SUPPLEMENTARY INFORMATION

Genetic Encoding of Bicyclononynes and *trans*-Cyclooctenes for Site-Specific Protein Labeling in Vitro and in Live Mammalian Cells via Rapid Fluorogenic Diels-Alder Reactions

Kathrin Lang[1], Lloyd Davis[1], Stephen Wallace[1], Mohan Mahesh[1], Daniel J. Cox[1], Melissa L. Blackman[2], Joseph M. Fox[2], & Jason W. Chin[1,*]

[1]Medical Research Council Laboratory of Molecular Biology, Hills Rd, Cambridge, CB2 0QH, United Kingdom

[2]Brown Laboratory, Department of Chemistry & Biochemistry, University of Delaware, Newark, Delaware 19716, United States

Correspondence:chin@mrc-lmb.cam.ac.uk

Full References cited in the main text:

1. Devaraj, N.K., Weissleder, R. & Hilderbrand, S.A. Tetrazine-based cycloadditions: application to pretargeted live cell imaging. *Bioconjug Chem* **19**, 2297-9 (2008).
2. Devaraj, N.K. & Weissleder, R. Biomedical applications of tetrazine cycloadditions. *Accounts of chemical research* **44**, 816-27 (2011).
3. Blackman, M.L., Royzen, M. & Fox, J.M. Tetrazine ligation: fast bioconjugation based on inverse-electron-demand Diels-Alder reactivity. *J Am Chem Soc* **130**, 13518-9 (2008).
4. Taylor, M.T., Blackman, M.L., Dmitrenko, O. & Fox, J.M. Design and synthesis of highly reactive dienophiles for the tetrazine-trans-cyclooctene ligation. *Journal of the American Chemical Society* **133**, 9646-9 (2011).
5. Liu, D.S. et al. Diels-Alder cycloaddition for fluorophore targeting to specific proteins inside living cells. *Journal of the American Chemical Society* **134**, 792-5 (2012).
6. Seitchik, J.L. et al. Genetically Encoded Tetrazine Amino Acid Directs Rapid Site-Specific in Vivo Bioorthogonal Ligation with trans-Cyclooctenes. *Journal of the American Chemical Society* **134**, 2898-901 (2012).
7. Lang, K. et al. Genetically encoded norbornene directs site-specific cellular protein labelling via a rapid bioorthogonal reaction. *Nature chemistry* **4**, 298-304 (2012).
8. Kaya, E. et al. A genetically encoded norbornene amino Acid for the mild and selective modification of proteins in a copper-free click reaction. *Angewandte Chemie* **51**, 4466-9 (2012).
9. Plass, T. et al. Amino Acids for Diels-Alder Reactions in Living Cells. *Angewandte Chemie-International Edition* **51**, 4166-4170 (2012).
10. Dommerholt, J. et al. Readily Accessible Bicyclononynes for Bioorthogonal Labeling and Three-Dimensional Imaging of Living Cells. *Angewandte Chemie-International Edition* **49**, 9422-9425 (2010).
11. Chen, W.X., Wang, D.Z., Dai, C.F., Hamelberg, D. & Wang, B.H. Clicking 1,2,4,5-tetrazine and cyclooctynes with tunable reaction rates. *Chemical Communications* **48**, 1736-1738 (2012).

12. McKay, C.S., Blake, J.A., Cheng, J., Danielson, D.C. & Pezacki, J.P. Strain-promoted cycloadditions of cyclic nitrones with cyclooctynes for labeling human cancer cells. *Chemical Communications* **47**, 10040-2 (2011).
13. McKay, C.S., Chigrinova, M., Blake, J.A. & Pezacki, J.P. Kinetics studies of rapid strain-promoted [3 + 2]-cycloadditions of nitrones with biaryl-aza-cyclooctynone. *Organic & biomolecular chemistry* **10**, 3066-70 (2012).
14. Ning, X. et al. Protein modification by strain-promoted alkyne-nitrone cycloaddition. *Angewandte Chemie* **49**, 3065-8 (2010).
15. Agard, N.J., Prescher, J.A. & Bertozzi, C.R. A strain-promoted [3 + 2] azide-alkyne cycloaddition for covalent modification of biomolecules in living systems. *Journal of the American Chemical Society* **126**, 15046-7 (2004).
16. Sletten, E.M. & Bertozzi, C.R. From mechanism to mouse: a tale of two bioorthogonal reactions. *Accounts of chemical research* **44**, 666-76 (2011).
17. Karver, M.R., Weissleder, R. & Hilderbrand, S.A. Bioorthogonal reaction pairs enable simultaneous, selective, multi-target imaging. *Angewandte Chemie* **51**, 920-2 (2012).
18. Devaraj, N.K., Hilderbrand, S., Upadhyay, R., Mazitschek, R. & Weissleder, R. Bioorthogonal Turn-On Probes for Imaging Small Molecules inside Living Cells. *Angew Chem Int Ed Engl* **49**, 2869-2872 (2010).
19. Fekner, T., Li, X., Lee, M.M. & Chan, M.K. A pyrrolysine analogue for protein click chemistry. *Angew Chem Int Ed Engl* **48**, 1633-5 (2009).
20. Nguyen, D.P., Garcia Alai, M.M., Kapadnis, P.B., Neumann, H. & Chin, J.W. Genetically encoding N(epsilon)-methyl-L-lysine in recombinant histones. *J Am Chem Soc* **131**, 14194-5 (2009).
21. Nguyen, D.P. et al. Genetic encoding and labeling of aliphatic azides and alkynes in recombinant proteins via a pyrrolysyl-tRNA Synthetase/tRNA(CUA) pair and click chemistry. *J Am Chem Soc* **131**, 8720-1 (2009).
22. Nguyen, D.P., Elliott, T., Holt, M., Muir, T.W. & Chin, J.W. Genetically Encoded 1,2-Aminothiols Facilitate Rapid and Site-Specific Protein Labeling via a Bio-orthogonal Cyanobenzothiazole Condensation. *J Am Chem Soc* **133**, 11418-21 (2011).

23. Neumann, H., Peak-Chew, S.Y. & Chin, J.W. Genetically encoding N(epsilon)-acetyllysine in recombinant proteins. *Nat Chem Biol* **4**, 232-4 (2008).
24. Polycarpo, C.R. et al. Pyrrolysine analogues as substrates for pyrrolysyl-tRNA synthetase. *FEBS Lett* **580**, 6695-700 (2006).
25. Li, X., Fekner, T., Ottesen, J.J. & Chan, M.K. A pyrrolysine analogue for site-specific protein ubiquitination. *Angew Chem Int Ed Engl* **48**, 9184-7 (2009).
26. Wang, Y.S., Fang, X., Wallace, A.L., Wu, B. & Liu, W.R. A Rationally Designed Pyrrolysyl-tRNA Synthetase Mutant with a Broad Substrate Spectrum. *Journal of the American Chemical Society* **134**, 2950-3 (2012).
27. Mukai, T. et al. Adding l-lysine derivatives to the genetic code of mammalian cells with engineered pyrrolysyl-tRNA synthetases. *Biochem Biophys Res Commun* **371**, 818-22 (2008).
28. Hancock, S.M., Uprety, R., Deiters, A. & Chin, J.W. Expanding the genetic code of yeast for incorporation of diverse unnatural amino acids via a pyrrolysyl-tRNA synthetase/tRNA pair. *J Am Chem Soc* **132**, 14819-24 (2010).
29. Greiss, S. & Chin, J.W. Expanding the genetic code of an animal. *Journal of the American Chemical Society* **133**, 14196-9 (2011).
30. Lin, S.X. et al. Site-Specific Incorporation of Photo-Cross-Linker and Bioorthogonal Amino Acids into Enteric Bacterial Pathogens. *Journal of the American Chemical Society* **133**, 20581-20587 (2011).
31. Gautier, A. et al. Genetically encoded photocontrol of protein localization in mammalian cells. *J Am Chem Soc* **132**, 4086-8 (2010).
32. Virdee, S. et al. Traceless and site-specific ubiquitination of recombinant proteins. *Journal of the American Chemical Society* **133**, 10708-11 (2011).

Protein expression and purification

To express sfGFP with incorporated unnatural amino acid **1**, we transformed *E. coli* DH10B cells with *pBKBCNRS* (which encodes *Mb*BCNRS) and p*sfGFP*150TAG*PylT*-His_6 (which encodes *Mb*$tRNA_{CUA}$ and a C-terminally hexahistidine tagged sfGFP gene with an amber codon at position 150). Cells were recovered in 1 ml of S.O.B media (supplemented with 0.2 % glucose) for 1 h at 37 ºC, before incubation (16 h, 37 ºC, 230 r.p.m) in 100 ml of LB containing ampicillin (100 μg/mL) and tetracycline (25 μg/mL). 20 ml of this overnight culture was used to inoculate 1 L of LB supplemented with ampicillin (50 μg/mL) and tetracycline (12 μg/mL) and incubated at 37 ºC. At OD_{600} = 0.4 to 0.5, a solution of **1** in H_2O was added to a final concentration of 2 mM. After 30 min, protein expression was induced by the addition of arabinose to a final concentration of 0.2 %. After 3 h of induction, cells were harvested by centrifugation and and frozen at -80 ºC until required. Cells were thawed on ice and suspended in 30 ml of lysis buffer (10 mM Tris-HCl, 20 mM imidazole, 200 mM NaCl, pH 8, 1mM phenylmethanesulfonylfluoride, 1 mg/mL lysozyme, 100 μg/mL DNaseA, Roche protease inhibitor). Proteins were extracted by sonication at 4 ºC. The extract was clarified by centrifugation (20 min, 21.000 g, 4 ºC), 600 μL of Ni^{2+} - NTA beads (Qiagen) were added to the extract and the mixture was incubated with agitation for 1 h at 4 ºC. Beads were collected by centrifugation (10 min, 1000 g). The beads were three times resuspended in 30 mL wash buffer (20 mM Tris-HCl, 30 mM imidazole, 300 mM NaCl, pH 8) and spun down at 1000g. Subsequently, the beads were resuspended 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 and further purified by size-exclusion chromatography employing a HiLoad 16/60 Superdex 75 Prep Grade column (GE Life Sciences) at a flow rate of 1 mL/min (buffer: 20 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, pH 7.4). Fractions containing the protein were pooled and concentrated with an Amicon Ultra-15 3 kDa MWCO centrifugal filter device (Millipore). Purified proteins were analyzed by 4-12 % SDS-PAGE and their mass confirmed by mass spectrometry (see Supplementary Information). SfGFP with incorporated **2** and **3**, sfGFP-**2**, sfGFP-**3** were prepared in the same way, expect that cells were transformed with pBKTCORS (which encodes MbTCORS) and and p*sfGFP*150TAG*PylT*-His_6 (which encodes *Mb*$tRNA_{CUA}$ and a C-terminally hexahistidine tagged sfGFP gene with an amber codon at position 150). SfGFP with incorporated **4** and **5**, sfGFP-**4**, sfGFP-**5** were prepared in the same way,

expect that cells were transformed with pBKPylRS (which encodes MbPylRS) and and p*sfGFP*150TAG*PylT*-His_6 (which encodes *Mb*$tRNA_{CUA}$ and a C-terminally hexahistidine tagged sfGFP gene with an amber codon at position 150). Yields of purified proteins were up to 6-12 mg/L.

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).

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.

Protein labelling via tetrazine-BCN or tetrazine-TCO cycloaddition

In vitro labelling of purified proteins with different tetrazines

To 40 μL of purified recombinant protein (~10 μM in 20 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, pH 7.4) 4 μL of a 1 mM solution of tetrazine compounds **6**, **7**, **8**, or **9** in MeOH were added (~ 10 or 20 equivalents). After 30 minutes of incubation at room temperature, the solutions were analyzed by LC-ESI-MS. (Supplementary Figure **S9**)

In vitro labelling of purified proteins with tetrazines and tetrazine-dye conjugates: Purified recombinant sfGFP with site-specifically incorporated **1** or **2**, sfGFP-**1** or sfGFP-**2** (~10 μM in 20 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, pH 7.4), was incubated with 10 equivalents of the tetrazine-dye conjugates **11**, **12**, **13**, **14**, **15** or **16**, respectively (2 mM in DMSO). The solution was incubated at room temperature and aliquots were taken after 30 min to 3 hours and analyzed by SDS PAGE and - after desalting with a C4-ZIPTIP - by ESI-MS. The SDS PAGE gels were either stained with coomassie or scanned with a Typhoon imager to visualize in-gel fluorescence (**Figure 4** and **Supplementary Figure S8**).

In vitro labelling of purified proteins with tetrazines-dye conjugates as a function of time:

2 nmol of purified sfGFP-**1**, sfGFP-**2** or sfGFP-**4** (10 μM in 20 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, pH 7.4) were incubated with 20 nmol of tetrazine-dye conjugate **11** (10 μl of a 2 mM solution in DMSO). At different time points (0, 30 s, 1 min, 2 min, 5 min, 10 min, 30 min, 1 h, 2 h, 3 h) 8 μL aliquots were taken from the solution and quenched with a 700-fold excess of BCN or TCO and plunged into liquid nitrogen. Samples were mixed with NuPAGE LDS sample buffer supplemented with 5 % β-mercaptoethanol, heated for 10 min to 90ºC and analyzed by 4-12% SDS page. The amounts of labelled proteins were quantified by scanning the fluorescent bands with a Typhoon Trio phosphoimager (GE Life Sciences). Bands were quantified with the ImageQuant^{TM} TL software (GE Life Sciences) using rubber band background subtraction. In gel fluorescence shows that labelling is complete within 1 h for sfGFP-**4** using 10 equivalents tetrazine-fluorophore **11** (**Figure 4e**), whereas the labelling of sfGFP-**1** and sfGFP-**2** was complete within the few seconds it took to measure the first time point.

