database of 4 protein sequences, with the lysine modification incorporated among the typically used variable modifications.

The Mascot search engine was utilised with the Scaffold program used for collation and analysis of the data.

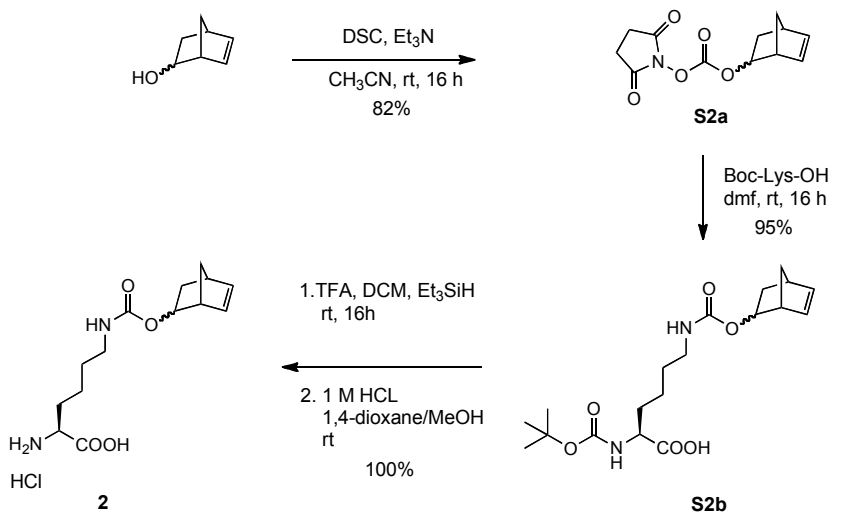
For the in house analysis, the protein solution was reduced and alkylated using standard methods prior to overnight digest with Promega procine Trypsin. The generated peptides were separated on a Dionex Ultimate 3000 HPLC system with a 15 cm, 75 Um, C18 acclaim pep-map column and analysed on a Thermo Scientific LTQ XL Orbitrap mass spectrometer. Protein identification was carried out using an in-house Mascot database.

Chemical Syntheses:

General Methods

^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 MHz instrument. Chemical shifts (δ) are reported relative to TMS and referenced to the residual proton signal in the deuterated solvents: $CDCl_3$ (7.26 ppm), d_6-DMSO (2.49 ppm) for ^{1}H-NMR spectra, $CDCl_3$ (77.0 ppm) of d_6-DMSO (39.5 ppm) for ^{13}C-NMR spectra. J values are given in Hertz, and the splitting patterns are designed as follows: s, singlet; s, br, broad singlet; d, doublet; t, triplet; 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/or by potassium permanganate staining. Flash column chromatography was carried out on silica gel 60 (230-400 mesh or 70-230 mesh). Using an Agilent 1200 LC-MS system, ESI-MS was carried out 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.

Synthesis of Norbornene Lysine 2



Supplementary Scheme 1. Synthetic route for *N*ε-5-norbornene-2-yloxy-carbonyl-L-lysine (**2**)

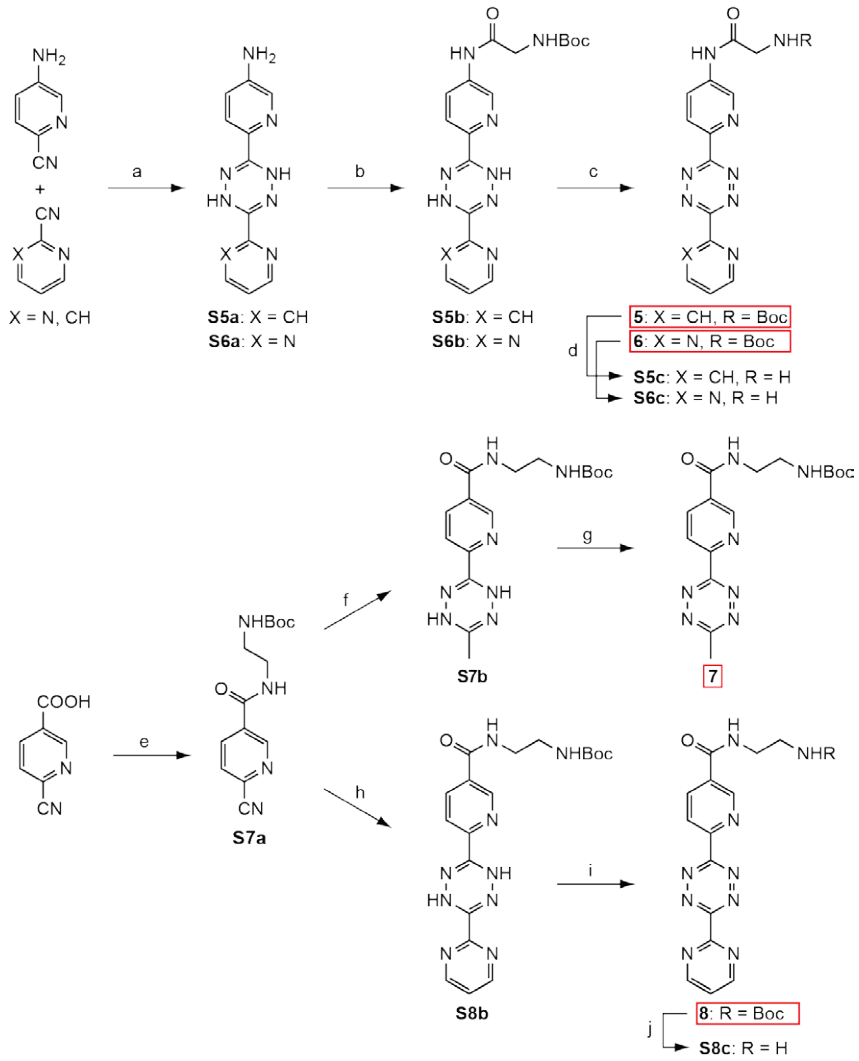
Disuccinimide carbonate (6.3 g, 0.024 mol) was added to a solution of (1R,4R)-5-norbornene-2-ol (*endo*/*exo* mixture, 1.5 g, 0.014 mol) and triethylamine (5.7 mL, 0.041 mol) in dry acetonitrile (50 mL) at room temperature. The resulting mixture was stirred overnight and then concentrated under vacuum. The product was purified by column chromatography on SiO_2 (1-5% diethyl ether in dichloromethane) to deliver **S2a** as a white solid in 82%, 7:3 *endo*/*exo* (2.8 g, 0.011 mol). R_f (Et_2O/DCM, 1/99): 0.4; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 6.32 and 6.23 (m_{endo}, dd_{exo}, J = 2.7 Hz, 1H), 5.94 and 5.89 (m_{endo}, t_{exo}, J = 3.6 Hz, 1H), 5.28 and 4.66 (m_{endo}, d_{exo}, J = 5.7 Hz, 1H), 3.19 and 3.00 (s_{endo}, s_{exo}, 1H), 2.84 (s, 1H), 2.80 (s, 4H), 2.21-2.13 and 1.81-1.57 (m_{endo}, m_{exo}, 1H), 1.52-1.49 (m, 1H), 1.32 (d, J = 9.0 Hz, 1H), 1.14-1.08 (dt, J_1 = 12.9 Hz, J_2 = 2.4 Hz, 1H) ppm; ^{13}C-NMR (300 MHz, $CDCl_3$): δ 169.02, 168.95, 151.25, 142.10, 139.16, 131.69, 130.90, 83.20, 82.76, 47.58, 47.23, 46.23, 45.72, 42.16, 40.52, 34.43, 25.44 ppm; ESI-MS (m/z): $[M + Na]^+$ calcd for $C_{12}H_{13}NO_5$ 274.0686, found 274.0683.

Boc-Lys-OH (3.2 g, 0.013 mol) was added to a stirred solution of **S2a** (2.5 g, 0.010 mol) in dry dimethylformamide (35 mL). The reaction was allowed to proceed overnight at room temperature. The mixture was diluted in water (150 mL) and extracted with ethyl acetate (150 mL x 3). The combined organic layers were washed with water (100 mL x 3) and brine (75 mL). The resulting organic layer was dried

over Na_2SO_4, filtered and concentrated under vacuum to dryness. Compound **S2b** was obtained in 95% yield (3.6 g, 9.40 mmol) as an off-white foam. R_f (Et_2O/DCM, 5/95): 0.1; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 9.11 (s, br, 1H), 8.03 (s, br, 1H), 6.30-6.21 (m, 1H), 5.95-5.93 (m, 1H), 5.30 and 4.59 (d, br_{endo}, J = 7.2 Hz; d, br_{exo}, J = 6.9 Hz, 1H), 5.24 (s, br, 1H), 4.86 (m, br, 1H), 4.77 (m, br, 1H), 4.28 (s, br, 1H), 4.09 (m, br, 1H), 3.12 (m, br, 2H), 2.80 (m, br, 1H), 2.09 (m, 1H), 1.81-1.28 (m, br, 15H), 0.90 (d, br, J = 12.9 Hz, 1H) ppm; ^{13}C-NMR (300 MHz, $CDCl_3$): δ 175.95, 156.76, 155.58, 140.74, 138.19, 132.49, 131.43, 79.76, 75.35, 75.14, 52.90, 47.39, 47.20, 45.91, 45.74, 41.95, 40.30, 40.14, 34.28, 31.73, 29.14, 28.09, 22.10, 21.75 ppm; ESI-MS (m/z): $[M + Na]^+$ calcd for $C_{19}H_{30}N_2O_6$ 405.1996, found 405.1983.

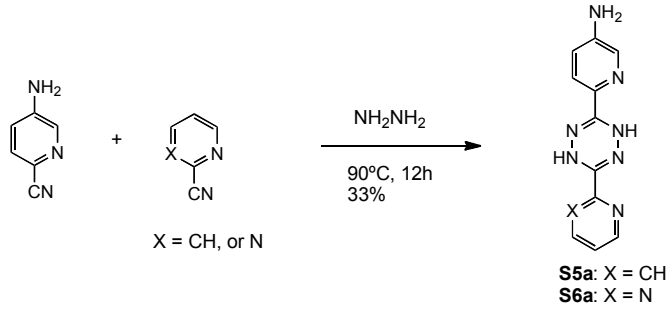
To a solution of **S2b** (3.3 g, 8.60 mmol) and Et_3SiH (2.7 ml, 0.017 mol) in dry dichloromethane (120 mL), trifluoroacetic acid (6.4 mL, 0.086 mol) was added dropwise, and the reaction mixture was allowed to stir at room temperature overnight. The solvents were evaporated under reduced pressure. The residue was re-dissolved in a 1M HCl solution (5 mL 4N HCl in 1,4-dioxane, 15 mL dry methanol), allowed to stir for 10 min and then concentrated. The latter process was repeated two more times to ensure complete HCl salt exchange. The concentrated residue was re-dissolved in a minimal amount of methanol and was precipitated into ice-cold diethyl ether, filtered and dried under vacuum, affording the amino acid **2** as a white solid in quantitative yield (2.7g, 8.50 mmol). ^{1}H-NMR (300 MHz, CD_3OD): δ 6.30-6.25 (m, 1H), 6.00-5.93 (m, 1H), 5.15 and 4.52 (m_{endo}, m_{exo}, 1H), 4.85 (m, 1H), 3.55 (t, J = 5.4 Hz, 1H), 3.07 (q, J = 6.7 Hz, 2H), 2.81 (d, J = 6.6 Hz, 1H), 2.13-2.05 (m, 1H), 1.93-1.74 (m, 2H), 1.68-1.63 (m, 1H), 1.53-1.28 (m, 5H), 0.93-0.87 (dt, J_1 = 12.3 Hz, J_2 = 2.7 Hz, 1H) ppm; ^{13}C-NMR (300 MHz, CD_3OD): δ 174.82, 159.52, 142.37, 139.36, 133.84, 132.80, 76.73, 76.73, 56.16, 47.43, 47.13, 43.63, 41.93, 41.42, 35.67, 32.80, 32.07, 30.74, 28.90, 24.22, 23.63 ppm; ESI-MS (m/z): $[M + Na]^+$ calcd for $C_{14}H_{22}N_2O_4$ 305.1472; found: 305.1475.

Synthesis of the Tetrazine Probes



Supplementary Scheme 2. Overview of synthetic route to various tetrazines used in this study. **a**) 4 eq hydrazine hydrate (64%), 12 h, 90ºC, 33%; **b**) 4 eq *N-tert*-butoxycarbonylglycine, 5 eq *N*-methylpyrrolidone, 3.3 eq isobutylchloroformate in THF, 0ºC – rt, 3h, 80%; **c**) 1.5 eq sodiumnitrate in acetic acid, 10 min, rt, 65%; **d**) 4 N HCl in dioxane/DCM, 30 min, rt, 100%; **e**) 0.5 eq 4-dimethylaminopyridine, 1.5 eq *N-tert*-butyloxycarbonylethylenediamine, 1.5 eq 1-ethyl-3-(3-dimethulaminopropyl)carbodiimide, in DCM, 0ºC – rt, 5 h, 90%; **f**) 5 eq hydrazine hydrate (64%) in acetonitrile, 90ºC, 12 h, 45%; **g**) 1.5 eq sodiumnitrate in acetic acid, 10 min, rt, 55%; **h**) 1 eq pyrimidine-2-carbonitrile, 5 eq hydrazine hydrate (64%) in ethanol, 90ºC, 12 h, 40%; **i**) 1.5 eq sodiumnitrate in acetic acid, 10 min, rt, 50%; **j**) 4 N HCl in dioxane/DCM, 30 min, rt, 100%. THF, tetrahydrofurane; DCM, dichloromethane; Boc, *tert*-butyloxycarbonyl.