Labelling of the whole E. coli proteome with tetrazine-dye conjugates:

E. coli DH10B cells containing either p*sfGFP*150TAG*PylT*-His_6 and *pBKBCNRS* or p*sfGFP*150TAG*PylT*-His_6 and *pBKPylRS* were inoculated into LB containing ampicillin (for *pBKBCNRS*, 100 μg/mL) or kanamycin (for *pBKPylRS* 50 μg/mL) and tetracycline (25 μg/mL). The cells were incubated with shaking overnight at 37 ºC, 250 rpm. 2 mL of overnight culture was used to inoculate into 100 mL of LB supplemented with ampicillin (50 μg/mL) and tetracycline (12 μg/mL) or kanamycin

(25 μg/mL) and tetracycline (12 μg/mL) and incubated at 37 ºC. At OD_{600} = 0.5, 3 ml culture aliquots were removed and supplemented with different concentrations (1 mM, 2 mM and 5 mM) of **1** and 1 mM of **5**. After 30 min of incubation with shaking at 37 ºC, protein expression was induced by the addition of 30 μL of 20 % arabinose. After 3.5 h of expression, cells were collected by centrifugation (16000 g, 5 min) of 1 mL of cell suspension. The cells were resuspended in PBS buffer, spun down again and the supernatant was discarded. This process was repeated twice more. Finally, the washed cell pellet was suspended in 100 μL PBS and incubated with 3 μL of tetrazine-dye conjugate **11** (2 mM in DMSO) at rt for 30 minutes. After adding a 200-fold excess of BCN in order to quench non-reacted tetrazine-dye, the cells were resuspended in 100 μL of NuPAGE LDS sample buffer supplemented with 5 % β-mercaptoethanol, heated at 90 ºC for 10 min and centrifuged at 16000 g for 10 min. The crude cell lysate was analyzed by 4-12 % SDS-PAGE to assess protein levels. Gels were either Coomassie stained or scanned with a Typhoon imager to make fluorescent bands visible (**Supplementary Figure S9 and S10**). Western blots were performed with antibodies against the hexahistidine tag (Cell Signaling Technology, His tag 27E8 mouse mAb #2366).

Stopped-flow determination of Kinetic Rate Constants for Small Molecule Cycloadditions

Rate constants k for different tetrazines were measured under pseudo first order conditions with a 10- to 100-fold excess of BCN or TCO in methanol/water mixtures by following the exponential decay in UV absorbance of the tetrazine at 320, 300 or 280 nm over time with a stopped-flow device (Applied Photophysics, **Supplementary Figure S2** and **S3** and **Supplementary Table 1**). Stock solutions were prepared for each tetrazine (0.1 mM in 9/1 water/methanol) and for BCN and TCO (1 to 10 mM in methanol). Both tetrazine and BCN and TCO solutions were thermostatted in the syringes of the stopped flow device before measuring. Mixing equal volumes of the prepared stock solutions via the stopped-flow apparatus resulted in a final concentration of 0.05 mM tetrazine and of 0.5 to 5 mM BCN or TCO, corresponding to 10 to 100 equivalents of BCN or TCO. Spectra were recorded using the following instrumental parameters: wavelength, 320 nm for **6** and **7**; 300 nm for **8**, 280 nm for **9**; 500 to 5000 datapoints per second). All measurements were conducted

at 25 ºC. Data were fit to a single-exponential equation for BCN-tetrazine reactions and to a sum of two single exponential equations for TCO-tetrazine reactions. Each measurement was carried out three to five times and the mean of the observed rates k' (the first exponential equation in case of the TCO-tetrazine reaction) was plotted against the concentration of BCN or TCO to obtain the rate constant k from the slope of the plot. For all four tetrazines complete measurement sets were done in duplicate and the mean of values is reported in **Supplementary Table 1**. All data processing was performed using Kaleidagraph software (Synergy Software, Reading, UK).

Cloning for Mammalian Cell Applications

The plasmids *pMmPylS-mCherry-TAG-EGFP-HA*[1,2] and *pMmPylRS-EGFR-(128TAG)-GFP-HA*[2] were both digested with the enzymes AflII and EcoRV (NEB) to remove the wild-type *Mm*PylRS. A synthetic gene of the mutant synthetase MbBCNRS and *MbTCORS* was made by GeneArt with the same flanking sites. The synthetic *MbBCNR*S and *MbTCORS* were also digested with AflII and EcoRV and cloned in place of the wild-type synthetase (*MmPylS*). Using a rapid ligation kit (Roche) vectors *pMbBCNRS-mCherry-TAG-EGFP-HA, pMbBCNRS-EGFR(128TAG)-GFP-HA* and *pMbTCORS-EGFR(128TAG)-GFP-HA* were created. The *pCMV-cJun-TAG-mCherry-MbBCNRS* plasmid was created from a *pCMV-cJun-TAG-mCherry-MmPylRS* plasmid (created by Fiona Townsley) by exchanging *MmPylRS* for *MbBCNRS.* This was carried out as for the *pMbBCNRS-mCherry-TAG-EGFP-HA* plasmid.

Incorporation of amino acid 1, 2 and 3 in HEK293 cells

HEK293 cells were plated on poly-lysine coated μ-dishes (Ibidi). After growing to near confluence in 10% fetal bovine serum (FBS) Dulbecco's modified eagle medium (DMEM) cells were transfected with 2μg of *pMbBCNRS-EGFR(128TAG)-GFP-HA* and 2μg of *p4CMVE-U6-PylT* (which contains four copies of the wild-type pyrrolysyl tRNA)[1,2] using lipofectamin 2000 (Life Technologies). After transfection cells were left to grow overnight in 10% FBS DMEM at 37°C and 5% CO_2. For a western blot, cells were plated on 24 well plates and grown to near confluence. Cells were transfected using lipofectamine 2000 with the *pMbBCNRS-mCherry-TAG-EGFP-HA* or *pMmPylRS-mCherry-TAG-EGFP-HA* or *pTCORS-mCherry-TAG-EGFP-HA*

construct and the *p4CMVE-U6-PylT* plasmid. After 16 hours growth with or without 0.5 mM **1**, 1 mM **2** or 1 mM **5** cells were lysed on ice using RIPA buffer (Sigma). The lysates were spun down and the supernatant was added to 4x LDS sample buffer (Life technologies). The samples were run out by SDS-PAGE, transferred to a nitrocellulose membrane and blotted using primary rat anti-HA (Roche) and mouse anti-FLAG (Ab frontier), secondary antibodies were anti-rat (Santa Cruz Biotech) and anti-mouse (Cell Signaling) respectively.

Labelling of mammalian cell surface protein

Cells were plated onto a poly-lysine coated μ-dish and after growing to near confluence were transfected with 2μg each of *pMbBCNRS-EGFR(128TAG)-GFP-HA* or *pMbTCORS-EGFR(128TAG)-GFP-HA* and *p4CMVE-U6-PylT*. After 8-16 hours growth at 37°C and at 5% CO_2 in DMEM with 0.1%FBS in the presence of 0.5 mM **1** (0.5% DMSO), 1 mM **2** or 1 mM **3** cells were washed in DMEM with 0.1% FBS and then incubated in DMEM with 0.1%FBS overnight. The following day cells were washed once more before 400 nM terazine-dye conjuagate **11** was added for 2-60 minutes. The media was exchanged twice and cells were then imaged. Imaging was carried out on a Zeiss 780 laser scanning microscope with a Plan apochromat 63X oil immersion objective; scan zoom: 1x or 2x; scan resolution: 512 x 512; scan speed: 9; averaging: 16x. EGFP was excited at 488 nm and imaged at 493 to 554 nm; TAMRA was excited and detected at 561nm and 566-685 nm respectively.

Controls were performed similarly but transfected with *pMmPylRS-EGFR(128TAG)-GFP-HA* instead of *pMbBCNRS-EGFR(128TAG)-GFP-HA*. Cells were grown overnight in the presence of 1 mM **5** and in the absence or presence of 0.5% DMSO (as would be the case for amino acid **1**).

Labeling of mammalian nuclear protein

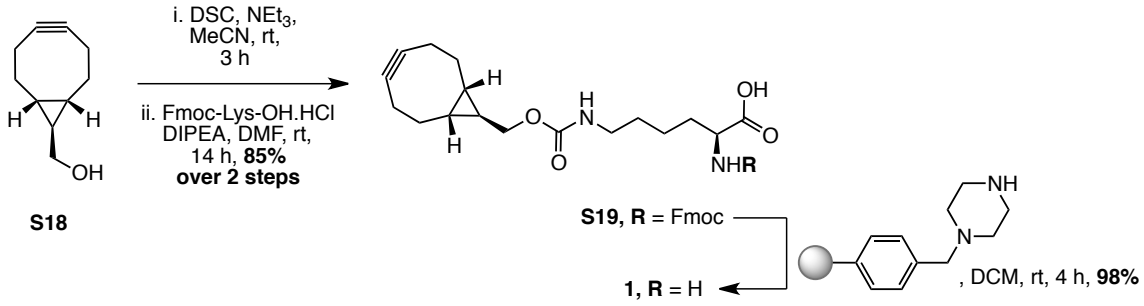
Cells were plated onto a poly-lysine coated μ-dish and after growing to near confluence were transfected with 2μg each of *pCMV-cJun-TAG-mCherry* and *p4CMVE-U6-PylT*. After approximately 16hrs growth at 37°C and at 5%CO_2 in DMEM with 0.1%FBS in the presence of 0.5 mM **1** (0.5% DMSO) cells were washed in DMEM 0.1% FBS and then incubated in DMEM 0.1%FBS overnight. The following day cells were washed repeatedly, using two media exchanges followed by

30 minutes incubation over 2 hours. 200 nM tetrazine-dye conjugate **11** was added for 15 minutes, the cells were then repeatedly washed again for 90mins. Imaging was carried out as for the cell surface labeling

Chemical Syntheses:

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) for ^{1}H-NMR spectra, $CDCl_3$ (77.0 ppm), d_6-DMSO (39.5 ppm) for ^{13}C-NMR spectra. ^{13}C- and ^{31}P-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/or by potassium permanganate 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. Bicyclo[6.1.0]non-4-yn-9-ylmethanol (BCN, exo/endo mixture ~ 4/1) was purchased from SynAffix, Netherlands. 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.

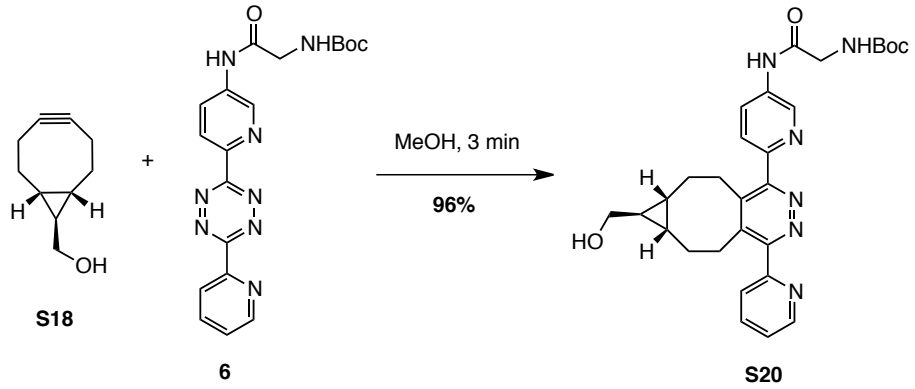


exo-Bicyclo[6.1.0]non-4-yn-9-ylmethanol (*exo*-BCN, **S18**) was synthesised according to a literature procedure.[3]

N,*N*'-disuccinimidyl carbonate (1.38 g, 5.37 mmol) was added to a stirring solution of *exo*-BCN-OH **S18** (538 mg, 3.58 mmol) and triethylamine (2.0 mL, 14.3 mmol) in MeCN (10 mL) at 0 ˚C. The solution was warmed to room temperature and stirred for 3 h and concentrated under reduced pressure. The crude oil was purified through a short pad of silica gel chromatography (eluting with 60% EtOAc in hexane) to yield the *exo*-BCN-succinimidyl carbonate, which was used without further purification. *exo*-BCN-OSu (1.25 g, 4.29 mmol) in DMF (4 mL) was added *via* cannula to a stirring solution of Fmoc-Lys-OH.HCl (2.61 g, 6.45 mmol) and DIPEA (1.49 mL, 8.58 mmol) in DMF (10 mL). The solution was stirred at room temperature for 14 h, diluted with Et_2O (100 mL) and washed with H_2O (3 x 100 mL). The organic phase was dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude oil was purified by silica gel chromatography (0-5% MeOH in DCM (0.1% AcOH)) to yield *exo*-Fmoc-BCNK-OH **S19** as a white solid (1.65 g, 85% over 2 steps). δ_H (400 MHz, d_6-DMSO) 12.67-12.31 (1H, br s), 7.90 (2H, d, J 7.5), 7.73 (2H, d, J 7.4), 7.63 (1H, d, J 7.8), 7.42 (2H, t, J 7.4), 7.34 (2H, t, J 7.4), 7.10 (1H, t, J 5.7), 4.31-4.19 (3H, m), 3.95-3.87 (1H, m), 3.84 (1H, d, J 6.4), 3.45-3.25 (br s, 1H), 3.01-2.91 (2H, m), 2.52-2.50 (1H, m), 2.33-2.15 (4H, m), 2.11-2.02 (2H, m), 1.75-1.54 (2H, m), 1.46-1.23 (6H, m), 0.70-0.58 (2H, m); δ_C (101 MHz, d_6-DMSO) 174.4, 156.9, 156.6, 144.30, 144.27, 141.2, 128.1, 127.5, 125.7, 120.6, 99.4, 68.1, 66.1, 54.3, 47.1, 33.3, 30.9, 29.5, 23.9, 23.4, 22.7, 21.3; LRMS (ESI^+): m/z 543 (100% $[M–H]^–$).

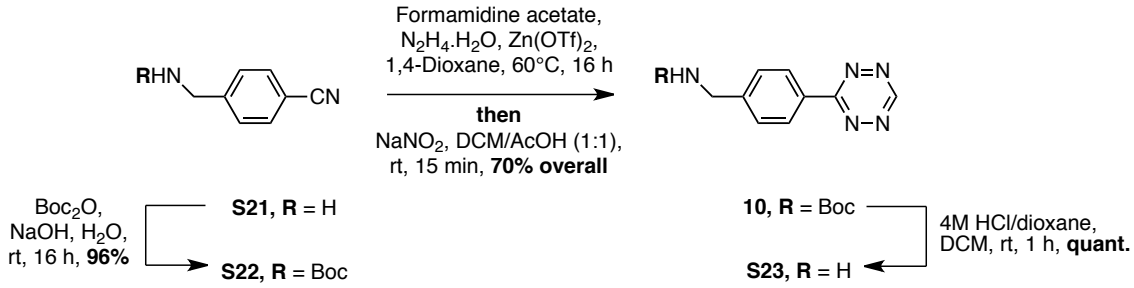
Polymer-bound piperazine (1.28 g, 1.28 mmol, 200-400 mesh, extent of labeling: 1.0-2.0 mmol/g loading, 2% cross-linked with divinylbenzene) was added to a stirring

solution of *exo*-Fmoc-BCNK-OH **S19** (174 mg, 0.32 mmol) in DCM (10 mL). The resulting mixture was stirred for 4 h at room temperature, filtered and the reagent washed with $CHCl_3$/MeOH (3:1, 3 x 50 mL). The filtrate was evaporated under reduced pressure, dissolved in H_2O (100 mL) and washed with EtOAc (3 x 100 mL). The aqueous phase was evaporated under reduced pressure and freeze-dried to yield *exo*-H-BCNK-OH **1** as a white solid (101 mg, 98%). For all subsequent labeling experiments using mammalian cells *exo*-H-BCNK-OH **1** was further purified by reverse-phase HPLC (0:1 H_2O:MeCN to 9:1 H_2O:MeCN gradient). 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% $[M+H]^+$). *endo*-Bicyclo[6.1.0]non-4-yn-9-ylmethanol (*endo*-BCN) was synthesised according to a literature procedure[3] and elaborated to the corresponding amino acid in an analogous fashion to **1**.



A glass vial (Biotage® Ltd.) equipped with a magnetic stirring bar was charged with compound **6** (39.2 mg, 0.096 mmol) and was sealed with an air-tight aluminium/rubber septum. The contents in the vial were dried *in vacuo* and purged with argon gas (× 3). MeOH (1 ml) was added to the vial, followed by addition of a solution of *exo*-Bicyclo[6.1.0]non-4-yn-9-ylmethanol (*exo*-BCN, **S18**) (20.2 mg in 1 ml of MeOH, 0.1344 mmol). The mixture was stirred at room temperature. Within 2 min, the reaction mixture decolorised and the contents were left stirring for additional 1 min. The mixture was then evaporated under reduced pressure and purified by silica gel chromatography (5% MeOH in DCM) to afford pyridazine **S20** as a faint yellow semi-solid (49 mg, 96%). δ_H (400 MHz, $CDCl_3$) 9.16 (1H, br s), 8.77-8.71

(1H, m), 8.67 (1H, app. d, J 2.1), 8.01 (1H, br s), 7.97 (1H, d, J 7.8), 7.89 (1H, ddd, J 7.8, 7.6, 1.7), 7.75 (1H, app. d, J 8.4), 7.40 (1H, ddd, J 7.4, 4.9, 1.1), 5.93 (1H, br s), 4.02 (2H, d, J 5.0), 3.49-3.31 (2H, m), 3.12-2.88 (4H, m), 2.68-2.49 (2H, m), 1.88-1.60 (1H, br s), 1.60-1.50 (1H, m), 1.48 (9H, s), 0.92-0.72 (4H, m); δ_C (101 MHz, $CDCl_3$) 169.0, 159.2, 159.0, 156.9, 156.8, 155.7, 152.1, 148.9, 143.0, 140.9, 137.0, 134.4, 128.0, 125.1, 124.9, 123.5, 80.7, 66.4, 45.7, 30.7, 29.9, 29.6, 29.5, 28.5 (3 x CH_3 (^{t}Bu)), 28.0, 27.8, 21.7; LRMS (ESI^+): m/z 531 (100% $[M+H]^+$).



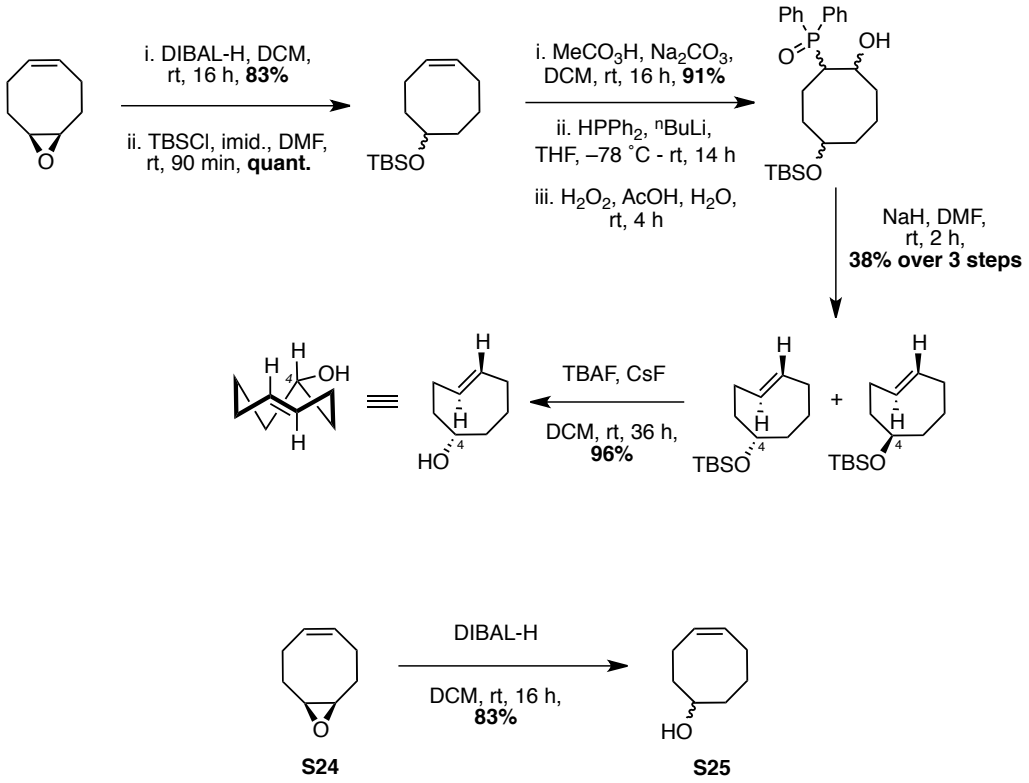
Commercially available 4-(Aminomethyl)benzonitrile hydrochloride **S21** (2.11 g, 12.50 mmol) in H_2O (10 mL) was added to a stirring solution of NaOH (1.50 g, 37.50 mmol) and di-*tert*-butyl dicarbonate (3.00 g, 13.75 mmol) in H_2O (10 mL) at room temperature. The mixture was stirred for 16 h, after which time a white precipitate had formed. The mixture was filtered, washed with H_2O (50 mL), and the resulting solid dried under vacuum to yield *tert*-butylcarbamate **S22** as a white solid (2.78 g, 96%). δ_H (400 MHz, $CDCl_3$) 7.62 (2H, d, J 8.2), 7.39 (2H, d, J 8.2), 5.00 (1H, br s), 4.37 (2H, d, J 5.8), 1.46 (9H, s); δ_C (101 MHz, $CDCl_3$) 155.9, 144.7, 132.4, 127.8, 118.9, 111.1, 80.1, 44.2, 28.4; LRMS (ESI^+): m/z 233 (100% $[M+H]^+$).

Tetrazine **10** was synthesised by modification of a literature procedure.[4] Hydrazine monohydrate (1.024 mL, 21.10 mmol) was added to a stirring suspension of *tert*-butylcarbamate **S22** (98 mg, 0.44 mmol), formamidine acetate (439 mg, 4.22 mmol), and $Zn(OTf)_2$ (77 mg, 0.22 mmol) in 1,4-dioxane (0.5 mL) at room temperature. The reaction was heated to 60 °C and stirred for 16 h. The reaction was cooled to room temperature and diluted with EtOAc (10 mL). The reaction was washed with 1M HCl (10 mL) and the aqueous phase extracted with EtOAc (2 x 5 mL). The organic phase was dried over sodium sulfate, filtered and evaporated under reduced pressure. The resulting crude residue was dissolved in a mixture of DCM and acetic acid (1:1, 5

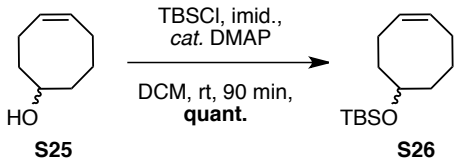
mL), and $NaNO_2$ (584 mg, 8.44 mmol) was added slowly over a period of 15 minutes, during which time the reaction turned bright red. The nitrous fumes were chased with an active air purge and the reaction then diluted with DCM (25 mL). The reaction mixture was washed with sodium bicarbonate (sat., aq., 25 mL) and the aqueous phase extracted with DCM (2 x 10 mL). The organic phase was dried over sodium sulfate, filtered and evaporated under reduced pressure. The resulting residue was purified by silica gel chromatography (20% EtOAc in hexane) to yield tetrazine **10** as a pink solid (85 mg, 70%). δ_H (400 MHz, $CDCl_3$) 10.21 (1H, s), 8.60 (2H, d, J 8.2), 7.53 (2H, d, J 8.2), 4.97 (1H, br s), 4.45 (2H, d, J 6.0), 1.49 (9H, s); δ_C (101 MHz, $CDCl_3$) 149.4, 142.6, 141.1, 132.1, 120.8, 119.2, 118.8, 51.8, 39.0; LRMS (ESI^+): m/z 188 (100% $[(M–Boc)+2H]^+$).

4M HCl in dioxane (2 mL, 8.0 mmol) was added to a stirring solution of tetrazine **10** (75 mg, 0.26 mmol) in DCM (4 mL). After 1 h the reaction was complete and the solvent was removed under reduced pressure to yield primary amine hydrochloride **S23** as a pink solid (61 mg, 100%). δ_H (400 MHz, d_6-DMSO) 10.64 (1H, s), 8.54 (2H, d, J 8.4), 7.79 (2H, d, J 8.4), 4.18 (2H, d, J 5.5); δ_C (101 MHz, d_6-DMSO) 165.2, 158.2, 138.9, 131.9, 129.8, 127.9, 41.8; LRMS (ESI^+): m/z 188 (100% $[M+H]^+$).

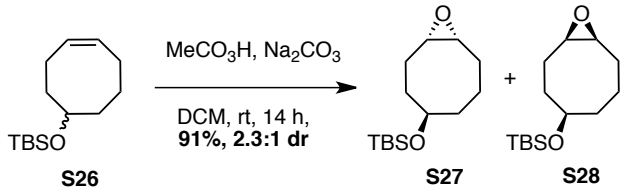
E-5-hydroxycyclooctene and *E-exo*-Bicyclo[6.1.0]non-4-ene-9-ylmethanol were either made by previously described photochemical procedures[5,6], or by the non-photochemical protocols described below.