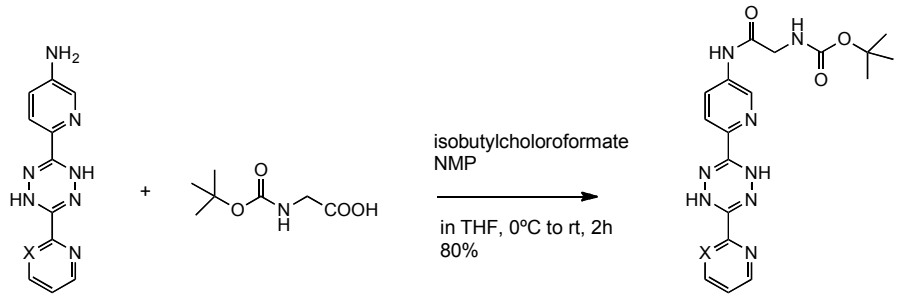
Synthesis of S5a and S6a



Equimolar amounts of 5-amino-2-cyanopyridine (1.14 g, 9.6 mmol) and 2-cyanopyridine (1.00 g, 9.6 mmol) were mixed with 64% aqueous hydrazine (1.85 ml, 38.4 mmol) and heated for 12 h to 90ºC behind a blast shield. The mixture was allowed to cool to room temperature (rt), the orange precipitate was isolated by filtration, washed with cold water and dried under vacuum. The crude solid was dissolved in methanol, concentrated onto silica gel and **S5a** was purified by chromatography on SiO_2 (0% to 3% methanol in dichloromethane) as an orange solid (802 mg, 33%). R_f (CH_2Cl_2/MeOH, 92/8): 0.50; ^{1}H-NMR (400 MHz, d_6-DMSO): δ 8.77 (s, 1H), 8.72 (s, 1H), 8.66-8.68 (m, 1H), 7.93-8.03 (m, 3H), 7.71 (d, J = 8.4 Hz, 1H), 7.54-7.57 (m, 1H), 7.04-7.07 (dd, J_1 = 8.8 Hz, J_2 = 2.8 Hz. 1H), 5.93 (s, 2H) ppm; ^{13}C-NMR (400 MHz, d_6-DMSO): δ 148.52 (CH), 147.48 (C), 146.65 (C), 146.62 (C), 146.59 (C), 137.29 (CH), 134.15 (C), 134.06 (CH), 125.12 (CH), 121.81 (CH), 120.76 (CH), 120.27 (CH) ppm; ESI-MS (m/z): $[M+H]^+$ calcd for $C_{12}H_{11}N_7$ 253.11, found 253.3.

In a similar experiment 5-amino-2-cyanopyridine (1.51 g, 9.52 mmol) and pyrimidine-2-carbonitrile (1.00 g, 9.52 mmol) were mixed with 64% hydrazine hydrate (2.3 ml, 47.6 mmol) for 12 h at 90ºC and compound **S6a** was isolated by column chromatography on SiO_2 (750 mg, 31%). R_f (CH_2Cl_2/MeOH, 92/8): 0.50; ^{1}H-NMR (400 MHz, d_6-DMSO): δ 8.95 (d, J = 4.8 Hz, 2H), 8.88 (s, 1H), 8.71 (s, 1H), 7.99 (d, J = 2.4 Hz, 1H), 7.70 (d, J = 8.4 Hz, 1H), 7.64 (t, J = 4.8, 1H), 7.04-7.07 (dd, J_1 = 8.4, J_2 = 2.4, 1H), 5.94 (s, 2H) ppm; ^{13}C-NMR (400 MHz, d_6-DMSO): δ 157.62 (CH), 156.12 (C), 146.66 (C), 146.11 (C), 146.00 (C), 134.09 (CH), 133.96 (C), 121.96 (CH), 121.92 (CH), 120.28 (CH) ppm; ESI-MS (m/z): $[M+H]^+$ calcd for $C_{11}H_{10}N_8$ 254.10, found 254.3.

Synthesis of S5b and S6b

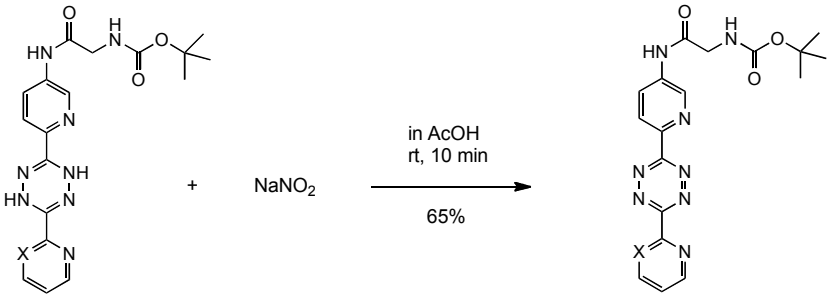


To a stirred solution of N-(*tert*-butoxycarbonyl)glycine (1.66g, 9.48 mmol) in dry THF N-methylpyrrolidone (1.3 ml, 11.85 mmol) was added. The reaction mixture was chilled to 0 ºC before isobutylchloroformate (1.0 ml, 7.82 mmol) was added dropwise. A white precipitate was formed instantaneously and the mixture was stirred at 0ºC before the portion-wise addition of 3-(5-aminopyridin-2-yl)-6-(pyridin-2-yl)-1,4-dihydro-*s*-tetrazine **S5a** (600 mg, 2.37 mmol) in dry THF (15 ml). The reaction was allowed to warm to rt with stirring and after 3 h the reaction was adjudged complete by TLC analysis. The solvent was evaporated and the residue dissolved in dichloromethane. The solution was extracted with 5% citric acid, water and saturated sodium bicarbonate solution. The organic layer was dried over Na_2SO_4 and the product **S5b** (778 mg, 80%) was isolated by column chromatography on SiO_2 (0% to 4% methanol in dichloromethane). R_f (CH_2Cl_2/MeOH, 95/5): 0.70; ^{1}H-NMR (400 MHz, d_6-DMSO): δ 10.41 (s 1H), 8.94 (s, 1H), 8.88 (s, 1H), 8.24-8.29 (d, J = 2.0 Hz, 1H), 8.63-8.65 (m, 1H), 8.15-8.17, dd, J_1 = 8.8, J_2 = 2.4 Hz, 1H), 7.92-7.99 (m, 3H), 7.52-7.55 (m, 1H), 7.13 (t, J = 6.0 Hz, 1H), 3.78 (d, J = 6.0 Hz, 2H), 1.39 (s, 9H) ppm; ^{13}C-NMR (400 MHz, d_6-DMSO): δ 169.12 (C), 155.80 (C), 148.56 (CH), 147.27 (C), 146.30 (C), 146.02 (C), 141.57 (C), 138.91 (CH), 137.35 (CH), 136.95 (C), 126.75 (CH), 125,265 (CH), 121.39 (CH), 120.92 (CH), 78.13 (C), 43.81 (CH_2), 28.16 (3 x CH_3) ppm; ESI-MS (m/z): $[M+H]^+$ calcd for $C_{19}H_{22}N_8O_3$ 410.18, found 410.2.

Compound **S6b** (605mg, 75%) was synthesized in a similar way by reacting **S6a** (500 mg, 1.96 mmol) with *N*-*tert*-butyloxycarbonylglycine (1.37 g, 7.84 mmol), isobutylchloroformate (883 mg, 840 µl, 6.47 mmol) and *N*-methylpyrrolidone (991 mg, 1.08 ml, 9.8 mmol) in dry THF. R_f (CH_2Cl_2/MeOH, 95/5): 0.70; ^{1}H-NMR (400 MHz, d_6-DMSO): δ 10.42 (s, 1H), 9.05 (s, 1H), 8.93 (d, J = 4.8 Hz, 2H), 8.89 (s, 1H),

8.82 (m, 1H), 8.14-8.19 (m, 1H), 7.93-7.96 (m, 1H), 7.62 (t, J = 4.8 Hz, 1H), 7.13 (t, J = 6.0 Hz, 1H), 3.79 (d, J = 6.0 Hz, 2H), 1.41 (s, 9H) ppm; ^{13}C-NMR (400 MHz, d_6-DMSO): δ 169.14 (C), 157.66 (2 x CH), 155.98 (C), 155.91 (C), 145.64 (C), 145.55 (C), 141.40 (C), 138.95 (CH), 136.98 (C), 126.77 (CH), 122.08 (CH), 121.49 (CH), 78.14 (C), 43.82 (CH_2), 27.34 (3 x CH_3) ppm; ESI-MS (m/z): $[M+H]^+$ calcd for $C_{18}H_{21}N_9O_3$ 411.18, found 411.3.

Synthesis of 5 and 6

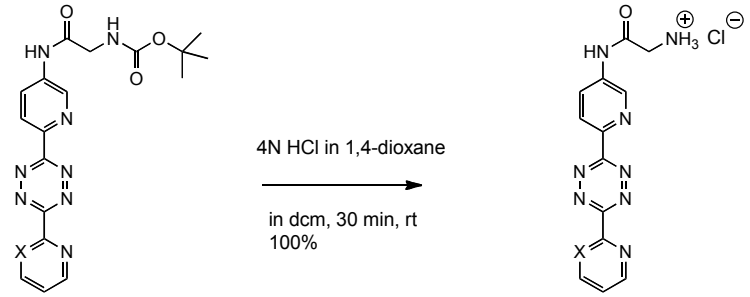


To a stirred solution of **S5b** (200 mg, 0.49 mmol) in acetic acid (10 ml) sodium nitrite (50 mg, 0.73 mmol) was added at rt. After 10 min the reaction mixture was diluted with dichloromethane and extracted several times with a half-saturated sodium bicarbonate solution. The organic layer was dried over Na_2SO_4 and the solvent evaporated. Column chromatography on SiO_2 (0% to 8% methanol in dichloromethane) afforded **5** as a pink solid (130 mg, 65%). R_f (CH_2Cl_2/MeOH, 9/1): 0.50; ^{1}H-NMR (400 MHz, d_6-DMSO): δ 10.62 (s, 1H), 9.06 (d, J = 2.28, 1H), 8.94 (m, 1H), 8.65 (d, J = 8.68, 1H), 8.60 (d, J = 7.88, 1H), 8.43 (dd, J_1 = 8.68, J_2 = 2.36, 1H), 8.16 (dt, J_1 = 7.76, J_2 = 1.72, 1H), 7.73 (ddd, J_1 = 7.76, J_2 = 1.72, 1H), 7.18 (t, J = 6.0 Hz, 1H), 3.85 (d, J = 6.0 Hz, 1.42, s 9H) ppm; ^{13}C-NMR (400 MHz, d_6-DMSO): δ 169.5 (C), 163.0 (C), 162.7 (C), 156.0 (C), 150.6 (CH), 150.2 (C), 144.0 (C), 141.3 (CH), 138.2 (C), 137.8 (CH), 126.5 (CH), 126.3 (CH), 124.9 (CH), 124.2 (CH), 78.2 (CH_2), 43.9 (C), 28.2 (CH_3) ppm; ESI-MS (m/z): $[M+H]^+$ calcd for $C_{19}H_{20}N_8O_3$ 408.17, found 408.2.

Oxidation of **S6b** (150 mg, 0.36 mmol) with $NaNO_2$ (38 mg, 0.55 mmol) under similar conditions gave 88 mg (60%) of compound **6**. R_f (CH_2Cl_2/MeOH, 9/1): 0.50; ^{1}H-NMR (400 MHz, d_6-DMSO): δ 10.64 (s, 1H), 9.21 (d, J = 4.8 Hz, 2H), 9.07 (d, J = 2.4 Hz, 1H), 8.67 (d, J = 8.8 Hz, 1H), 8.43-8.46 (dd, J_1 = 8.8 Hz, J_2 = 2.4 Hz, 1H),

7.84 (t, J = 4.8, 1H), 7.18 (t, J = 6.0, 1H), 3.84 (d, J = 6.0 Hz, 1H), 1.42 (s, 9H) ppm; ^{13}C-NMR (400 MHz, d_6-DMSO): δ 169.4 (C), 162.76 (C), 162.68 (C), 159.09 (C), 158.47 (CH), 155.95 (C), 143.78 (C), 141.34 (C), 138.33 (C), 126.22 (CH), 125.30 (CH), 122.95 (CH), 78.18 (C), 43.93 (CH_2), 28.18 (3 x CH_3) ppm; ESI-MS (m/z): $[M+H]^+$ calcd for $C_{18}H_{19}N_9O_3$ 409.16, found 409.4.