Diisobutylaluminium hydride (1.0 M solution in cyclohexane, 89 mL, 89 mmol) was added drop-wise to a stirring solution of commercially available 9-oxabicyclo[6.1.0]non-4-ene **S24** (10 g, 80.53 mmol) in DCM (300 mL) at 0 ˚C. The solution was stirred at 0 ˚C for 30 min, warmed to room temperature and stirred for 16 h. After this time, the reaction was cooled to 0 ˚C and propan-2-ol (50 mL) was added slowly followed by HCl (1M, aq., 100 mL). The aqueous phase was extracted with DCM (3 x 200 mL). The combined organics were washed with brine, dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude material was purified by silica gel chromatography (10-20% EtOAc in hexanes) to yield cyclooctene-4-ol **S25** as a colorless oil (8.42 g, 83%). Spectral data was in accordance with the literature.[7]

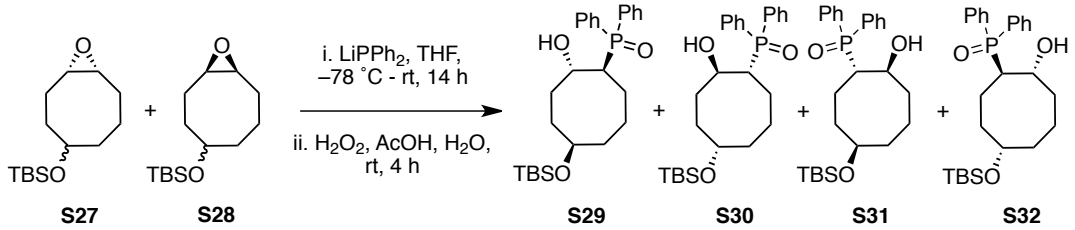


tert-Butyl(chloro)dimethylsilane (13.3 g, 88.0 mmol) was added to a stirring solution of cyclooctene-4-ol **S25** (5.6 g, 44.0 mmol), imidazole (7.5 g, 0.11 mol) and DMAP (1 crystal) in DCM (30 mL) at 0 ˚C. The solution was warmed to room temperature and stirred for 90 min, during which time a white precipitate formed. The reaction was cooled to 0 ˚C, diluted with DCM (100 mL) and sodium bicarbonate (sat., aq., 100 mL) was added. The phases were separated and the aqueous phase was extracted with DCM (3 x 100 mL). The combined organics were washed with brine (200 mL), dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude material was purified by silica gel chromatography (10-20% DCM in hexane) to yield silyl ether **S26** as colorless oil (10.55 g, quant.). δ_H (400 MHz, $CDCl_3$) 5.71-5.63 (1H, m), 5.60-5.52 (1H, m), 3.80 (1H, app td, J 8.6, 4.2), 2.34 (1H, dtd, J 13.8, 8.2, 3.8), 2.25-2.15 (1H, m), 2.13-2.05 (1H, m), 2.02-1.93 (1H, m), 1.87-1.52 (5H, m), 1.47-1.35 (1H, m), 0.88 (9H, s), 0.04 (3H, s), 0.03 (3H, s); δ_C (101 MHz, $CDCl_3$) 130.4, 129.4, 73.1, 38.0, 36.5, 26.1, 25.8, 25.1, 22.7, 18.4, –3.4; LRMS (ESI^+): m/z 241 (11% $[M+H]^+$).

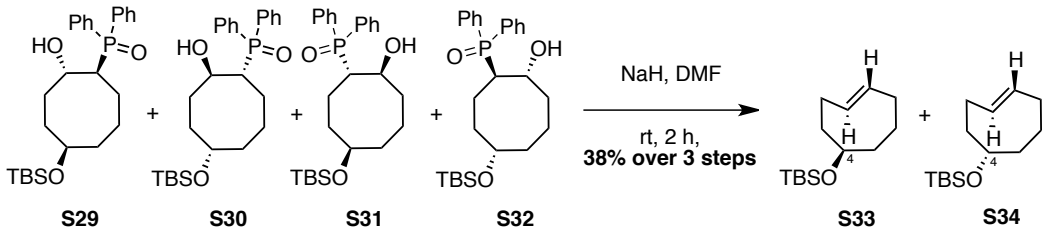


Peracetic acid (39% in acetic acid, 10.3 ml, 52.7 mmol) was added drop-wise to a stirred solution of silyl ether **S26** (10.6 g, 43.9 mmol) and sodium carbonate (7.0 g, 65.8 mmol) in DCM (80 mL) at 0 ˚C. The mixture was warmed to room temperature and stirred for 14 h. The reaction was cooled to 0 ˚C, diluted with DCM (50 mL) and sodium thiosulfate (sat., aq., 100 mL) was added. The mixture was stirred at room temperature for 10 min and then basified to pH 12 with NaOH (2M, aq.). The phases were separated and the organic phase washed with H_2O (100 mL), brine (100 mL), dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude material was purified by silica gel chromatography (80%-90% DCM in hexane)

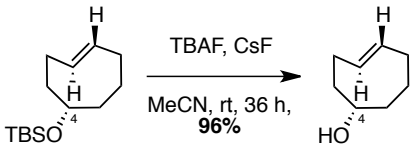
to yield epoxides **S27**/**S28**, as an inseparable mixture of diastereomers (2.3:1 by ^{1}H-NMR) and as a colorless oil (10.2 g, 91%). *Major diastereomer*: δ_H (400 MHz, $CDCl_3$) 3.90 (1H, app sext, J 4.2), 2.90 (2H, ddd, J 16.7, 8.3, 4.4), 2.21-2.09 (1H, m), 1.85-1.60 (6H, m), 1.50-1.38 (2H, m), 1.34-1.23 (1H, m), 0.88 (9H, s), 0.04 (3H, s), 0.03 (3H, s); δ_C (101 MHz, $CDCl_3$) 171.9, 55.5, 55.4, 36.3, 34.3, 27.7, 26.0, 25.8, 22.6, 18.3, –3.4; LRMS (ESI^+): m/z 257 (8% $[M+H]^+$).



n-Butyllithium (2.5 M in hexanes, 14.8 mL, 37.0 mmol) was added drop-wise over 15 min to a stirring solution of epoxides **S27**/**S28** (7.9 g, 30.8 mmol) and diphenylphosphine (6.43 mL, 37.0 mmol) in THF (80 mL) at –78 ˚C. The resulting mixture was stirred at –78˚C for 1 h, warmed to room temperature and stirred for 14 h. The reaction mixture was diluted with THF (80 mL) and cooled to 0˚C. Acetic acid (5.54 mL, 92.4 mmol) was added followed by hydrogen peroxide (30% solution in H_2O, 7.68 mL, 67.7 mmol). The reaction mixture was warmed to room temperature and stirred for 4 h. Sodium thiosulfate (sat., aq., 100 mL) was added and the mixture stirred for 10 min. The aqueous phase was extracted with EtOAc (3 x 200 mL). The combined organics were washed with brine (3 x 200 mL), dried over sodium sulfate, filtered and concentrated under reduced pressure to yield phosphine oxides **S29**/**S30**/**S31**/**S32** as a mixture of four diastereomers, which were used without further purification. δ_P (162 MHz, $CDCl_3$) 45.2, 44.8, 44.4, 43.8; LRMS (ESI^+): m/z 459 (100% $[M+H]^+$).

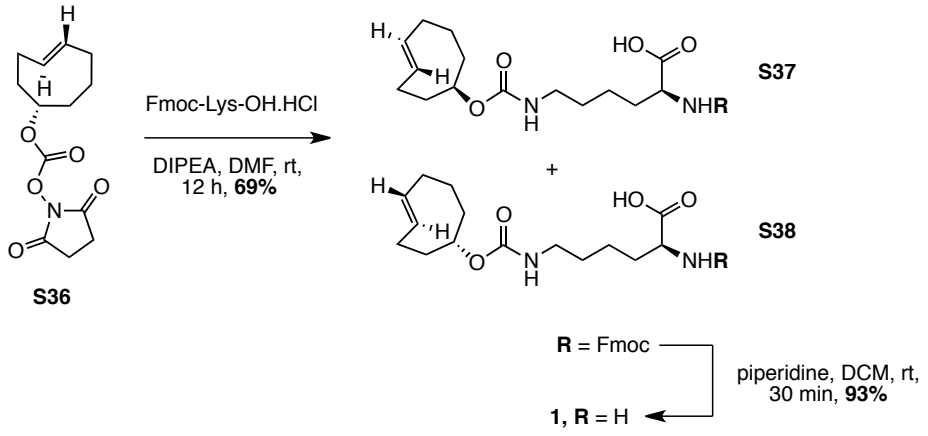


Sodium hydride (60% dispersion in mineral oil, 2.46 g, 61.5 mmol) was added to a stirring solution of crude hydroxyl phosphine oxides **S29**/**S30**/**S31**/**S32** in DMF (100 mL) at 0 ˚C. The resulting mixture was warmed to room temperature, wrapped in tin foil and stirred for 2 h. The reaction was cooled to 0 ˚C, diluted with Et_2O (200 mL) and H_2O (200 mL) was added. The phases were separated and the combined organics washed with brine (2 x 200 mL), dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude mixture was purified by silica gel chromatography (1-15% DCM in hexane) to yield *trans*-cyclooctenes **S33**/**S34** as a separable mixture of diastereomers, with exclusive *E*-selectivity, and as colorless oils (2.78 g, 1.2:1 dr, 38% over 3 steps). **S33**: δ_H (400 MHz, $CDCl_3$) 5.64 (1H, ddd, J 16.0, 10.8, 3.6), 5.45 (1H, ddd, J 15.9, 11.1, 3.2), 4.01 (1H, app dd, J 10.2, 5.4), 2.41 (1H, qd, J 11.5, 4.4), 2.26-2.19 (1H, m), 2.09-1.94 (3H, m), 1.92-1.73 (2H, m), 1.71-1.63 (1H, m), 1.54 (1H, tdd, J 14.0, 4.7, 1.1), 1.30-1.08 (1H, m), 0.94 (9H, s), 0.03 (3H, s), 0.01 (3H, s); δ_C (101 MHz, $CDCl_3$) 135.9, 131.5, 67.6, 44.0, 35.2, 34.8, 29.7, 27.7, 26.2, 18.4, –4.7, –4.8; LRMS (ESI^+): m/z 241 (8% $[M+H]^+$). **S34**: δ_H (400 MHz, $CDCl_3$) 5.55 (1H, ddd, J 15.9, 11.0, 3.6), 5.36 (1H, ddd, J 16.1, 10.8, 3.4), 3.42-3.37 (1H, m), 2.36-2.28 (2H, m), 2.22 (1H, app qd, J 11.2, 6.3), 2.02-1.87 (4H, m), 1.73 (1H, dd, J 14.9, 6.2), 1.67-1.45 (2H, m), 0.87 (9H, s), 0.03 (6H, s); δ_C (101 MHz, $CDCl_3$) 135.5, 132.5, 78.6, 44.9, 42.0, 34.6, 33.0, 31.3, 26.1, 18.3, –4.4, –4.5; LRMS (ESI^+): m/z 241 (12% $[M+H]^+$). For all further experiments *trans*-cyclooctene **S34** was used, where the *C*4-oxygen substituent occupies an equatorial position.



S34 **S35**

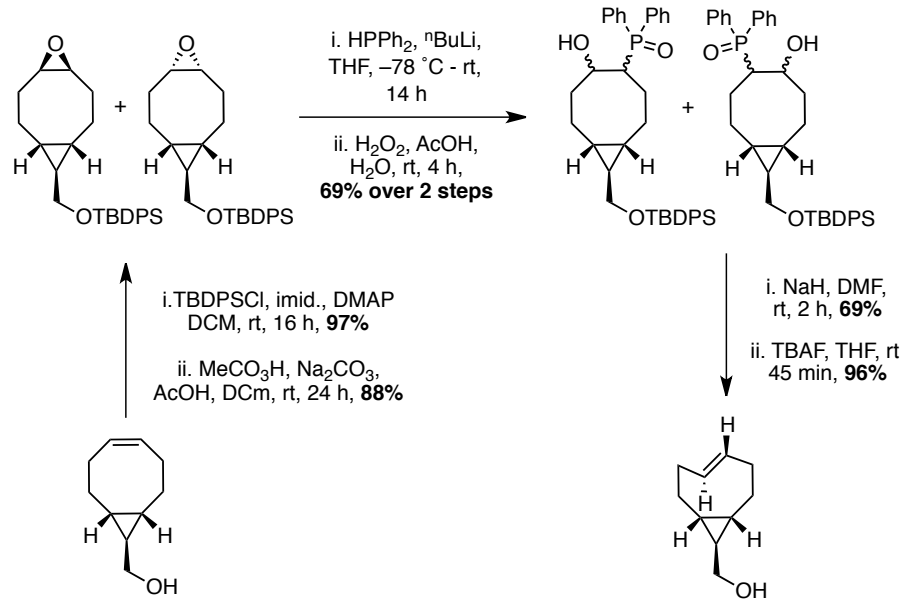
Tetrabutylammonium fluoride (1M solution in THF, 23.8 mL. 23.8 mmol) and cesium fluoride (1.08 g, 7.14 mmol) were added to a stirring solution of silyl ether **S34** (573 mg, 2.38 mmol) in MeCN (5 mL) at room temperature. The resulting mixture was wrapped in tin foil and stirred at room temperature for 36 h. After this period the reaction was cooled to 0 ˚C, diluted with DCM (100 mL) and H_2O (100 mL) was added. The phases were separated, the organic phase washed with brine (2 x 100 mL), dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude material was purified by silica gel chromatography (20% EtOAc in hexane) to yield secondary alcohol **S35** as a colorless oil (289 mg, 96%) δ_H (400 MHz, $CDCl_3$) 5.60 (1H, ddd, J 16.0, 10.7, 4.2), 5.41 (1H, ddd, J 16.0, 11.1, 3.7), 3.52-3.45 (2H, m), 2.40-2.25 (3H, m), 2.03-1.90 (4H, m), 1.75-1.53 (3H, m), 1.25-1.18 (1H, m); δ_C (101 MHz, $CDCl_3$) 135.1, 132.8, 77.7, 44.6, 41.1, 34.3, 32.6, 32.1; LRMS (ESI^+): m/z 127 (14% $[M+H]^+$).