Synthesis of S5c and S6c

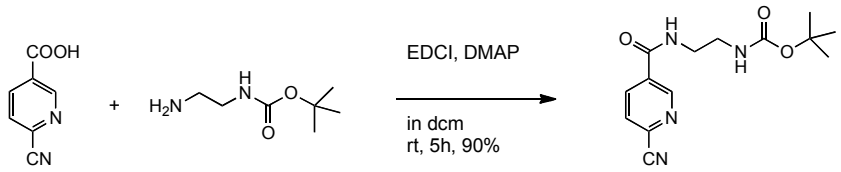


To a stirred solution of compound **5** (100 mg, 0.24 mmol) in dry dichloromethane (4 ml) a 4N HCl solution in dioxane (2 ml) was added and the reaction mixture was allowed to stir for 30 min at rt, after which time complete consumption of the starting material was observed by LC-MS and TLC analysis. The reaction mixture was concentrated to dryness under reduced pressure, to give compound **S5c** as HCl salt (85 mg, 100%). The crude material was deemed pure enough for subsequent reactions. ^{1}H-NMR (400 MHz, d_6-DMSO): δ 11.7 (s, 1H), 9.13 (d, J = 2.4 Hz, 1H), 8.87-8.89 (m, 1H), 8.61 (d, J = 8.8 Hz, 1H), 8.56 (d, J = 8.0 Hz, 1H), 8.38-8.41 (dd, J_1 = 8.8 Hz, J_2 = 2.4 Hz, 1H and s, br, 2H), 8.12-8.16 (dt, J_1 = 7.6 Hz, J_2 = 1.8 Hz, 1H), 7.69-7.72 (m, 1H), 3.88 (m, 2H) ppm; ^{13}C-NMR (400 MHz, d_6-DMSO): δ 166.08 (C), 162.81 (C), 162.67 (C), 150.24 (CH), 147.90 (C), 144.40 (C), 141.21 (CH), 138.35 (CH), 137.76 (C), 126.79 (CH), 126.61 (CH), 125.06 (CH), 124.32 (CH), 41.20 (CH_2) ppm; ESI-MS (m/z): $[M+H]^+$ calcd for $C_{14}H_{12}N_8O$ 308.11, found 308.3.

Deprotection of compound **6** (150 mg, 0.37 mmol) under similar acidic conditions afforded compound **S6c** as HCl salt (126 mg, 100%). ^{1}H-NMR (400 MHz, d_6-DMSO): δ 11.79 (s, 1H), 9.13 (m, 3H), 8.62 (d, J = 4.4 Hz, 1H), 8.38-8.41 (m, br, 3H), 7.77 (t, J = 4.8 Hz, 1H), 3.88 (m, 2H) ppm; ^{13}C-NMR (400 MHz, d_6-DMSO): δ 166.11 (C), 162.77 (C), 162.58 (C), 159.02 (C), 158.49 (2 x CH), 144.19 (C), 141.21

(CH), 137.90 (C), 126.61 (CH), 125.40 (CH), 122.99 (CH), 43.58 (CH_2) ppm; ESI-MS (m/z): $[M+H]^+$ calcd for $C_{13}H_{11}N_9O$ 309.11, found 309.5.

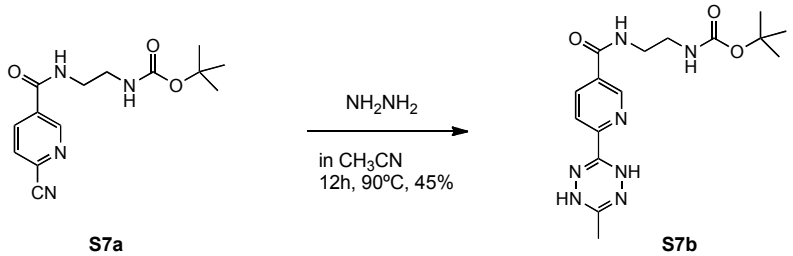
Synthesis of S7a



S7a

To a stirred solution of 6-cyanonicotinic acid (500 mg, 3.38 mmol) in dry dichloromethane (30 ml) 4-dimethylaminopyridine (DMAP, 206 mg, 1.69 mmol) was added and the solution was chilled to 0ºC. N-Boc-ethylenediamine (811 mg, 800 ul, 5.06 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI, 971 mg, 5.06 mmol) were added portion-wise and the reaction mixture was allowed to warm to rt and stirred for 5 h. The reaction mixture was diluted with dichloromethane, extracted with 5% citric acid and saturated sodium bicarbonate solution and the organic layer was dried over Na_2SO_4. The solvent was evaporated and compound **S7a** (882 mg, 90%) could be used without further purification for the next step. R_f (CH_2Cl_2/MeOH, 9/1): 0.50; ^{1}H-NMR (400 MHz, d_6-DMSO): δ 9.11 (s, 1H), 8.88 (t, *J* = 5.2 Hz, 1H), 8.37-8.40 (m, 1H), 8.14-8.19 (M, 1H), 6.93 (t, *J* = 5.6 Hz, 1H), 3.30-3.33 (m, 2H), 3.11-3.18 (m, 2H), 1.37 (s, 9H) ppm; ^{13}C-NMR (400 MHz, d_6-DMSO): δ 163.50 (C), 155.70 (C), 149.79 (CH), 136.61 (CH), 134.12 (C), 133.01 (C), 128.75 (CH), 117.12 (C), 77.66 (C), 39.92 (CH_2), 39.71 (CH_2), 28.18 (3 x CH_3) ppm; ESI-MS (m/z): $[M+H]^+$ calcd for $C_{14}H_{18}N_4O_3$ 290.14, found 290.5.

Synthesis of S7b

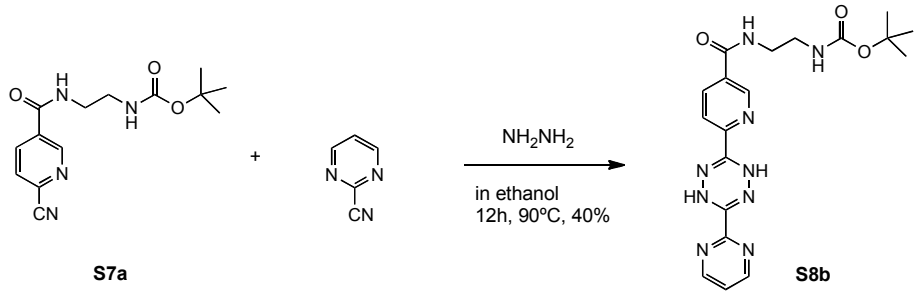


S7a **S7b**

A dry round-bottom flask was charged with compound **S7a** (150 mg, 0.52 mmol) and 64% hydrazine hydrate (130 ul, 2.58 mmol) in dry acetonitrile (2ml). The flask was fitted with a reflux condenser, and the mixture was heated to 90ºC for 12 h behind a

blast shield. The reaction mixture was allowed to cool to room temperature, the solvents were evaporated, the residue was dissolved in dichloromethane and extracted with 5% citric acid and saturated sodium bicarbonate solution. The organic layer was dried over sodium sulfate and concentrated under vacuum to dryness to afford compound S**7b** (84 mg, 45%) in sufficient purity for the next step. R_f (CH_2Cl_2/MeOH, 94/6): 0.50; ^{1}H-NMR (400 MHz, d_6-DMSO): δ 9.04 (s, 1H), 8.82 (t, *J* = 5.2 Hz, 1H), 8.31 (d, *J* = 8.0, 1H), 8.04 (d, *J* = 8.0, 1H), 7.00 (m, 1H), 3.36 (m, 2H), 3.18 (m, 2H), 1.87 (s, 3H), 1.42 (s, 9H) ppm; ^{13}C-NMR (400 MHz, d_6-DMSO): δ 164.28 (C), 155.69 (C), 149.43 (C), 147.51 (C), 147.42 (CH), 145.28 (C), 135.99 (CH), 130.61 (C), 120.11 (CH), 77.65 (C), 39.62 (CH_2), 39.37 (CH_2), 28.19 (3 x CH_3), 15.60 (CH_3) ppm; ESI-MS (m/z): $[M+H]^+$ calcd for $C_{16}H_{23}N_7O_3$ 361.19, found 361.5.

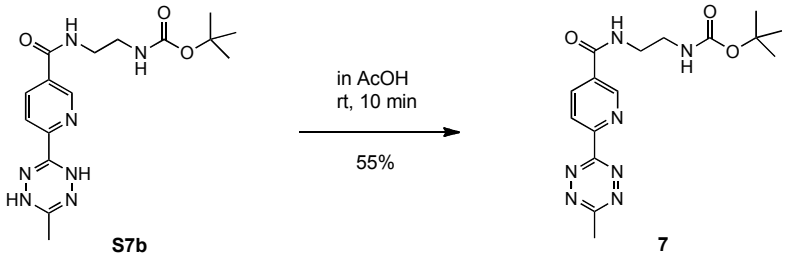
Synthesis of S8b



Equimolar amounts of compound **S7a** (1.28 g, 4.4 mmol) and pyrimidine-2-carbonitrile (462 mg, 4.4 mmol) were mixed with 64% hydrazine hydrate (1.06 ml, 22.0 mmol) in ethanol (5 ml) and heated for 12 h to 90ºC behind a blast shield. The mixture was allowed to cool to room temperature (rt), the solvents evaporated, the residue dissolved in ethylacetate and extracted with 5% citric acid and saturated sodium bicarbonate solution. The organic layer was dried over Na_2SO_4 and evaporated to dryness under vacuum to afford compound **S8b** (748 mg, 40%) which was deemed pure enough for the subsequent step. R_f (CH_2Cl_2/MeOH, 96/4): 0.50; ^{1}H-NMR (400 MHz, d_6-DMSO): δ 9.24 (s, 1H), 9.12 (s, 1H), 9.09 (m, 1H), 8.99 (d, *J* = 4.8 Hz, 2H), 8.82 (m, 1H), 8.33-8.72 (m, 1H), 8.10 (d, *J* = 8.4 Hz, 1H), 7.68 (t, *J* = 8.4 Hz, 1H), 7.68 (t, *J* = 4.8 Hz, 1H), 6.98 (t, *J* = 5.8 Hz, 1H), 3.25-3.38 (m, 2H), 3.18-3.20 (m, 2H), 1.41 (s, 9H) ppm; ^{13}C-NMR (400 MHz, d_6-DMSO): δ 171.18 (C), 164.25 (C), 157.69 (2 x CH), 155.86 (C), 155.70 (C), 148.84 (C), 148.75 (C), 147.52 (CH), 136.19 (CH), 131.15 (C), 122.17 (CH), 120.61 (CH), 77.66 (C), 39.65 (CH_2),

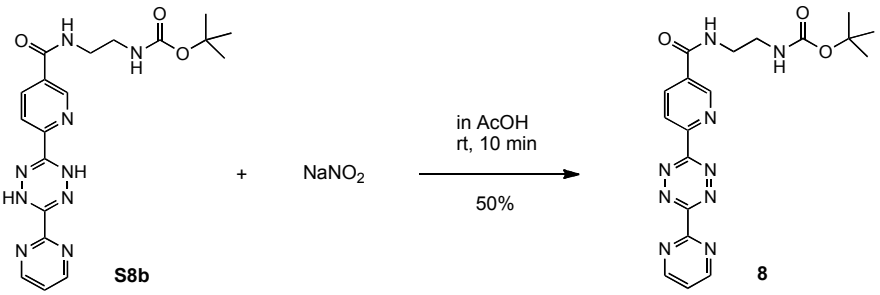
39.37 (CH_2), 28.19 (3 x CH_3) ppm; ESI-MS (m/z): $[M+H]^+$ calcd for $C_{19}H_{23}N_9O_3$ 425.19, found 425.5.

Synthesis of 7



To a stirred solution of **S7b** (75 mg, 0.21 mmol) in acetic acid (3 ml) sodium nitrite (22 mg, 0.31 mmol) was added at rt. After 10 min the reaction mixture was diluted with dichloromethane and extracted several times with a half-saturated sodium bicarbonate solution. The organic layer was dried over Na_2SO_4 and the solvent evaporated. Column chromatography on SiO_2 (0% to 4% methanol in dichloromethane) afforded **7** as a pink solid (40 mg, 55%). R_f (CH_2Cl_2/MeOH, 94/6): 0.40; ^{1}H-NMR (400 MHz, d_6-DMSO): δ 9.27 (s, 1H), 8.89 (t, J = 5.2 Hz, 1H), 8.61 (d, J = 8.4 Hz, 1H), 8.46-8.49 (dd, J_1 = 8.4 Hz, J_2 = 2.0 Hz, 1H), 6.97 (t, J = 5.8 Hz, 1H) 3.35 (m, 2H), 3.08 (s, 3H), 3.17 (m, 2H), 1.40 (s, 9H) ppm; ^{13}C-NMR (400 MHz, d_6-DMSO): δ 167.61 (C), 164.28 (C), 162.85 (C), 155.73 (C), 152.02 (C), 149.17 (CH), 136.59 (CH), 131.64 (C), 123.28 (CH), 77.67 (C), 39.74 (CH_2), 39.37 (CH_2), 28.21 (3x CH_3), 20.97 (CH_3) ppm; ESI-MS (m/z): $[M+H]^+$ calcd for $C_{16}H_{21}N_7O_3$ 359.17, found 359.6.

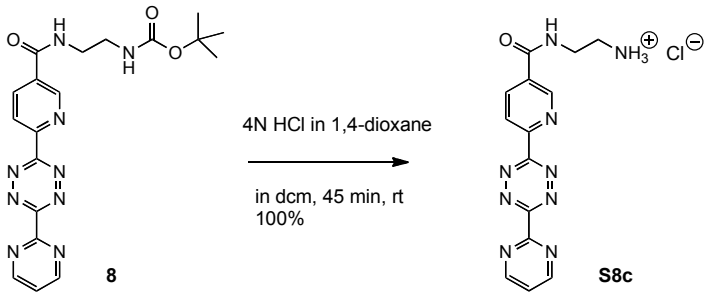
Synthesis of 8



To a stirred solution of **S8b** (200 mg, 0.47 mmol) in acetic acid (10 ml) sodium nitrite (48.6 mg, 0.71 mmol) was added at rt. After 10 min the reaction mixture was diluted with dichloromethane and extracted several times with a half-saturated sodium

bicarbonate solution. The organic layer was dried over Na_2SO_4 and the solvent evaporated. Column chromatography on SiO_2 (0% to 8% methanol in dichloromethane) afforded **8** as a pink solid (100 mg, 50%). R_f (CH_2Cl_2/MeOH, 9/1): 0.50; ^{1}H-NMR (400 MHz, d_6-DMSO): δ 9.38 (d, J = 1.2 Hz, 1H), 9.28 (d, J = 4.8 Hz, 2H), 8.98-9.01 (t, J = 5.4 Hz, 1H), 8.80 (d, J = 8.4 Hz, 1H), 8.57-8.59 (dd, J_1 = 8.2 Hz, J_2 = 1.8 Hz, 1H), 7.91-7.93 (t, J = 4.8 Hz, 1H), 7.03-7.05 (t, J = 5.8 Hz, 1H), 3.43-3.45 (m, 2H), 3.19-3.26 (m, 2H), 1.44 (s, 9H) ppm; ^{13}C-NMR (400 MHz, d_6-DMSO): δ 164.24 (C), 162.94 (2 x C), 158.98 (C), 158.54 (2 x CH), 155.74 (C), 151.64 (C), 149.34 (CH), 136.67 (CH), 132.16 (C), 124.17 (CH), 123.09 (CH), 77.68 (C), 39.77 (CH_2), 39.38 (CH_2), 28.22 (3 x CH_3) ppm; ESI-MS (m/z): $[M+H]^+$ calcd for $C_{19}H_{21}N_9O_3$ 423.18, found 423.5.

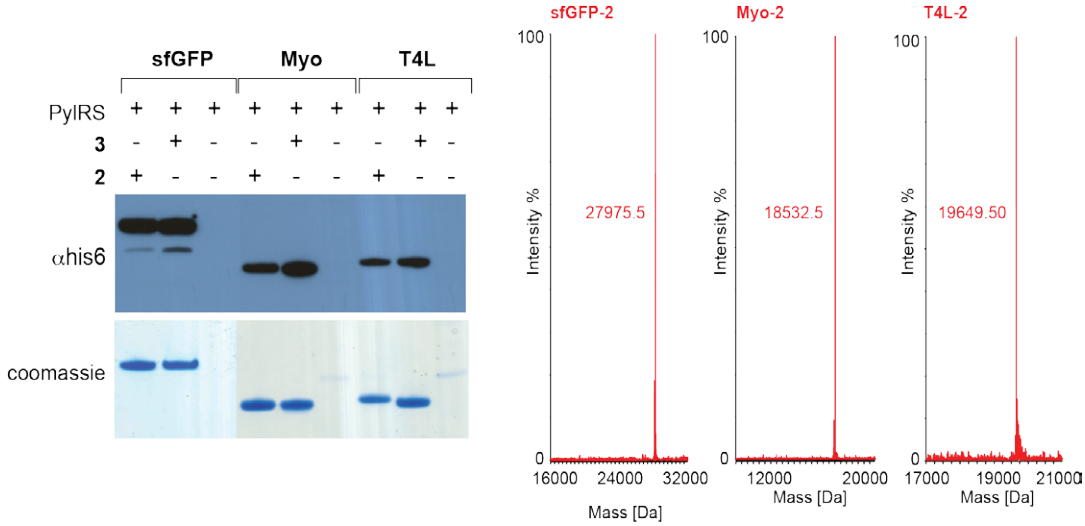
Synthesis of S8c



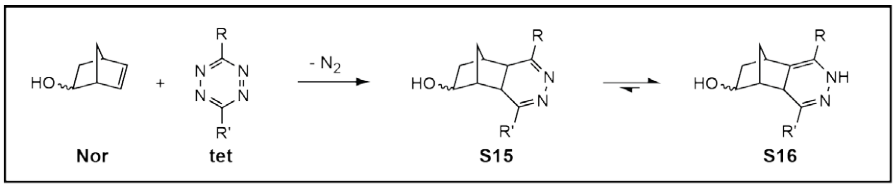
To a stirred solution of compound **8** (200 mg, 0.47 mmol) in dry dichloromethane (4 ml) a 4N HCl solution in dioxane (2 ml) was added and the reaction mixture was allowed to stir for 45 min at rt, after which time complete consumption of the starting material was observed by LC-MS and TLC analysis. The reaction mixture was concentrated to dryness under reduced pressure, to give compound **S8c** as HCl salt (170 mg, 100%). The crude material was deemed pure enough for subsequent reactions. ^{1}H-NMR (400 MHz, d_6-DMSO): δ 9.44 (s, 1H), 9.34-9.37 (t, J = 5.2 Hz, 1H), 9.24 (d, J = 4.8 Hz, 1H), 8.77 (m, 1H), 8.63-8.67 (m, 1H), 8.24 (s, br, 2H), 7.87-7.89 (t, J = 4.8 Hz, 1H), 3.62-3.66 (m, 2H), 3.06-3.09 (m, 2H) ppm; ^{13}C-NMR (400 MHz, d_6-DMSO): δ 164.66 (C), 162.93 (C), 158.95(C), 158.55 (2 x CH), 151.78 (C), 149.59 (CH), 136.90 (CH), 131.68 (C), 124.12 (CH), 124.12 (CH), 123.11 (CH), 66.31 (CH_2) ppm; ESI-MS (m/z): $[M+H]^+$ calcd for $C_{14}H_{13}N_9O$ 323.12, found 323.3.

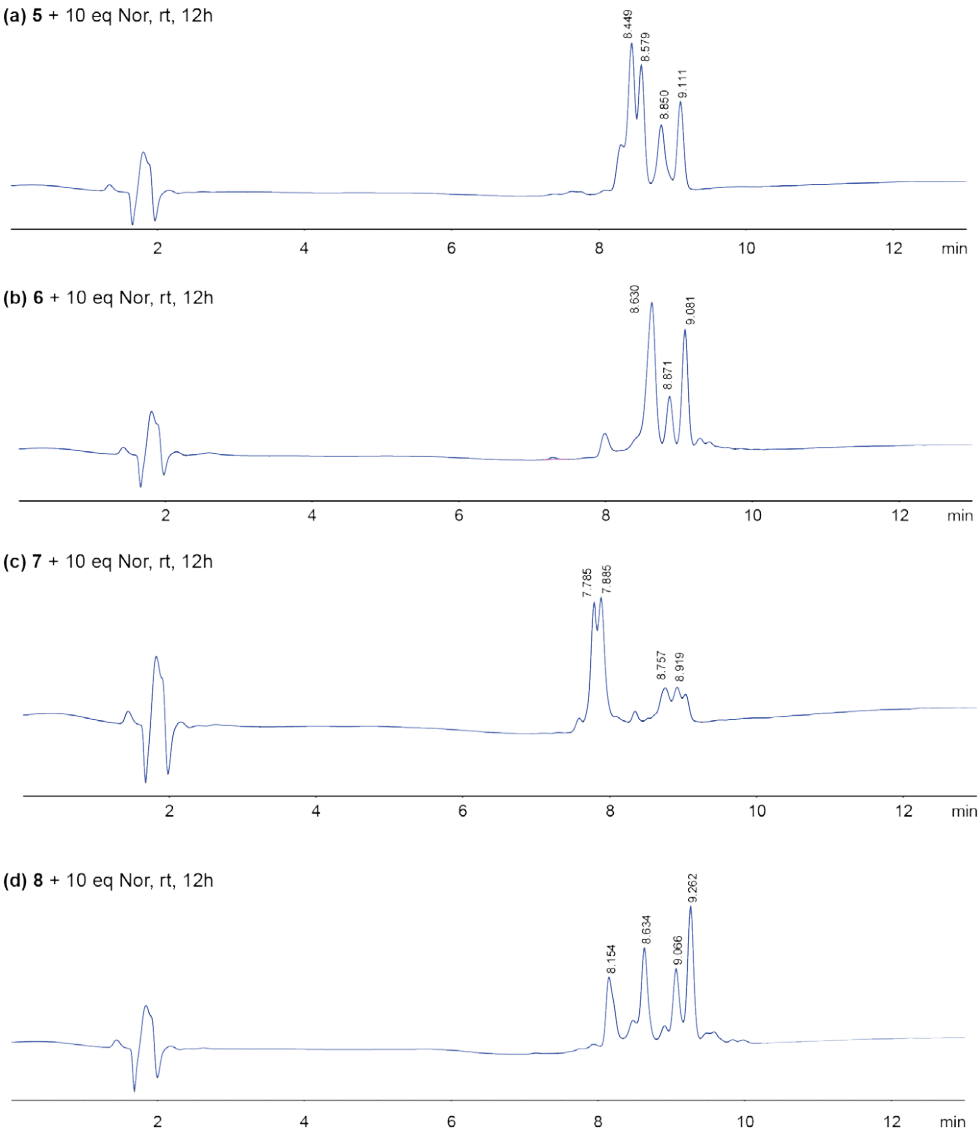
General procedure for the synthesis of tetrazine-fluorophore conjugates

To a solution of the succinimidyl ester or the isothiocyanate of the appropriate fluorophore (15 μmol) in anhydrous dmf, the corresponding tetrazine HCl salt **S5c**, **S6c** or **S8c** (30 μmol) and *N,N*-diisopropylethylamine (45 μmol) were added and the reaction mixture was stirred in the dark. The progress of the reaction was followed by LC-MS and after several hours the reaction was adjudged complete by consumption of the starting material. The solvent was evaporated and the residue dried under vacuum. The product was purified by preparative reverse phase HPLC using a gradient from 20% to 85% of buffer B in buffer A (buffer A: H_2O, 0.1% TFA; buffer B: acetonitril, 0.1% TFA). The identity and purity of the conjugates were confirmed by LC-MS (see **Supplementary Table 2** and **Supplementary Fig. 4**)