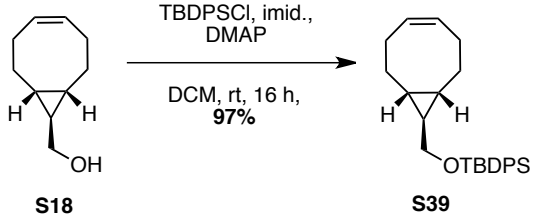
Succimidyl carbonate **S36** (200 mg, 0.75 mmol) was added to a stirring solution of Fmoc-Lys-OH.HCl (303 mg, 0.75 mmol) and DIPEA (0.19 g, 1.50 mmol) in DMF (7.5 mL) at 0 ˚C. The solution was warmed to room temperature, wrapped in tin foil and stirred for 12 h. After this period the solution was concentrated under reduced pressure and purified by silica gel chromatography (0-10% MeOH in DCM) to yield Fmoc-TCOK-OH **S37**/**S38** as a yellow oil that still contained DMF (350 mg, 81%).

δ_H (400 MHz, $CDCl_3$) 7.75-7.69 (2H, m), 7.63-7.52 (2H, m), 7.41-7.33 (2H, m), 7.32-7.25 (2H, m), 5.82-5.34 (3H, m), 5.27 (1H, br s), 4.90-4.50 (1H, m), 4.47-4.01 (5H, m), 3.32-3.30 (1H, m), 2.39-1.08 (17H, m); δ_C (100 MHz, $CDCl_3$) 174.3, 156.3, 155.9, 143.8, 143.6, 141.1, 135.0, 134.8, 132.8, 132.6, 127.5, 126.9, 125.0, 119.8, 80.3, 66.8, 53.4, 47.0, 41.0, 40.4, 38.5, 34.1, 32.5, 32.3, 32.1, 30.8, 29.3, 22.3; ESI-MS (m/z): [M+Na]+ calcd. for $C_{30}H_{36}N_2O_6Na$ 543.2471, found 543.2466.

Piperidine (1 mL) was added to a stirring solution of Fmoc-TCOK-OH **S37**/**S38** (0.269 g, 0.517 mmol) in DCM (4 mL). The mixture was wrapped in tin foil and stirred at room temperature for 30 min. The reaction mixture was concentrated under reduced pressure and the crude material was purified by silica gel chromatography (30-50% MeOH in DCM) to yield H-TCOK-OH **1** as an ivory-colored solid. δ_H (400 MHz, d_4-MeOD) 5.63-5.56 (1H, m), 5.50-5.43 (1H, m), 4.31-4.25 (1H, m), 3.60-3.53 (1H, m), 3.11-3.03 (2H, m), 2.37-2.26 (3H, m), 2.02-1.36 (13H, m); δ_C (100 MHz, d_4-MeOD) 174.3, 159.0, 136.3, 133.9, 81.8, 56.0, 42.4, 41.4, 39.8, 35.4, 33.7, 32.3, 32.1, 30.9, 23.6; ESI-MS (m/z): $[M–H]^-$ calcd. for $C_{15}H_{25}N_2O_4$ 297.1814, found 297.1811.



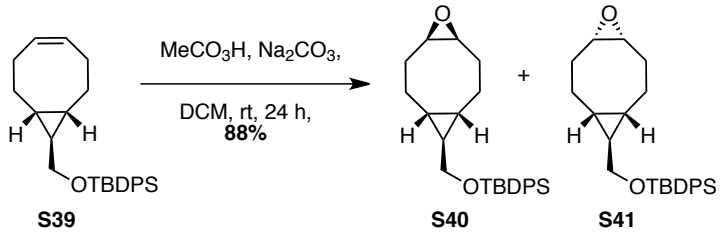
exo-Bicyclo[6.1.0]non-4-ene-9-ylmethanol **S18** was synthesised according to a literature procedure.[5]



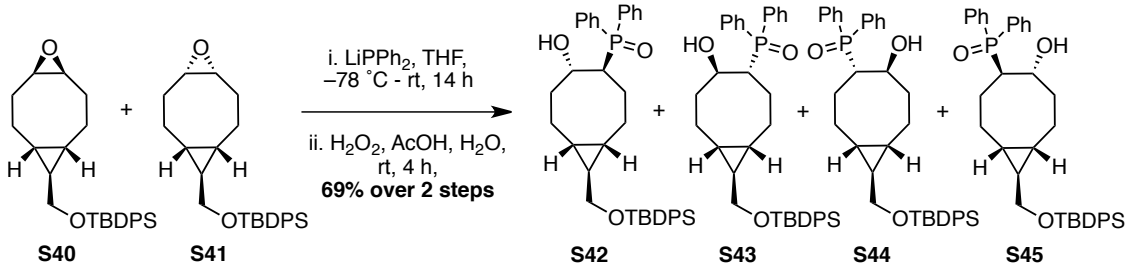
S18 **S39**

tert-Butyl(chloro)diphenylsilane (7.45 g, 27.1 mmol) was added to a stirring solution of exo-bicyclo[6.1.0]non-4-ene-9-ylmethanol **S18** (2.75 g, 18.1 mmol), imidazole (2.15 g, 31.6 mmol) and DMAP (2.21 g, 18.1 mmol) in DCM (35 ml) at 0 ˚C. The solution was warmed to room temperature and stirred for 24 h, during which a white precipitate formed. The reaction was cooled to 0 ˚C, diluted with DCM (100 mL) and sodium bicarbonate (sat., aq., 100 mL) was added. The phases were separated and the aqueous phase was extracted with DCM (3 × 100 mL). The combined organics were washed with brine (200 mL), dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude material was purified by silica gel chromatography (20% DCM in hexane) to yield silyl ether **S39** as a colorless oil (6.85 g, 97%), δ_H (400 MHz, $CDCl_3$) 7.79-7.64 (4H, m), 7.50-7.32 (6H, m), 5.63 (2H, dm, J 11.5), 3.59 (2H, d, J 6.2), 2.40-2.21 (2H, m), 2.18-1.96 (4H, m), 1.45-1.33 (2H, m), 1.07 (9H, s),

0.72-0.56 (3H, m); δ_C (101 MHz, $CDCl_3$) 135.7, 134.3, 130.2, 129.5, 127.6, 67.9, 29.1, 28.6, 27.2, 26.9, 22.0, 19.3; LRMS (ESI^+): m/z 408 (10%, $[M+NH_4]^+$).

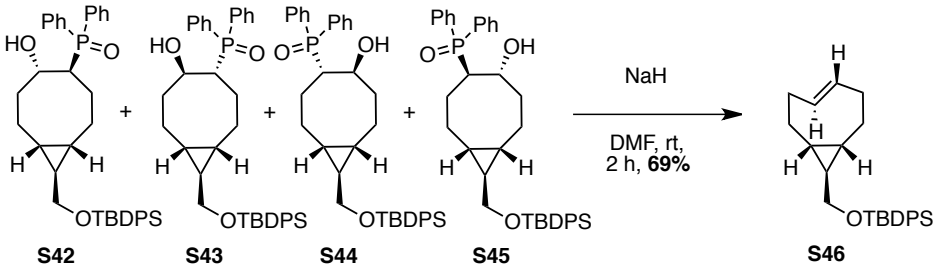


Peracetic acid (3.38 ml, 39% in acetic acid, 19.9 mmol) was added to a stirred solution of silyl ether **S39** (6.49 g, 16.6 mmol) and anhydrous sodium carbonate (2.64 g, 24.9 mmol) in DCM (65 mL) at 0 ˚C. The mixture was warmed to room temperature and stirred for 24 h. The reaction was then cooled to 0 ˚C, diluted with DCM (100 mL) and sodium thiosulfate (sat., aq., 150 mL) was added. The mixture was stirred at room temperature for 30 min and then basified to pH 12 with NaOH (2M, aq.,). The phases were separated and the organic phase was washed with H_2O (200 mL), brine (200 mL), dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude material was purified by silica gel chromatography (100% DCM) to yield epoxides **S40** and **S41** as an inseparable mixture of diastereomers (1:1 by ^1H NMR spectroscopy) and as a colorless oil (5.97 g, 88%). δ_H (400 MHz, $CDCl_3$) 7.72-7.63 (8H, m), 7.47-7.34 (12H, m), 3.57 (2H, d, J 5.6), 3.54 (2H, d, J 5.9), 3.03-3.10 (2H, m), 3.02-2.91 (2H, m), 2.36-2.24 (2H, m), 2.21-2.08 (2H, m), 2.06-1.85 (6H, m), 1.35-1.12 (4H, m), 1.06 (9H, s), 1.05 (9H, s), 0.92-0.80 (2H, m), 0.78-0.47 (6H, m); δ_C(101 MHz, $CDCl_3$) 135.65, 135.63, 134.2, 134.1, 129.6 (2 × CH), 127.6 (2 × CH), 67.4, 67.0, 56.91, 56.85, 29.7, 27.7, 26.9 (2 × $3CH_3$), 26.6, 26.5, 23.31, 23.25, 21.7, 20.4, 19.2 (2 × 2C); LRMS (ESI^+): m/z 407 (9%, $[M+H]^+$).



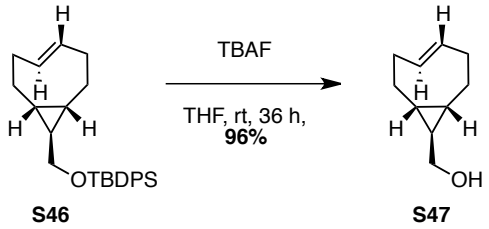
n-Butyllithium (2.5 M in hexanes, 5.92 mL, 14.8 mmol) was added drop wise over 15 min to a stirring solution of epoxides **S40**/**S41** (5.47 g, 13.5 mmol) and

diphenylphosphine (2.57 mL, 14.80 mmol) in THF (50 mL) at –78 ˚C. The resulting mixture was stirred at –78˚C for 1 h, warmed to room temperature and stirred for additional 14 h. The reaction mixture was diluted with THF (80 mL) and cooled to 0 ˚C. Acetic acid (1.54 mL, 26.9 mmol) was added followed by addition of hydrogen peroxide (30% solution in H_2O, 3.05 mL, 26.9 mmol). The reaction mixture was warmed to room temperature and stirred for 4 h. Sodium thiosulfate (sat., aq., 100 mL) was added and the mixture stirred for 10 min. The aqueous phase was extracted with EtOAc (3 x 200 mL). The combined organics were washed with brine (3 x 200 mL), dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude mixture was purified by silica gel chromatography (40-100% EtOAc in hexane) to yield phosphine oxides **S42**/**S43**/**S44**/**S45** as a 51:18 mixture of two diasteroisomers (5.61 g, 69% over 2 steps), each of which is a 1:1 mixture of regioisomers (**S42**/**S45** and **S43**/**S44**). *Major diastereomer*: δ_H (400 MHz, $CDCl_3$) 7.82-7.68 (4H, m), 7.68-7.58 (4H, m), 7.52-7.32 (12H, m), 4.58-4.45 (1H, m), 4.16 (1H, d, J 5.3), 3.54 (2H, d, J 6.0), 2.47 (1H, ddd, J 12.0, 11.7, 4.3), 2.21-2.07 (1H, m), 2.05-1.85 (2H, m), 1.78-1.55 (3H, m), 1.22-1.05 (1H, m), 1.03 (9H, s), 0.91-0.75 (1H, m), 0.62-0.35 (3H, m); δ_P(162 MHz, $CDCl_3$) 39.7; LRMS (ESI^+): *m/z* 609 [100%, $(M+H)^+$]. *Minor diastereomer*: δ_H (400 MHz, $CDCl_3$) 7.87-7.77 (2H, m), 7.74-7.60 (6H, m), 7.52-7.30 (12H, m), 4.26 (1H, d, J 4.0), 3.89-3.78 (1H, m), 3.63 (1H, dd, J 10.7, 5.8), 3.54 (1H, dd, J 10.7, 6.2), 3.26-3.10 (1H, m), 2.22-2.12 (1H, m), 2.00-1.78 (3H, m), 1.70-1.62 (1H, m), 1.42-1.28 (1H, m), 1.04 (9H, s), 1.04-0.92 (2H, m), 0.79-0.65 (1H, m), 0.55-0.41 (1H, m), 0.27-0.12 (1H, m); δ_P(162 MHz, $CDCl_3$) 39.6; LRMS (ESI^+): m/z 609 [100%, $(M+H)^+$].