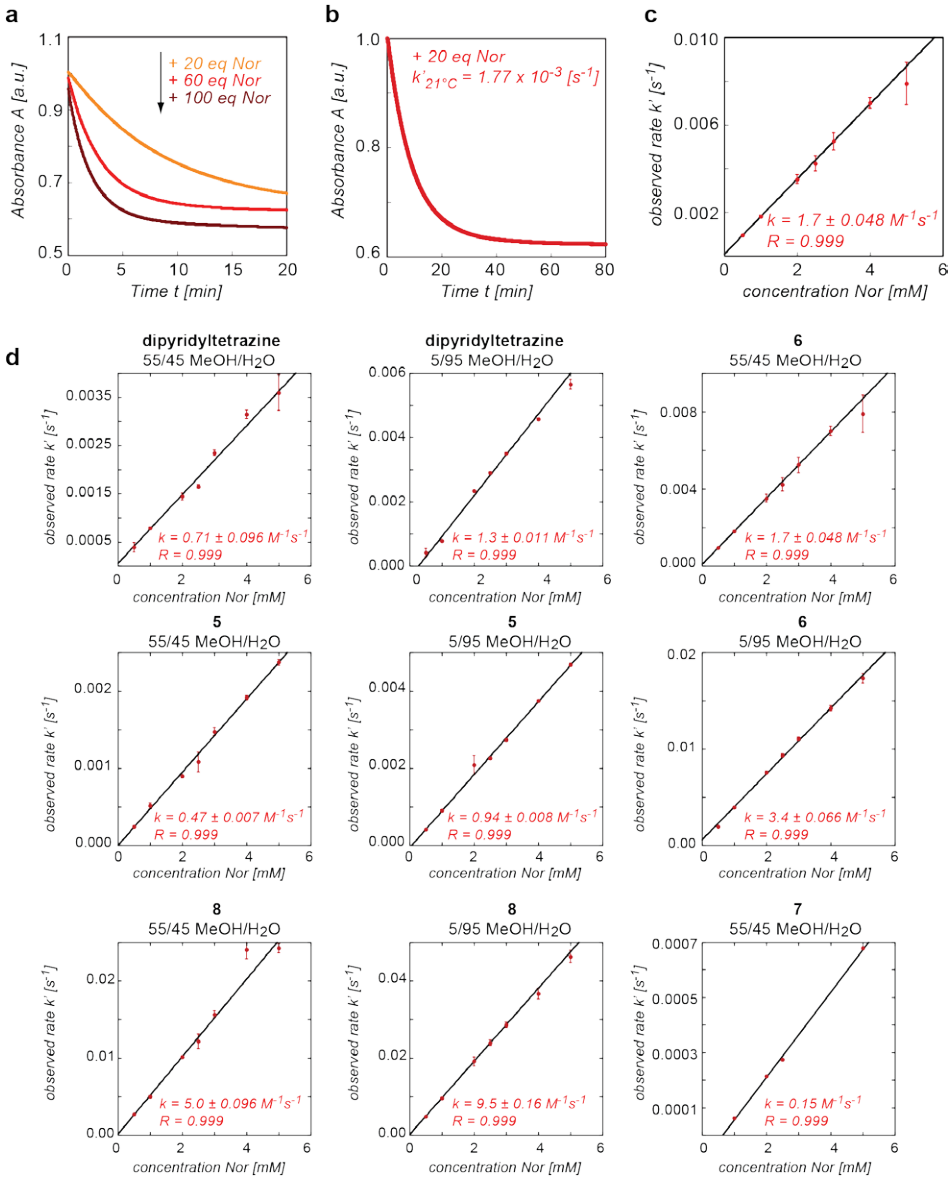


Supplementary Figure 1. Efficient, genetically directed incorporation of **2** and **3** using the PylRS/tRNA$_{CUA}$ pair in *E. coli*. Left: Amino acid dependent expression of sfGFP-His$_6$, bearing an amber codon at position 150, Myoglobin-His$_6$, bearing an amber codon at position 4 and T4-Lysozyme-His$_6$, bearing an amber codon at position 83. Coomassie stained SDS-PAGE gels and western blots performed with antibodies against the hexa-histidine tag are shown. Right: MS characterization of amino acid incorporation. sfGFP-**2**-His$_6$, found: 27975.5 ± 1.5 Da, calculated: 27977.5 Da; Myo-**2**-His$_6$, found: 18532.5 ± 1.5 Da, calculated: 18532.2; T4L-**2**-His$_6$, found: 19649.5 ± 1.5 Da, calculated: 19648.4.





Supplementary Figure 2. LC/MS trace (254 nm) showing the isomeric forms of dihydropyridazine products (**S15**, **S16**) formed from the reaction of the corresponding tetrazines **5**, **6**, **7**, and **8** with 10 equivalents of 5-norbornene-2-ol (Nor) in MeOH. The products for each reaction, eluting at different timepoints, have the same mass. (**a**) Reaction of **5** with Nor, $[M+H]^+$ calculated for $C_{26}H_{30}N_6O_4$, 491.55; found, 491.3. (**b**) Reaction of **6** with Nor, $[M+H]^+$ calculated for $C_{25}H_{29}N_9O_4$, 492.54; found, 491.5. (**c**) Reaction of **7** with Nor, $[M+H]^+$ calculated for $C_{23}H_{31}N_5O_4$, 442.52; found, 442.3. (**d**) Reaction of **8** with Nor, $[M+H]^+$ calculated for $C_{26}H_{31}N_7O_4$, 505.57; found, 505.6. The HPLC traces were taken after incubating the reactions for 12 hours at room temperature. The overall yield for conversion to the dihydropyridazine products is > 96%.



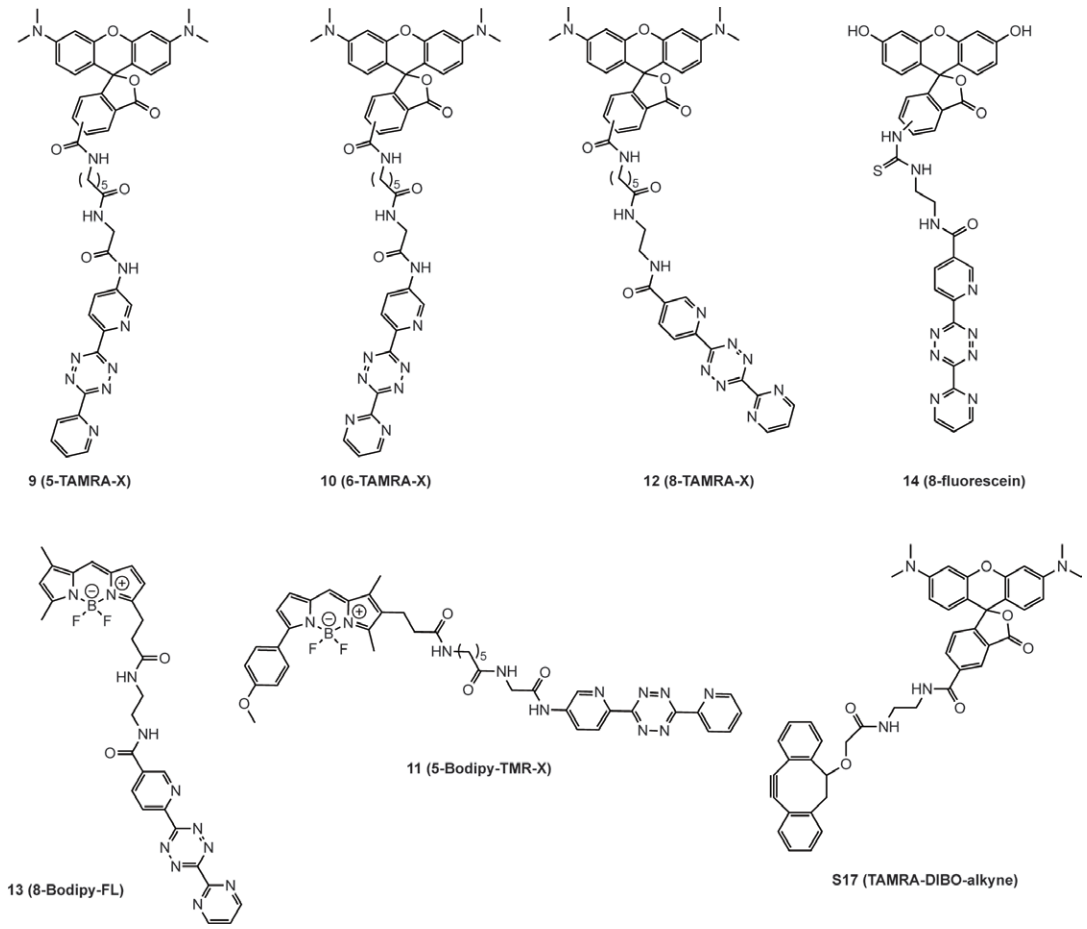
Supplementary Figure 3. Determination of rate constants k for the reaction of various tetrazines with 5-norbornene-2ol (Nor) by UV spectroscopy. (**a**) Dependence of the observed rate constant k' on the concentration of Nor; with increase in the concentration of Nor, the observed rate constant k' rises; (**b**) Response of the UV absorbance at 320 nm of compound **6** upon Nor addition (20 eq = 1 mM); by fitting the data to a single exponential equation, k' values were determined; (**c**) determined k' values for different concentrations were plotted against concentration of Nor and subjected to a linear fit, the slope of the plot yields the rate constant k. (**d**) Rate constants k obtained from plots of observed rate k' verss Nor concentration for various tetrazines (3,6-dipyridyl-1,2,4,5-tetrazine, **5**, **6**, **7**, and **8** in $MeOH/H_2O$ (either 55/45 or 5/95) mixtures. Observed rates k' were determined under pseudo-first order conditions from at least three different measurements (apart from **7**) and plotted against Nor concentration. Conditions: c_{tet} = 0.05 mM in 9/1 H_2O/MeOH, c_{Nor} = 0.5 to 5 mM in either MeOH or H_2O. All experiments were recorded at 21 ºC

Tetrazine	k_2 [$M^{-1}s^{-1}$][a]	k_2 [$M^{-1}s^{-1}$][b]
5	0.47 ± 0.0069	0.94 ± 0.0079
6	1.70 ± 0.048	3.41 ± 0.066
7	0.15	n.d
8	5.00 ± 0.096	9.46 ± 0.16
3,6-dipyridyltetrazine	0.71 ± 0.019	1.30 ± 0.011

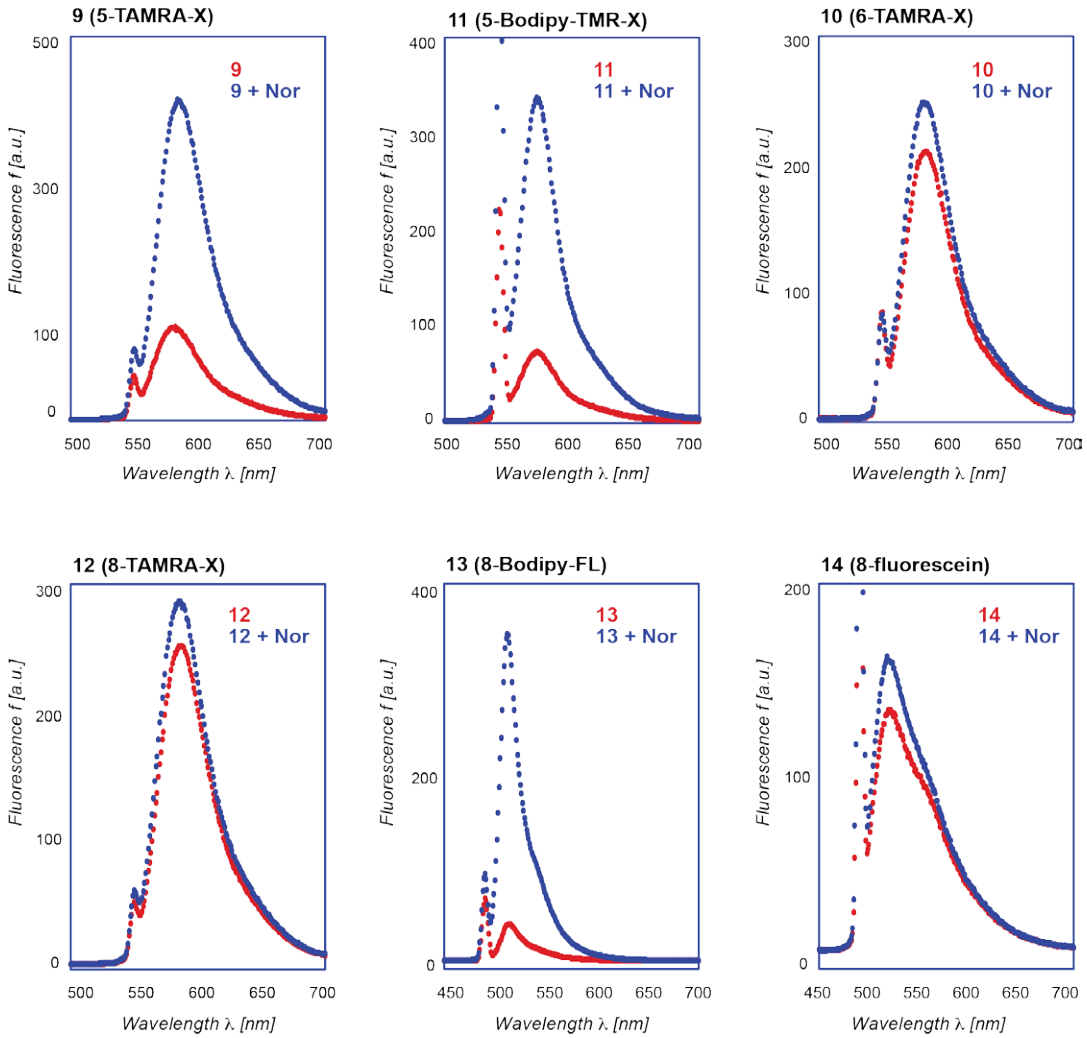
Supplementary Table 1. Rate constants for the reaction of various tetrazines (**5**, **6**, **7**, **8** and 3,6-dipyridyltetrazine) with 5-norbornene-2-ol at 21 ºC measured under pseudo first order conditions (Supplementary Figure 3). All values (apart from the one for **7**) were determined from at least three independent measurements. [a] solvent system: 55/45 methanol/water, [b] solvent system: 5/95 methanol/water.

Tetrazine-fluorophore	formula	Mass calculated for $[M+H]^+$	Mass found for $[M+H]^+$
9 (Tet1-TAMRA-X)	$C_{45}H_{43}N_{11}O_6$	834.34	834.5
10 (Tet2-TAMRA-X)	$C_{44}H_{42}N_{12}O_6$	835.35	836.0
11 (Tet1-Bodipy-TMR-X)	$C_{41}H_{42}BF_2N_{11}O_4$	802.65	802.8
12 (Tet4-TAMRA-X)	$C_{45}H_{44}N_{12}O_6$	849.35	849.5
13 (Tet4-Bodipy-FL)	$C_{28}H_{26}BF_2N_{11}O_2$	598.39	598.5
14 (Tet4-fluorescein)	$C_{35}H_{24}N_{10}O_6S$	713.69	713.3

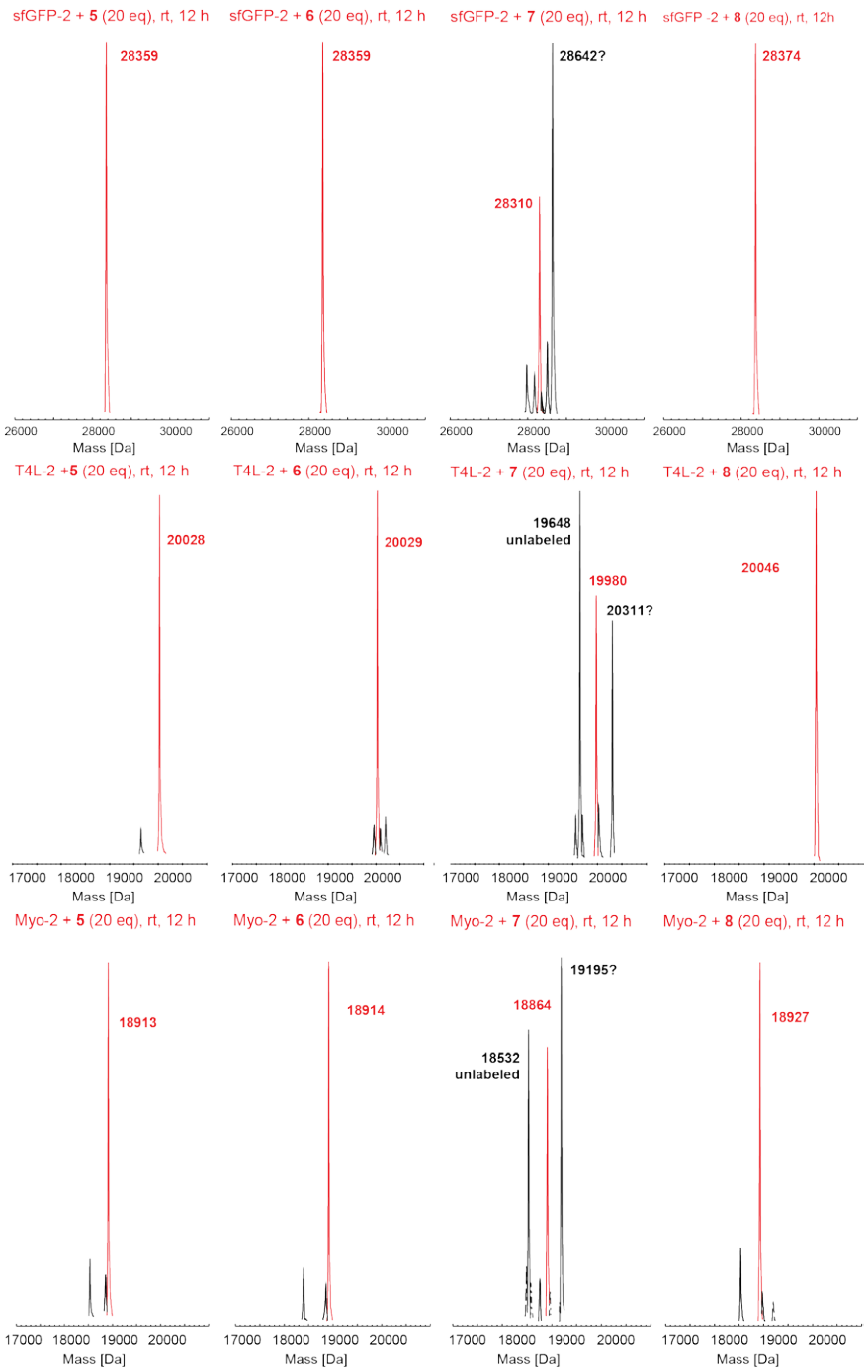
Supplementary Table 2. Mass spectrometry data for various tetrazine-fluorophores used in this study.



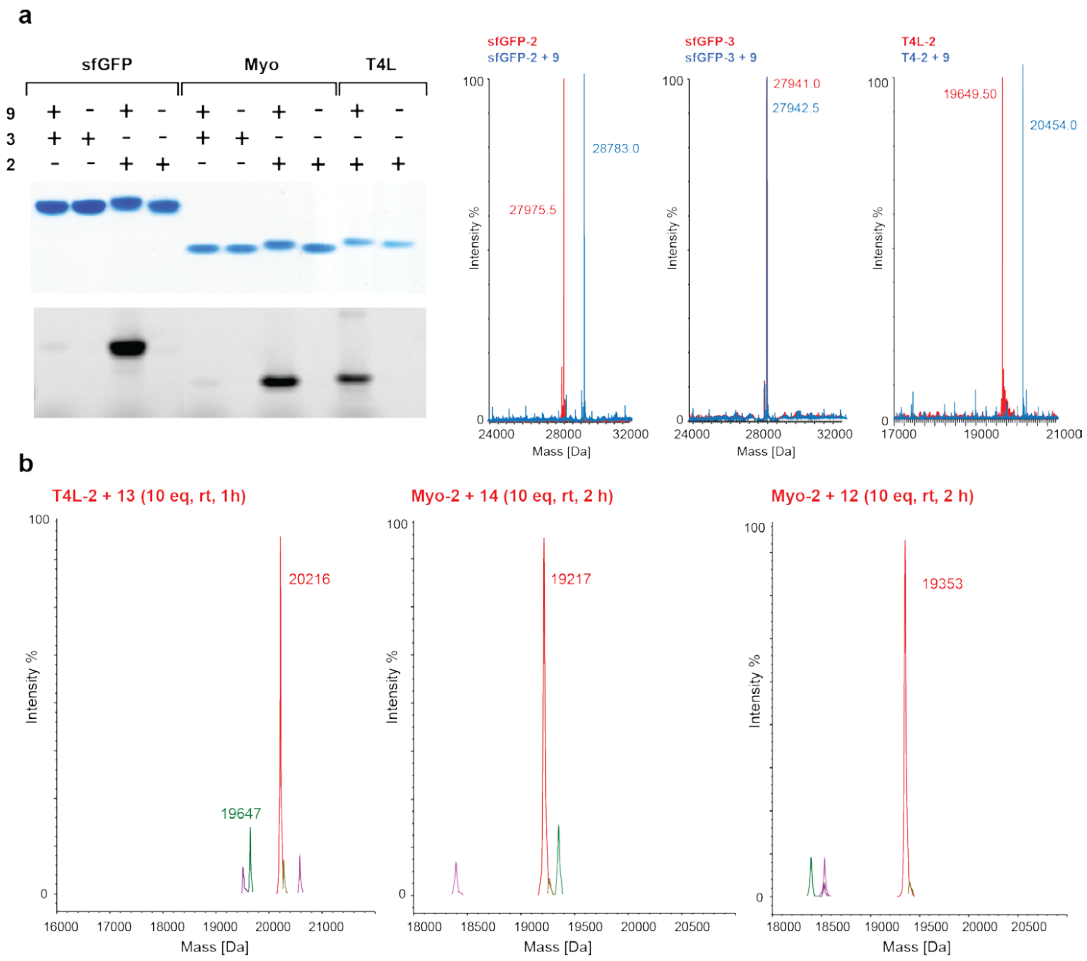
Supplementary Figure 4. Structural formulas of various tetrazine-fluorophores used in this study, as well as for the cyclooctyne DiBO-TAMRA dye (Invitrogen).



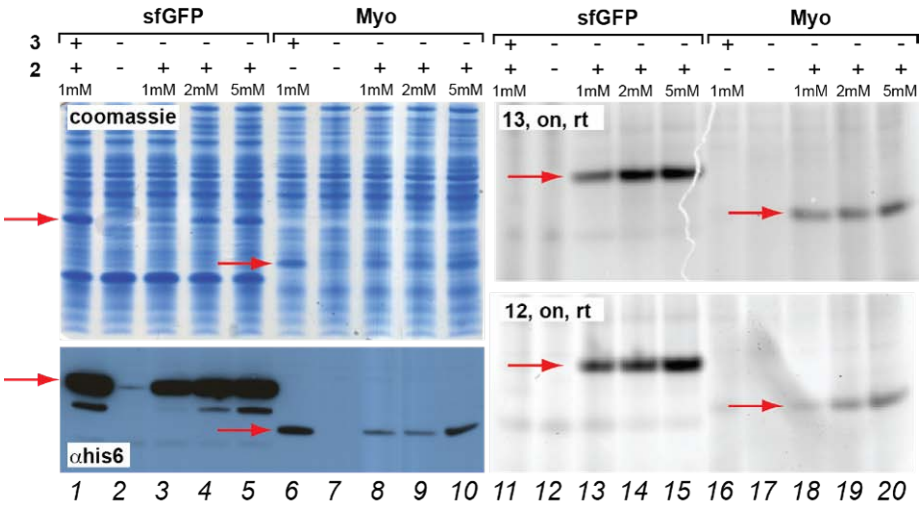
Supplementary Figure 5. "Turn on" fluorescence of tetrazine-fluorophores upon reaction with 5-norbornene-2-ol (Nor). A 2 μM solution of the corresponding tetrazine-fluorophore in water (2 mM stock solutions in DMSO) was reacted with 1000 equivalents of 5-norbornene-2-ol. Emission spectra were recorded before and 2h after the addtion of 5-norbornene-2-ol. Excitation wavelengths: TAMRA-dyes, 550 nm; Bodipy-FL, 490 nm; Bodipy-TMR-X, 544 nm; Fluorescein, 490 nm.



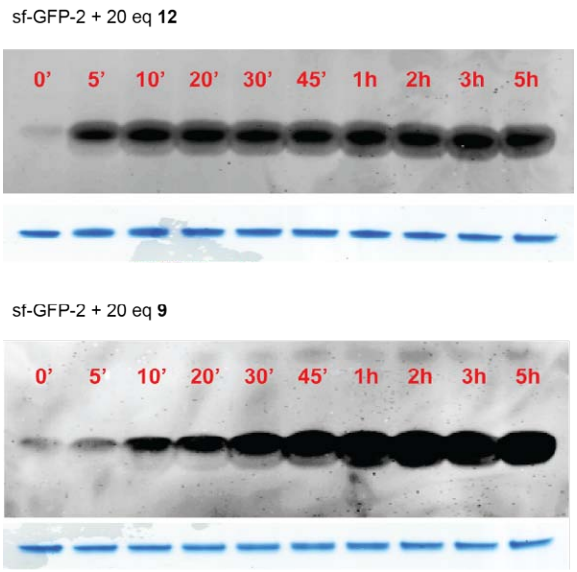
Supplementary Figure 6. LC-ESI-MS characterization of the labeling of sfGFP-**2**, T4L-**2** and Myo-**2** with various tetrazines. The red peaks denote the expected masses after reaction with **5**, **6**, **7**, and **8**, respectively. The reaction with **7** does not go to completion and leads to byproducts which were not analyzed further, while labeling with **5**, **6** and **8** is specific and quantitative. Reactions conditions: to a ~10 μM protein solution (in 20 mM Tris-HCl, 100 mM NaCl, pH 7.4) 20 equivalents of the corresponding tetrazine (1 mM stock solution in MeOH) were added and the reaction mixture incubated overnight at rt.