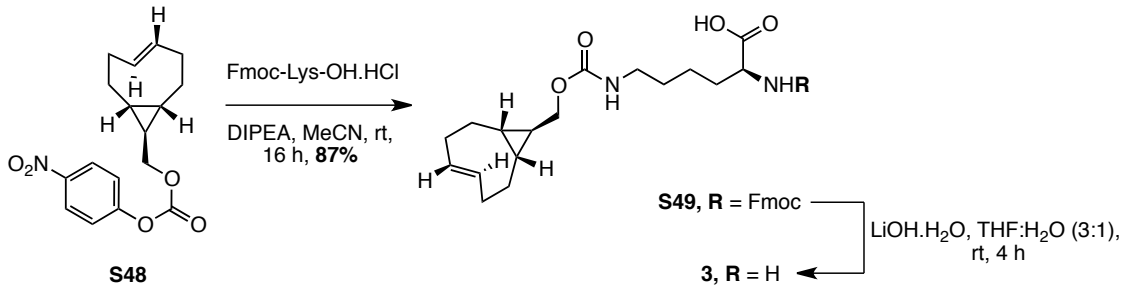
Sodium hydride (60% dispersion in mineral oil, 0.46 g, 11.5 mmol) was added to a stirring solution of hydroxyl phosphine oxides **S42**/**S43**/**S44**/**S45** (4.68 g, 7.69 mol) in anhydrous DMF (60 mL) at 0 ˚C. The resulting mixture was warmed to room temperature, wrapped in tin foil and stirred for 2 h. The reaction mixture was cooled

to 0 ˚C, diluted with Et_2O (200 mL) and H_2O (200 mL), the phases were separated and aqueous phase was extracted with hexane (150 mL). The combined organics were washed with brine (sat., aq., 5 × 250 mL), dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude mixture was purified by silica gel chromatography (1-20% DCM in hexane) to yield *trans*-cyclooctene **S46** as a single diastereomer and with exclusive *E*-selectivity (2.08 g, 69%); δ_H (400 MHz, $CDCl_3$) 7.72-7.62 (4H, m), 7.46-7.34 (6H, m), 5.83 (1H, ddd, J 16.1, 9.2, 6.2), 5.11 (1H, ddd, J 16.1, 10.6, 3.3), 3.59 (2H, d, J 5.7), 2.28-2.40 (1H, m), 2.12-2.27 (3H, m), 1.80-1.95 (2H, m), 1.04 (9H, s), 0.74-0.90 (1H, m), 0.46-0.60 (1H, dm, J 14.0), 0.31-0.42 (2H, m), 0.18-0.29 (1H, m); δ_C (101 MHz, $CDCl_3$) 138.6, 135.8, 134.4, 131.3, 129.6, 127.7, 68.1, 39.0, 34.1, 32.9, 28.2, 27.9, 27.0, 21.6, 20.5, 19.4.



S46 **S47**

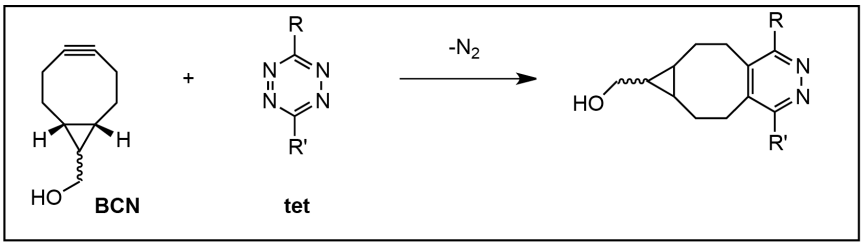
Tetrabutylammonium fluoride (1M solution in THF, 10.0 ml, 10.0 mmol) was added to a stirring solution of silyl ether **S46** (0.78 g, 2 mmol) in THF (5 mL) at room temperature, wrapped in tin foil and stirred for 45 min. After this period, the reaction mixture was concentrated under reduced pressure, diluted with DCM (100 mL) and washed with brine (100 mL). The phases were separated and the organic phase washed with brine (2 × 100 mL). The combined organics were dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude material was purified by silica gel chromatography (20% EtOAc in hexane) to yield primary alcohol **S47** as a colorless oil (0.29 g, 96%); δ_H (400 MHz, d_4-MeOD) 5.87 (1H, ddd, J 16.5, 9.3, 6.2), 5.13 (1H, dddd, J 16.5, 10.4, 3.9, 0.8), 3.39-3.47 (2H, dd, J 6.2, 1.5), 2.34-2.44 (1H, m), 2.12-2.33 (3H, m), 1.82-1.98 (2H, m), 0.90 (1H, dtd, J 12.5, 12.5, 7.1), 0.55-0.70 (1H, m), 0.41-0.55 (1H, m), 0.27-0.41 (2H, m); δ_C (101 MHz, d_4-MeOD) 139.3, 132.2, 67.5, 39.9, 34.8, 33.8, 29.2, 28.7, 23.0, 21.9; MS-CI (NH_3): m/z [M-OH] calcd. for $C_{10}H_{15}$, 135.1174; found 135.1173.

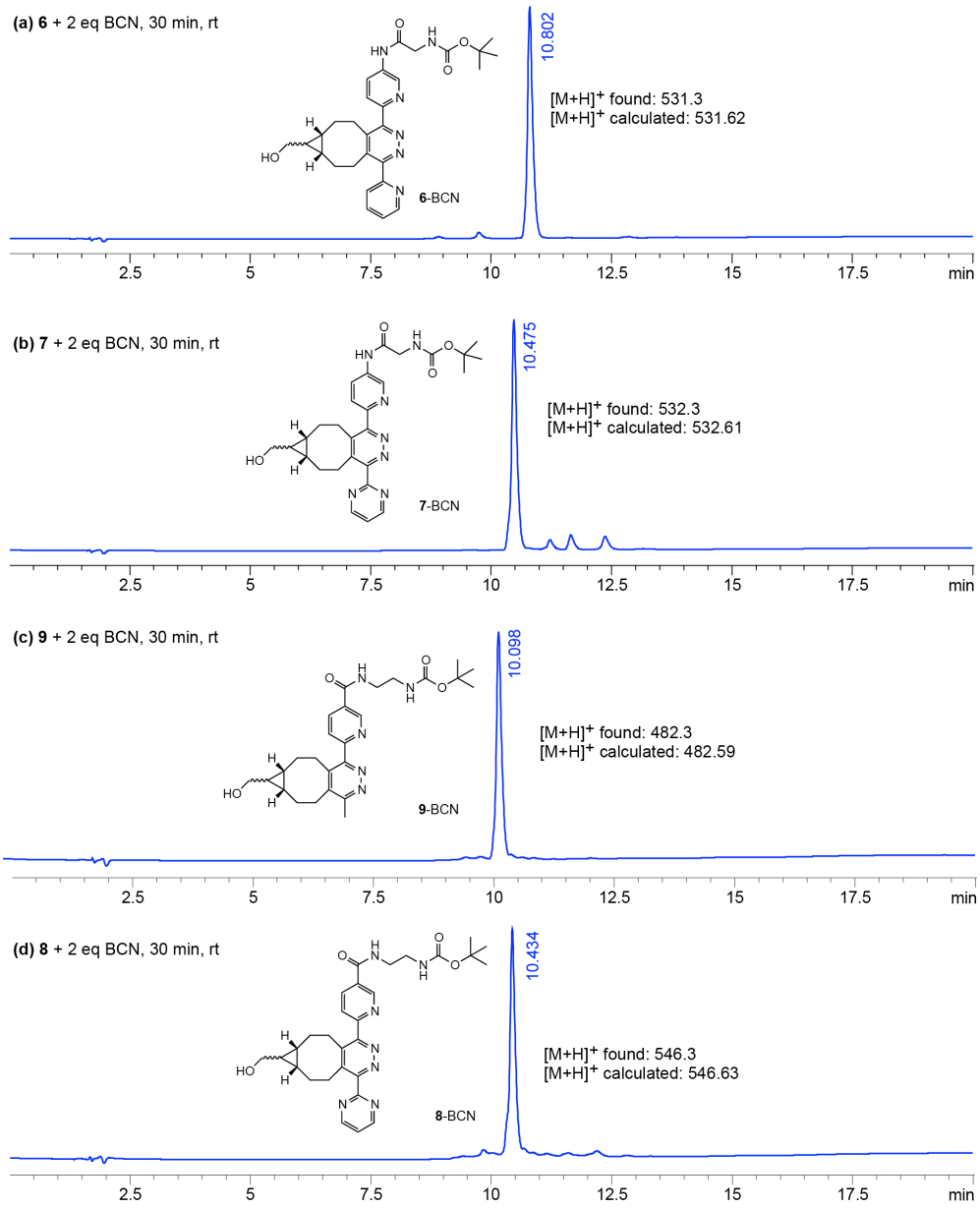


pNO_2-phenyl carbonate **S48** (250 mg, 0.79 mmol) was added to a stirring solution of Fmoc-Lys-OH.HCl (478 mg, 1.18 mmol) and DIPEA (0.27 mL, 1.58 mmol) in DMF (3 mL) at 0 ˚C. The solution was warmed to room temperature, wrapped in tin foil and stirred for 16 h. After this period the solution was concentrated under reduced pressure and purified by silica gel chromatography (0-5% MeOH in DCM) to yield Fmoc-*exo*-sTCOK **S49** as a white foam (373 mg, 87%). δ_H (400 MHz, d_6-DMSO) 13.09-12.06 (1H, br s), 7.90 (2H, d, J 7.5), 7.73 (2H, d, J 7.5), 7.66-7.56 (1H, m), 7.43 (2H, t, J 7.4), 7.34 (2H, J 7.4), 7.08 (1H, t, J 5.4), 5.84-5.72 (1H, m), 5.13-5.01 (1H, m), 4.31- 4.19 (3H, m), 3.93-3.79 (3H, m), 3.00-2.90 (2H, m), 2.31-2.07 (4H, m), 1.91-1.78 (2H, m), 1.75-1.49 (2H, m), 1.45-1.22 (4H, m), 0.91-0.75 (1H, m), 0.62-0.45 (2H, m), 0.43-0.32 (2H, m); δ_C (101 MHz, d_6-DMSO) 173.9, 156.4, 156.1, 143.8, 140.7, 137.9, 131.0, 127.6, 127.0, 125.2, 120.1, 79.1, 67.9, 65.6, 53.8, 46.6, 38.1, 33.4, 31.9, 30.4, 29.0, 27.2, 24.3, 22.8, 21.2, 20.2; LRMS (ESI^+): m/z 545 (100% $[M–H]^–$).

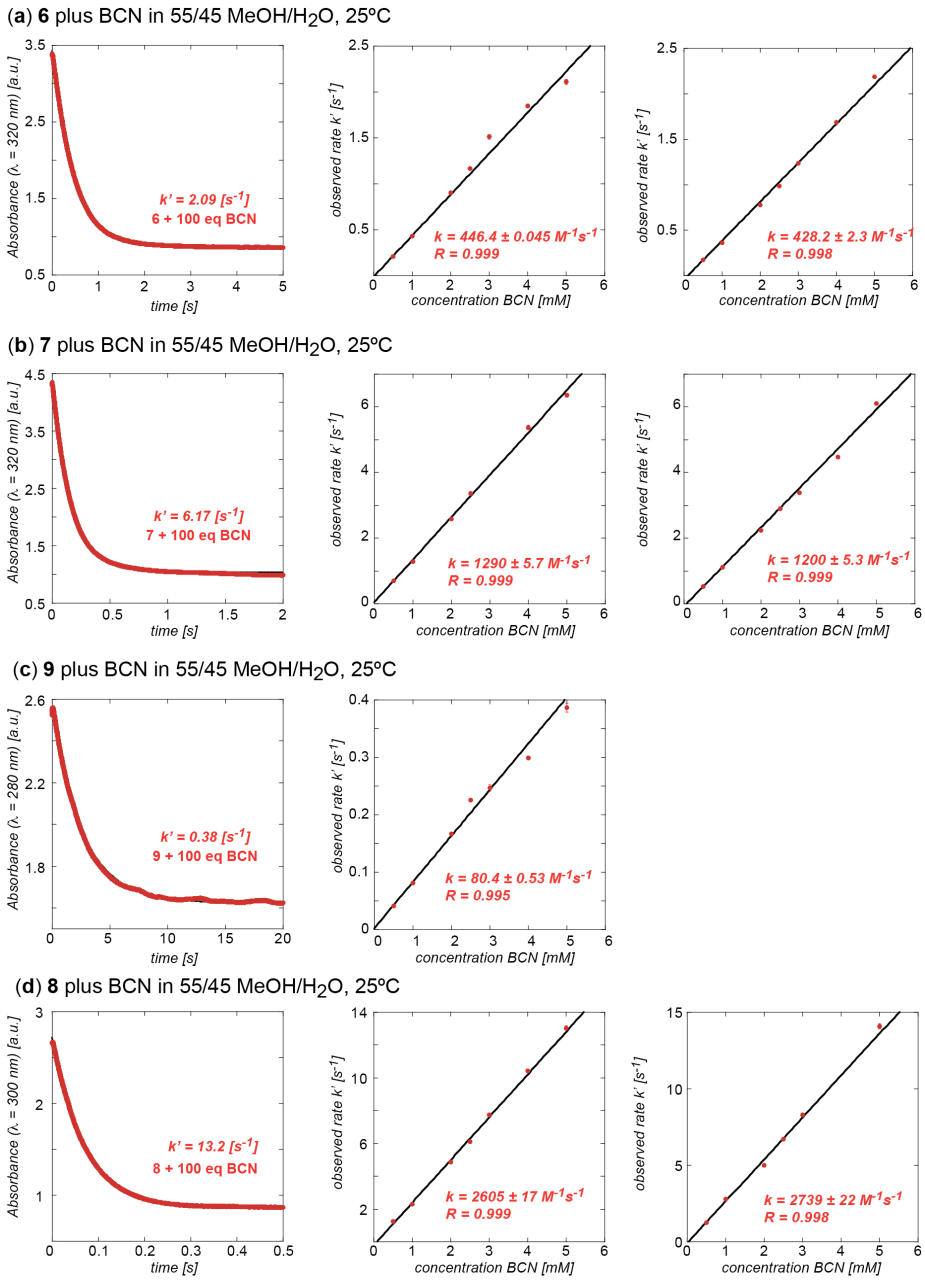
Lithium hydroxide monohydrate (94 mg, 0.75 mmol) was added to a stirring solution of *exo*-sTCOK **S49** in THF:H_2O (3:1, 8 mL). The solution was wrapped in tin foil, stirred for 4 h at room temperature and EtOAc (100 mL) and H_2O (100 mL) were added. The aqueous phase was carefully acidified to pH 4 by the addition of AcOH and extracted with EtOAc (4 x 100 mL). The aqueous phase was evaporated under reduced pressure and freeze-dried to yield *exo*-sTCOK **3** as a white solid. For all subsequent labeling experiments using mammalian cells *exo*-H-bcnK-OH **1** was further purified by reverse-phase HPLC (0:1 H_2O:MeCN to 9:1 H_2O:MeCN gradient). δ_H (400 MHz, d_6-DMSO) 7.21-7.09 (1H, br m), 5.85-5.72 (1H, m), 5.14-5.02 (1H, m), 3.80 (2H, d, J 2.6), 3.14-3.05 (1H, m), 2.98-2.86 (2H, m), 2.31-2.08 (4H, m), 1.92-1.78 (2H, m), 1.73-1.65 (1H, m), 1.55-1.44 (1H, m), 1.41-1.25 (4H, m), 0.90-0.62 (1H, m), 0.65-0.45 (2H, m), 0.43-0.32 (2H, m); δ_C (101 MHz, d_6-DMSO) 175.5,