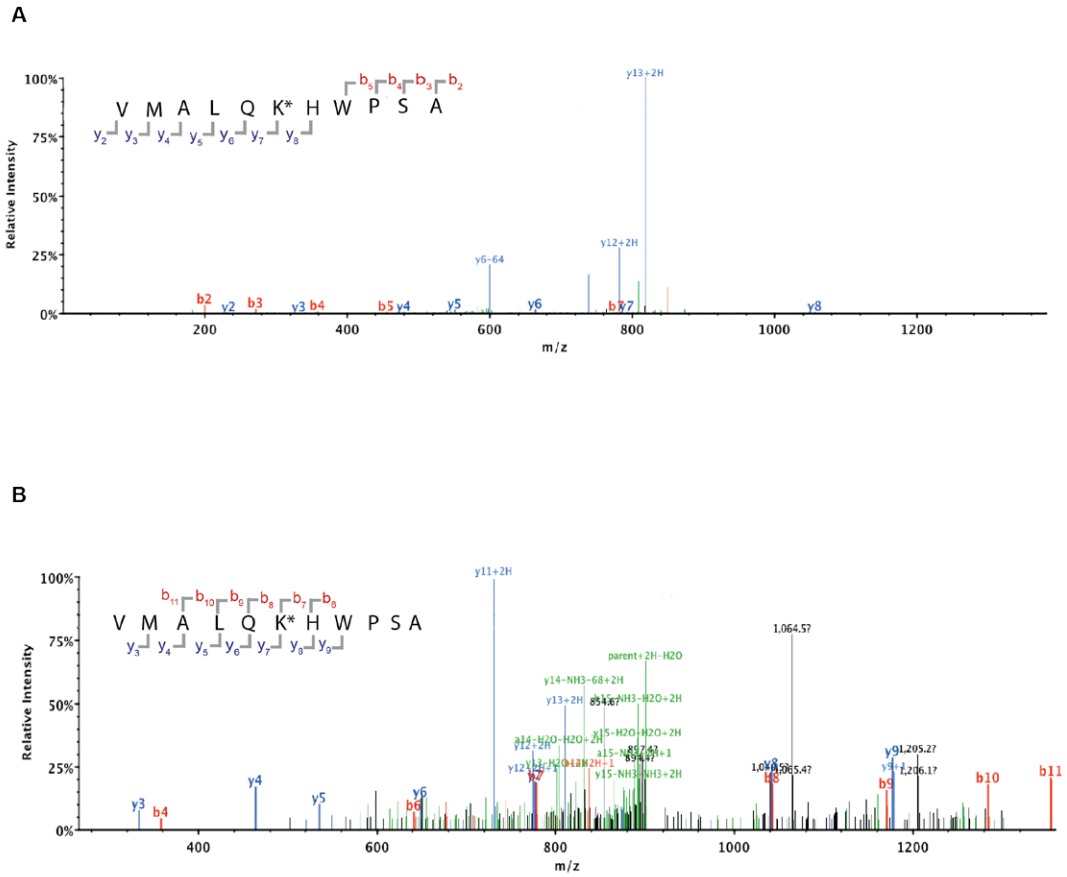
Supplementary Figure 7. (**A**) Specific and quantitative labeling of proteins containing **2** with tetrazine-fluorophore **9**, demonstrated by SDS PAGE (Coomassie staining and in gel fluorescence) and mass spectrometry. Reaction conditions: $c_{protein}$= 10 μM (in 20 mM Tris-HCl, 100 mM NaCl, pH 7.4), 10 equivalents of **9** added (stock solution 2 mM in dmso), 12 h, rt. Red mass spectrum shows pre-labeling (sfGFP-**2**, found 27975.5 ± 1.5 Da, expected 27977.5 Da; T4L-**2**, found: 19649.5 ± 1.5 Da, expected 19648.4). Blue mass spectrum is post-labeling (sfGFP-**2**, found 28783 ± 1.5 Da, expected 28784.4 Da; T4L-**2**, found: 20454 ± 1.5 Da, expected 20455.4 Da). No non-specific labeling was detected with a control protein containing **3**, sfGFP-**3**, mass expected 27941.4 Da, found 27941 ± 1.5 Da. (**B**) LC-ESI-MS characterization of the labeling of T4L-**2** and Myo-**2** with tetrazine-fluorophores **13**, **14** and **12**. T4L-**2**+**13**, expected mass, 20217.4 Da, found, 20216; Myo-**2**+**14**, expected mass, 19216.98 Da, found 19217 Da; Myo-**2**+**12**, expected mass, 19353.2 Da, found, 19353 Da. To a 10 μM protein solution (in 20 mM Tris-HCl, 100 mM NaCl, pH 7.4) 10 equivalents of tetrazine-fluorophore were added (2 mM stock solution in DMSO) and the reaction incubated at rt for the indicated time.



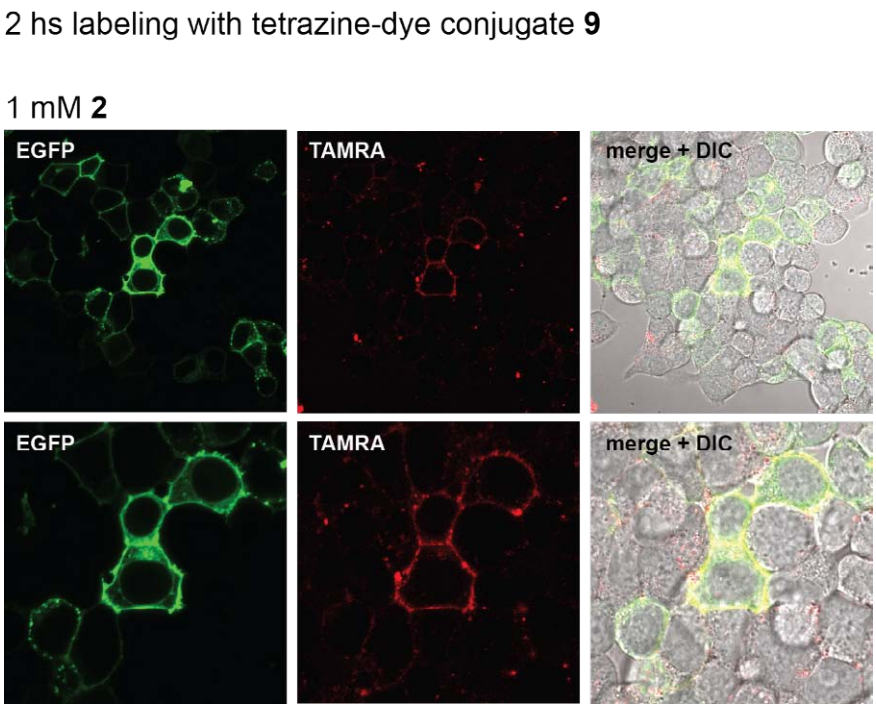
Supplementary Figure 8. Specificity of labeling **2** in sfGFP-**2** and Myo-**2** versus the *E. coli* proteome. sfGFP-**2** and Myo-**2** are marked with red arrows. Lanes 1-5: top: Coomassie stained gel showing proteins from *E. coli* producing sfGFP from p*sfGFP*150TAG*PylT*-His_6 in the presence of the indicated concentration of unnatural amino acids **2** or **3**; bottom: the expressed protein was detected in lysates using an anti His_6 antibody. Lanes 6-10: top: Coomassie stained gel showing proteins from *E. coli* producing myoglobin from p*Myo*4TAG*PylT*-His_6 in the presence of the indicated concentration of unnatural amino acids **2** or **3**; bottom: the expressed protein was detected in lysates using an anti His_6 antibody. Lanes 11-15: fluorescence images of sfGFP labeled with the indicated fluorophore **13** (top) and **12** (bottom), Lanes 16-20: fluorescence images of myoglobin labeled with the indicated fluorophore **13** (top) and **12** (bottom).



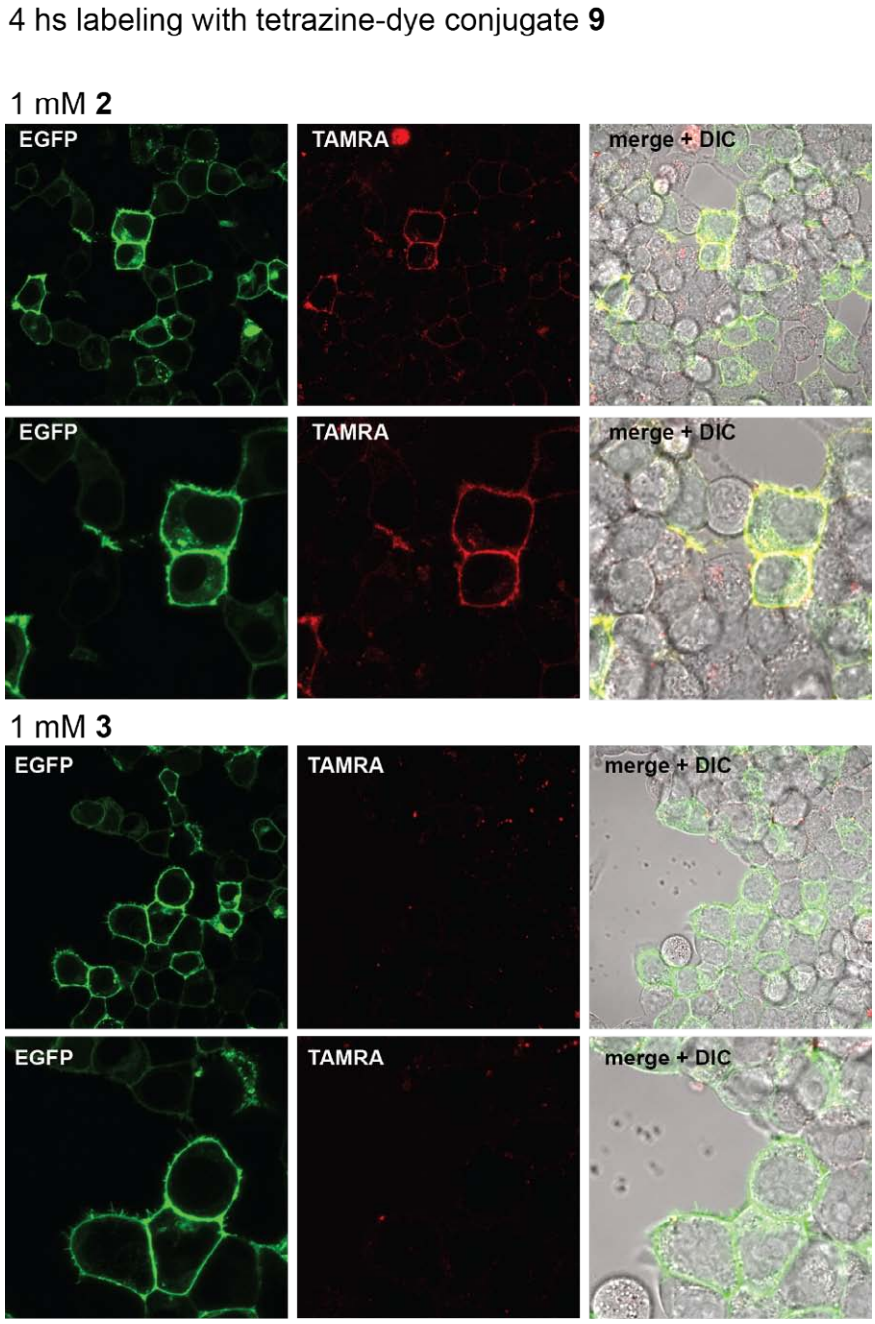
Supplementary Figure 9. The rapid labeling of sfGFP bearing **2** at position 150 with tetrazine-fluorophores **12** (top) and **9** (bottom). Fluorescence imaging SDS PAGE gels and Coomassie stained loading controls are shown.



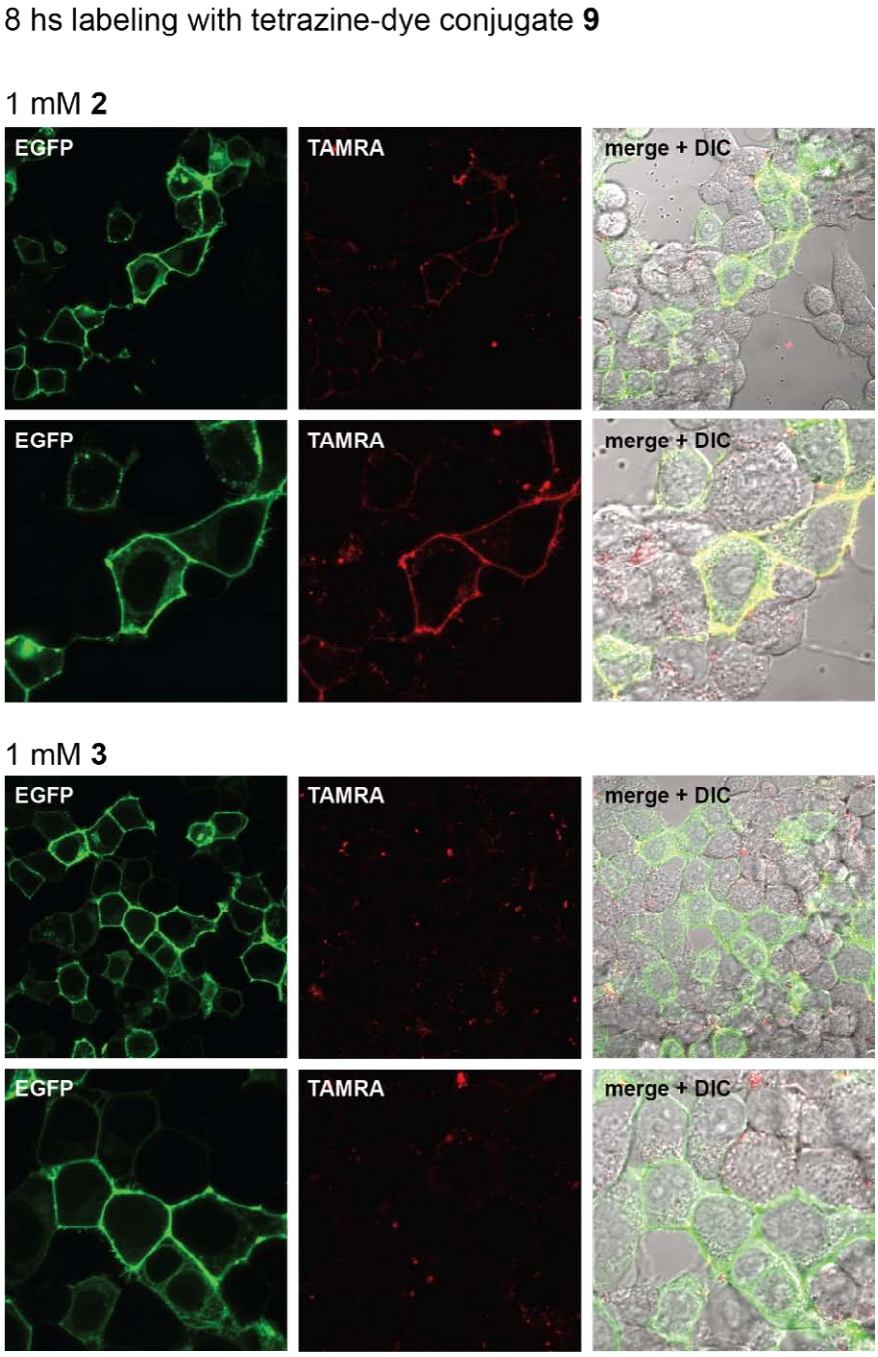
Supplementary Figure 10. MS/MS data showing the incorporation of **2** in the linker between GFP and mCherry.



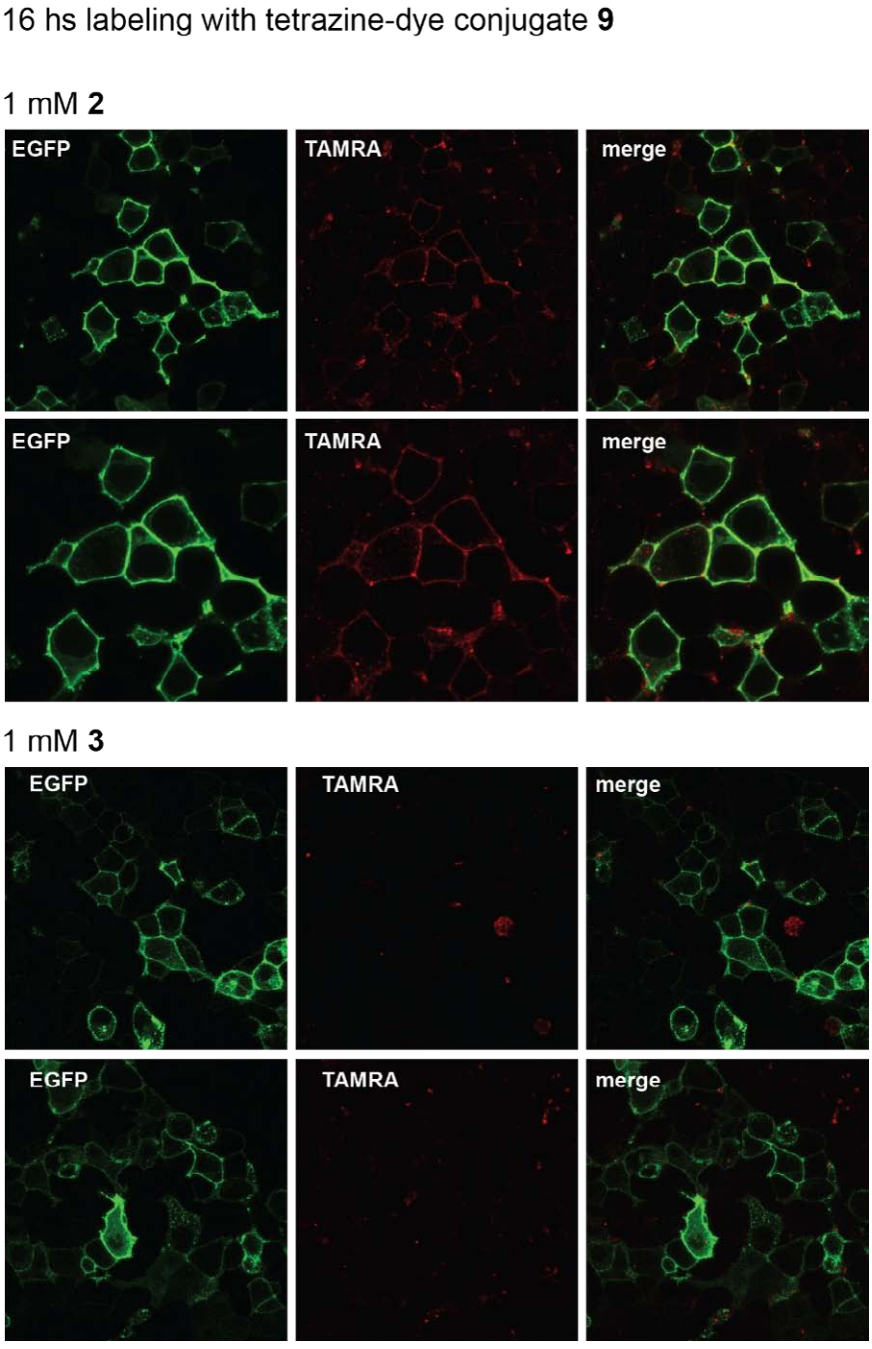
Supplementary Figure 11. Specific and rapid labeling of EGFR-GFP with tetrazine-fluorophore conjugate **9** for 2 hours. EGFR-GFP bearing **2** at position 128 is visible as green fluorescence at the membrane of transfected cells (left panels). Treatments of cells with **9** (200 nM) leads to selective labeling of EGFR-GFP containing **2** (middle panels). Cells were imaged two hours after addition of **9**. All images were taken using a 63 x oil immersion objective, lower images are at 2 x scanning zoom.



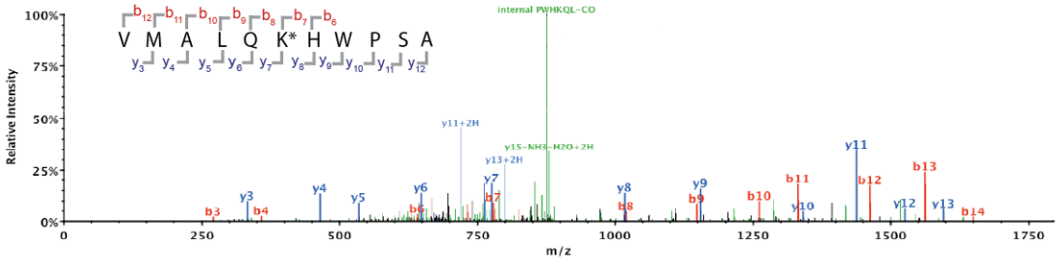
Supplementary Figure 12. Specific and rapid labeling of EGFR-GFP with tetrazine-fluorophore conjugate **9** for 4 hours. EGFR-GFP bearing **2** or **3** at position 128 is visible as green fluorescence at the membrane of transfected cells (left panels). Treatments of cells with **9** (200 nM) leads to selective labeling of EGFR-GFP containing **2** (middle panels). Cells were imaged 4 hours after addition of **9**. All images were taken using a 63 x oil immersion objective, lower images are at 2 x scanning zoom.



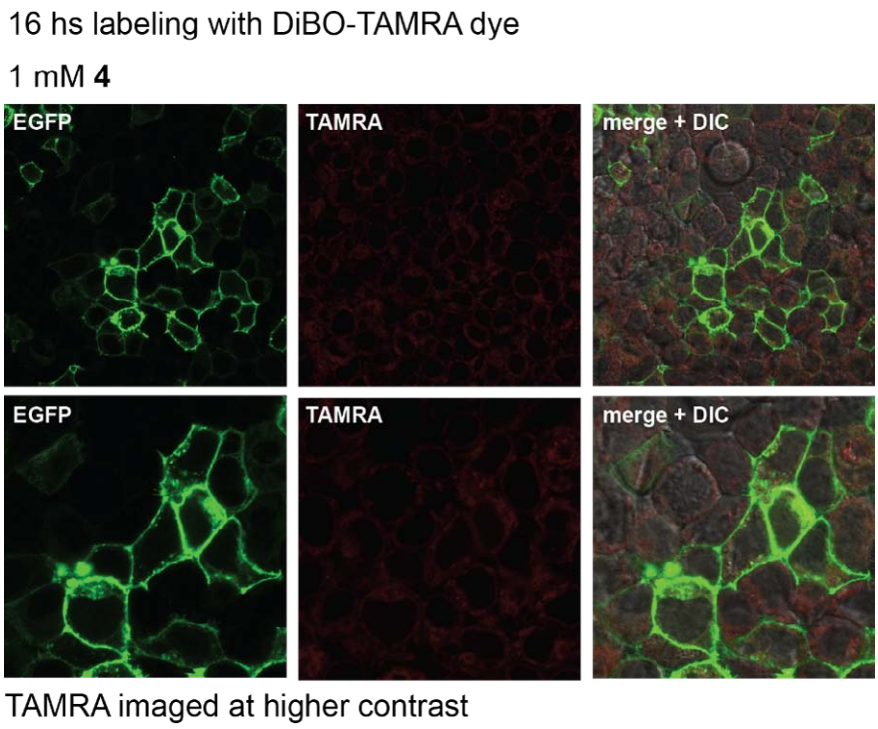
Supplementary Figure 13. Specific and rapid labeling of EGFR-GFP with tetrazine-fluorophore **9** for 8 hours. EGFR-GFP bearing **2** or **3** at position 128 is visible as green fluorescence at the membrane of transfected cells (left panels). Treatments of cells with **9** (200 nM) leads to selective labeling of EGFR-GFP containing **2** (middle panels). Cells were imaged 8 hours after addition of **9**.



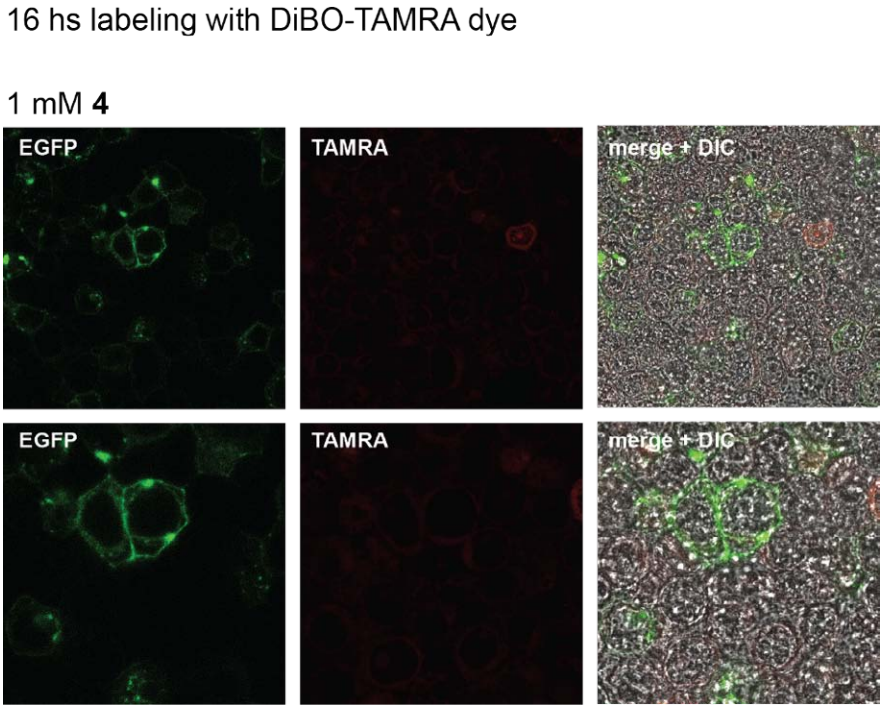
Supplementary Figure 14. Specific and rapid labeling of EGFR-GFP with tetrazine fluorophore **9** for 16 hours. EGFR-GFP bearing **2** or **3** at position 128 is visible as green fluorescence at the membrane of transfected cells (left panels). Treatments of cells with **9** (200 nM) leads to selective labeling of EGFR-GFP containing **2** (middle panels). Cells were imaged 16 hours after addition of **9**. All images were taken using a 63 x oil immersion objective, lower images are at 2 x scanning zoom.



Supplementary Figure 15. MS/MS data showing the incorporation of **4** in the linker between GFP and mCherry.



Supplementary Figure 16. Unsuccessful labeling of EGFR-GFP with the DIBO-TAMRA fluorophore for 16 hours. EGFR-GFP bearing **4** at position 128 is visible as green fluorescence at the membrane of transfected cells (left panels). However, when we attempted to label **4** with the cyclooctyne-based fluorophore **17** (200 nM) under identical conditions used to label **2** with **9**, we did not observe specific labeling. Cells were imaged 16 hours after addition of **17**. All images were taken using a 63 x oil immersion objective, lower images are at 2 x scanning zoom.



Supplementary Figure 17. Unsuccessful labeling of EGFR-GFP with the DIBO-TAMRA fluorophore for 16 hours. EGFR-GFP bearing **4** at position 128 is visible as green fluorescence at the membrane of transfected cells (left panels). We attempted to label **4** with a cyclooctyne based fluorophore **17** (200 nM) under conditions suggested by the supplier (see Materials and Methods), but did not observe specific labeling. Cells were imaged 16 hours after addition of **17**. All images were taken using a 63 x oil immersion objective, lower images are at 2 x scanning zoom.