156.3, 137.9, 131.1, 67.8, 54.5, 38.1, 33.4, 32.1, 32.0, 29.2, 27.2, 24.7, 24.3, 22.5, 21.2, 20.2; LRMS (ESI^+): m/z 325 (100% $[M+H]^+$).

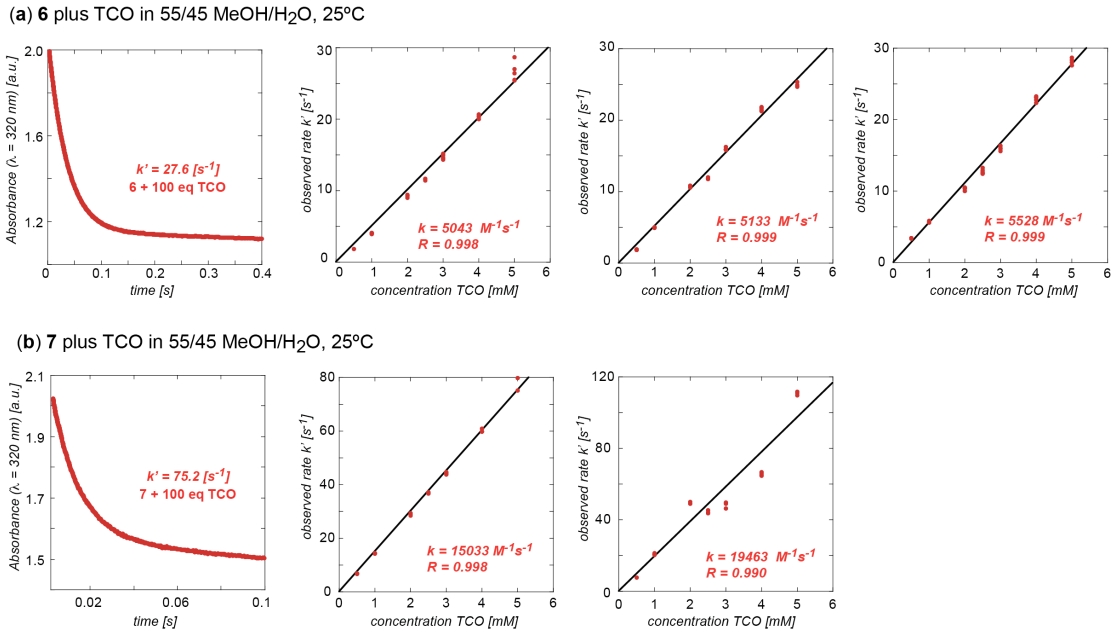




Supplementary Figure S1. LC/MS traces (254 nm) showing the formation of pyridazine products (**6**-BCN, **7**-BCN, **9**-BCN, **8**-BCN) from reaction of the corresponding tetrazines (**6**, **7**, **9** and **8**) with 2 equivalents of BCN (exo/endo mixture ~ 4/1) in MeOH. All masses are given in Daltons. The HPLC traces were taken after incubating the reactions for 10 to 30 minutes at room temperature. The overall yield for conversion to pyridazine products was > 98%.



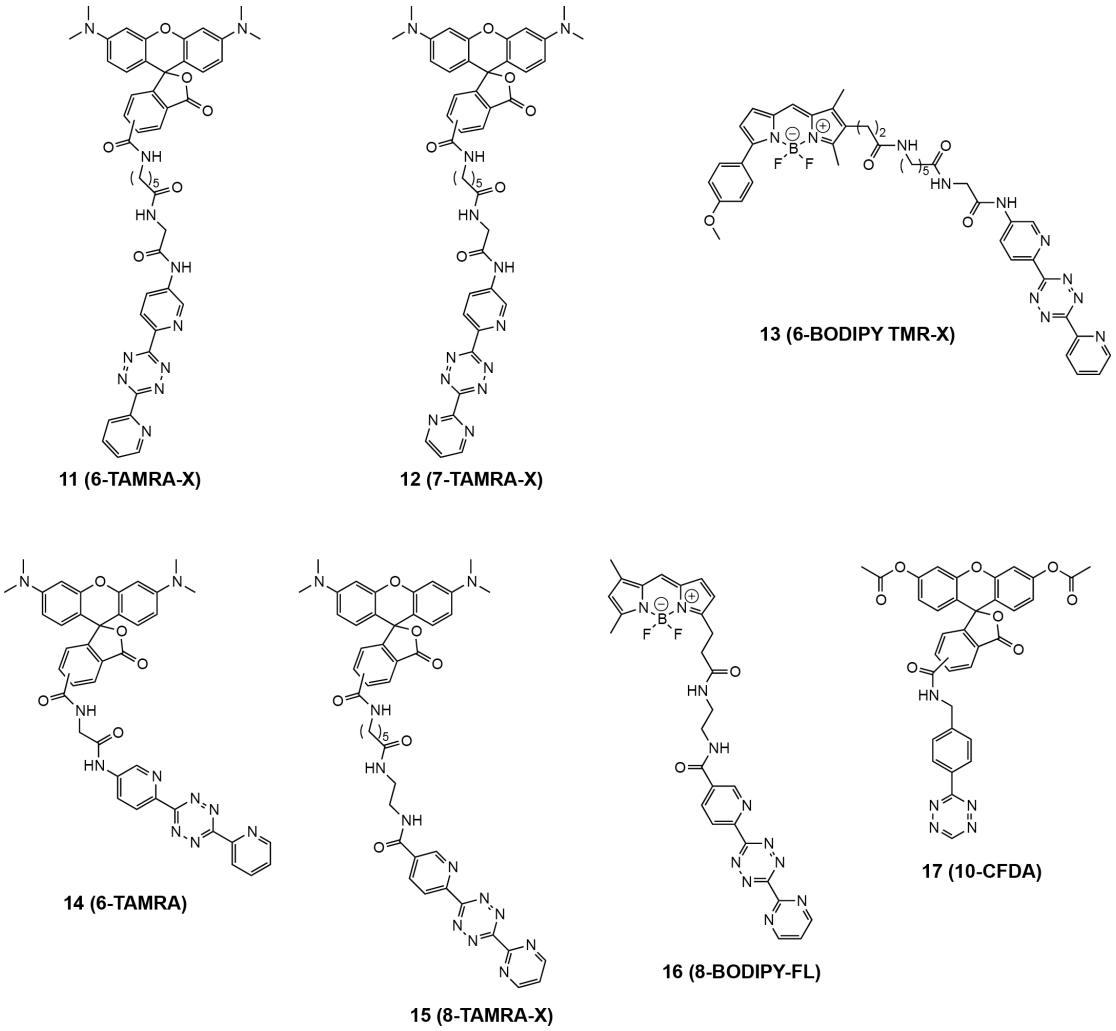
Supplementary Figure S2. Determination of rate constants k for the reaction of various tetrazines with BCN by UV-spectroscopy using a stopped-flow device. (**a**) Response of the UV absorbance at 320 nm of compound **6** upon BCN addition (100 eq = 5 mM); by fitting the data to a single exponential equation, k' values were determined (left panel); each measurement was carried out three to five times and the mean of the observed rates k' was plotted against the concentration of BCN to obtain the rate constant k from the slope of the plot. For all four tetrazines complete measurement sets were done in duplicate (middle and right panel) and the mean of values is reported in **Supplementary Table 1**. (**b-d**) same as (**a**) for tetrazines **7**, **9** and **8**. Conditions: $c_{tetrazine}$ = 0.05 mM in 9/1 H_2O/MeOH, c_{BCN} = 0.5 to 5 mM in MeOH, resulting in a final 55/45 MeOH/H_2O mixture. All experiments were recorded at 25°C.



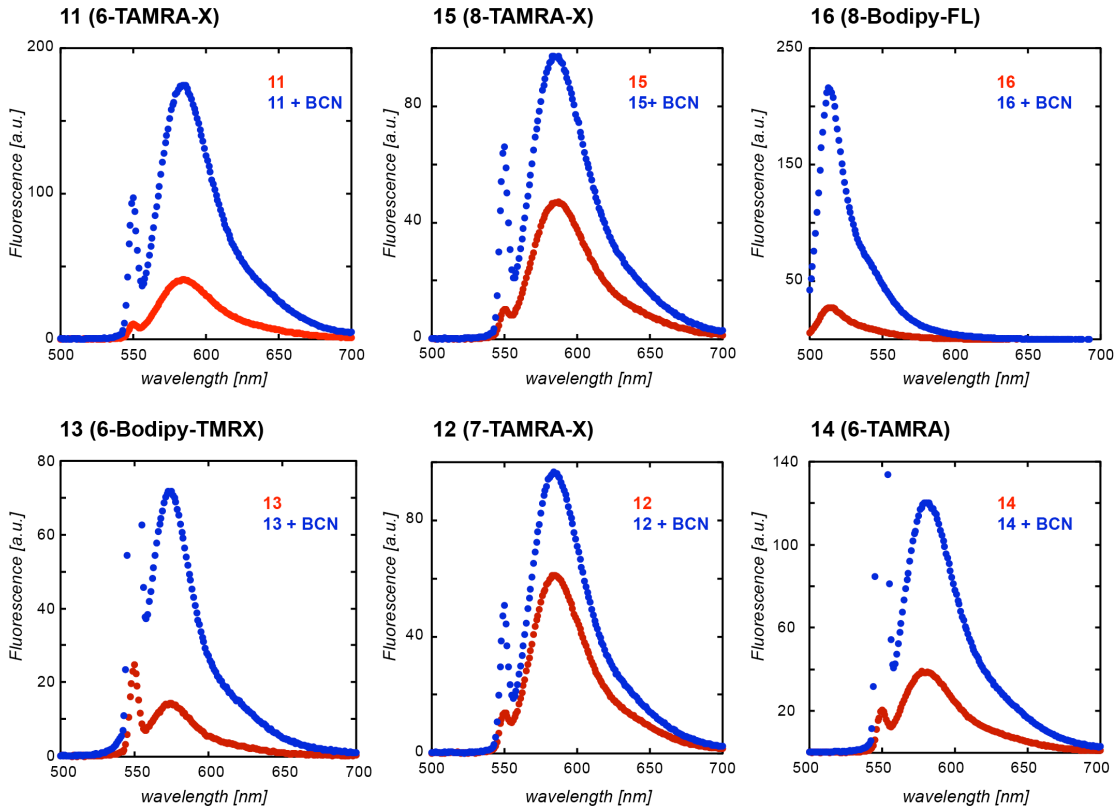
Supplementary Figure S3. Determination of rate constants k for the reaction of tetrazines **6** and **7** with TCO by UV-spectroscopy using a stopped-flow device. (**a**) Response of the UV absorbance at 320 nm of compound **6** upon TCO addition (100 eq = 5 mM); by fitting the data to the sum of two single exponential equations, k' values for the fast single exponential equations were determined (left panel); each measurement was carried out three to five times and observed rates k' were plotted against the concentration of TCO to obtain the rate constant k from the slope of the plot. For both tetrazines complete measurement sets were done at least in duplicate (middle and right panel) and the mean of values is reported in **Supplementary Table 1**. (**b**) same as (**a**) for tetrazine **7**. Conditions: $c_{tetrazine}$ = 0.05 mM in 9/1 H_2O/MeOH, c_{TCO} = 0.5 to 5 mM in MeOH, resulting in a final 55/45 MeOH/H_2O mixture. All experiments were recorded at 25ºC.

Tetrazine	BCN k_2 [$M^{-1}s^{-1}$][a]	Nor k_2 [$M^{-1}s^{-1}$][a]	TCO k_2 [$M^{-1}s^{-1}$][a]
6	437 ± 13	0.47 ± 0.0069	5235 ± 258
7	1245 ± 45	1.70 ± 0.048	17248 ± 3132
9	80	0.15	n.d.
8	2672 ± 95	5.00 ± 0.096	n.d.

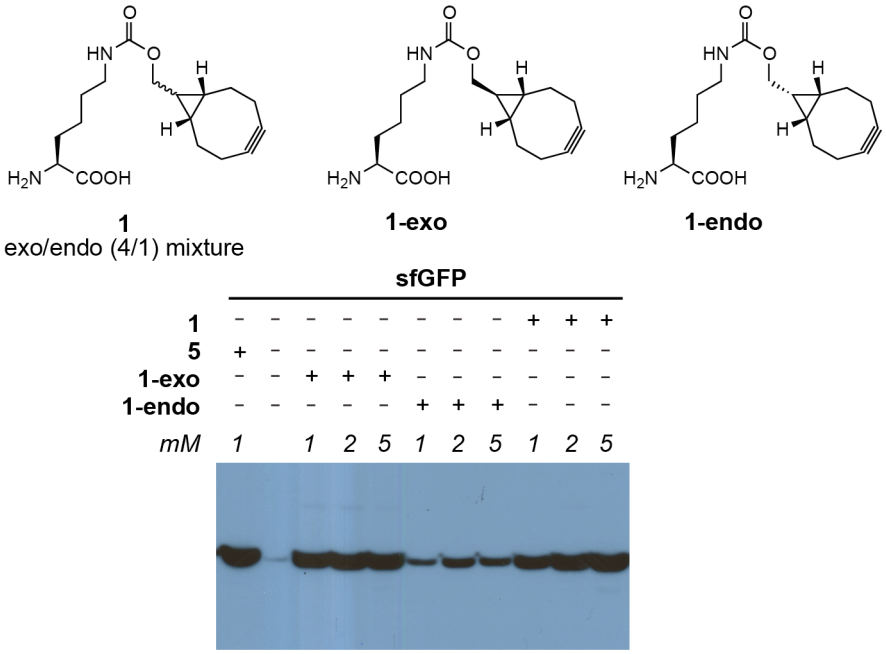
Supplementary Table 1. Rate constants k for the reaction of various tetrazines (**6**, **7**, **9** and **8**) with BCN and TCO at 25ºC measured under pseudo first order conditions using a stopped-flow device in comparison to rate constants for the reaction of the same tetrazines with 5-norbornene-2-ol at 21ºC.[2] Values were determined from at least two independent measurements. Solvent system: 55/45 methanol/water. The cycloaddition reaction of BCN to tetrazines is 500 to 1000 times faster than the one of 5-norbornene-2-ol, the reaction between TCO and tetrazines is 10 to 15 times faster than the one between BCN and tetrazines.



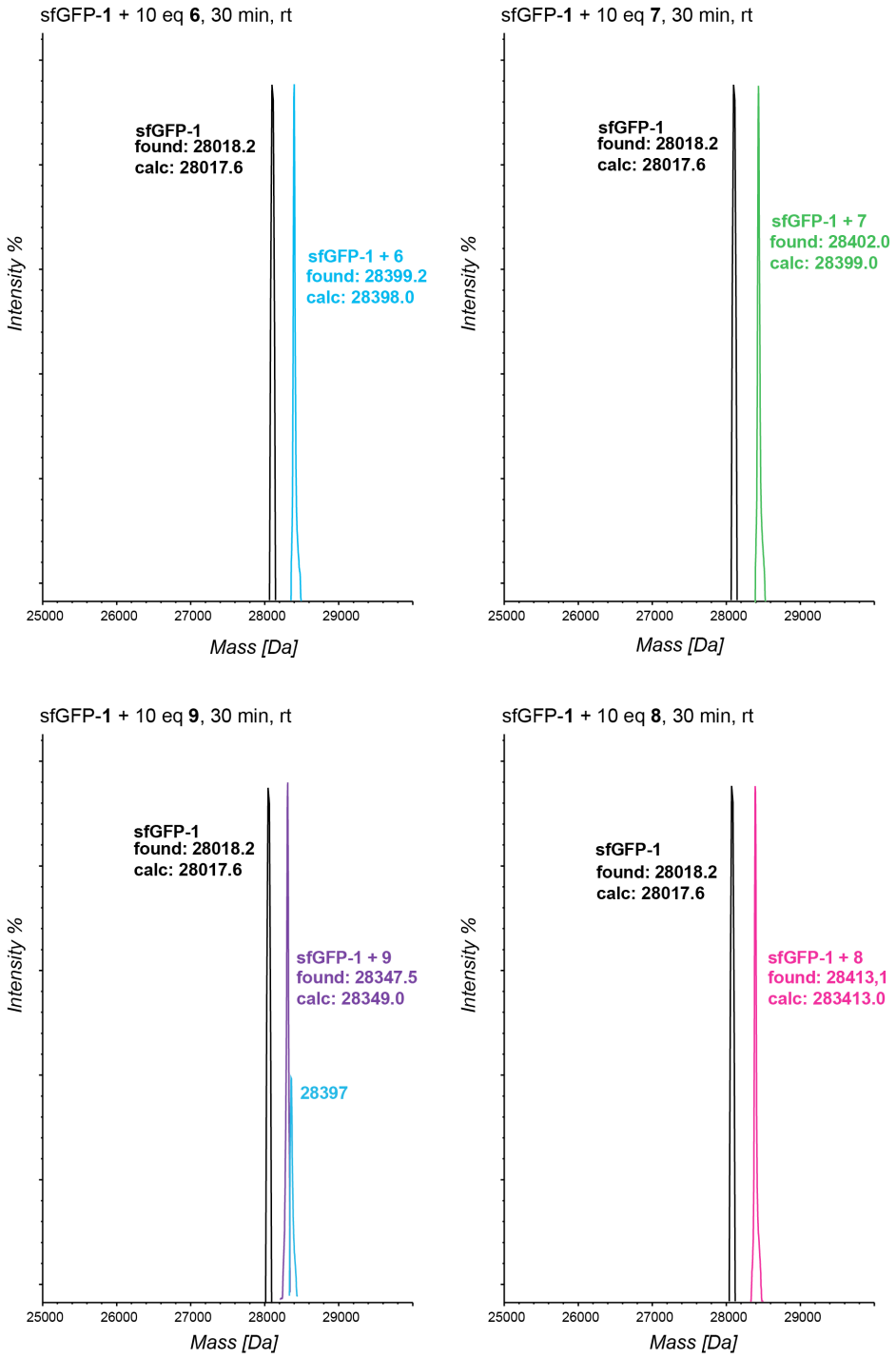
Supplementary Figure S4. Structural formulae of various tetrazine-fluorophores used in this study. Details on synthesis and characterization of these tetrazine-fluorophores can be found in reference 2.



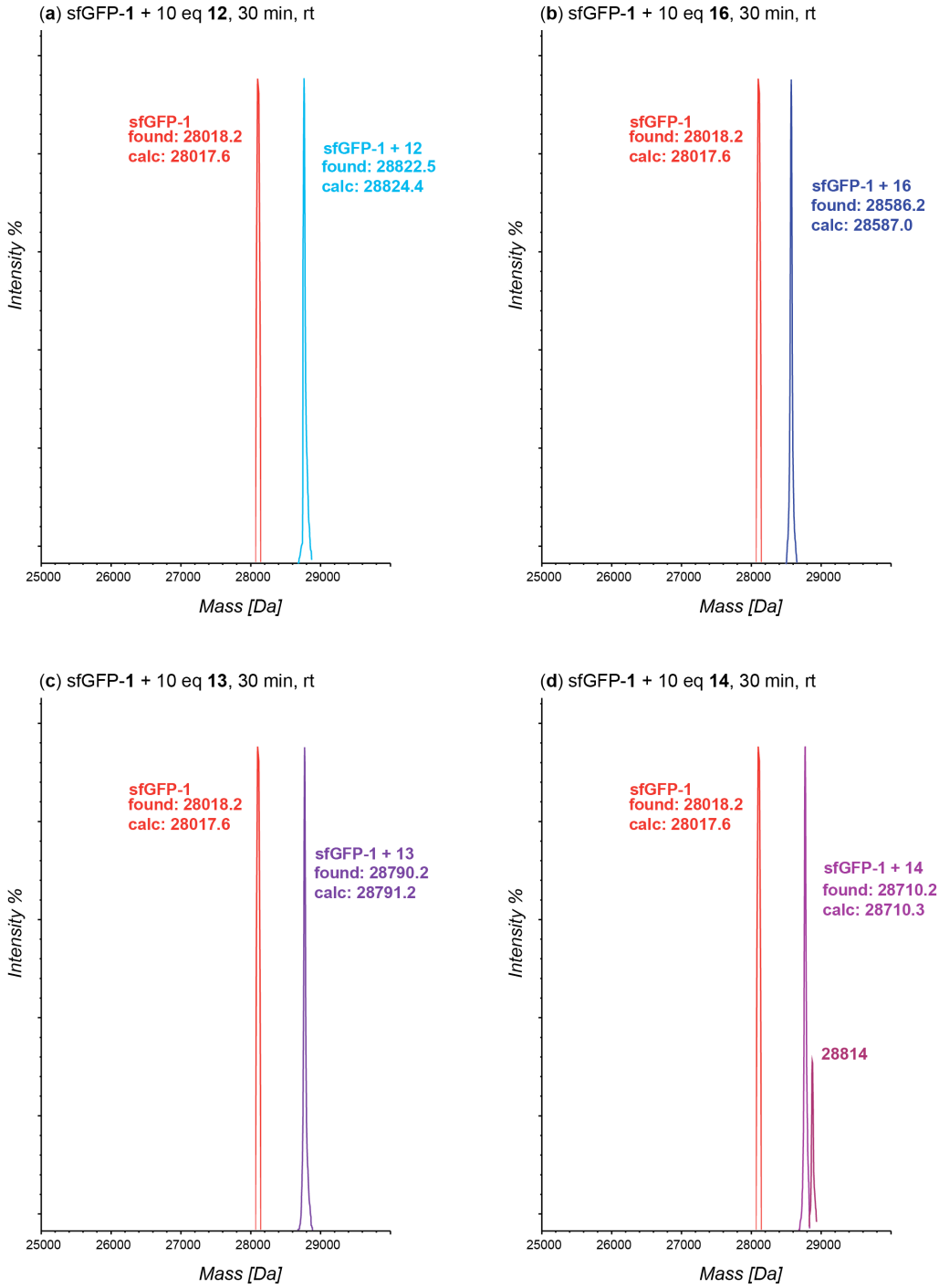
Supplementary Figure S5. "Turn on" fluorescence of tetrazine –fluorophores upon reaction with 9-hydroxymethylbicyclo[6.1.0]nonyne (BCN). A 2 μM solution of the corresponding tetrazine-fluorophore in water (2 mM in DMSO) was reacted with 300 equivalents of BCN. Emission spectra were recorded before and 30 min after the addition of BCN. Excitation wavelengths: TAMRA-dyes and Bodipy-TMR-X: 550 nm; Bodipy-FL: 490 nm.



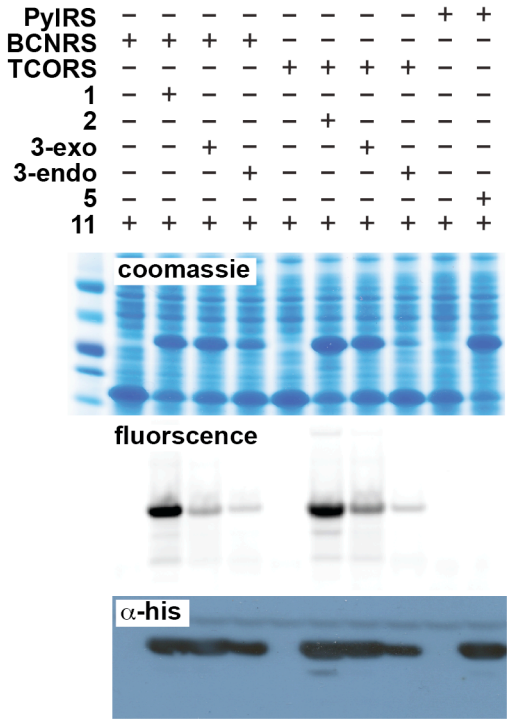
Supplementary Figure S6. Amino acid dependent expression of sfGFP-His_6 bearing an amber codon at position 150. The expressed protein was detected in lysates using an anti-His_6 antibody. Using purified exo or endo diastereomers of amino acid **1** demonstrated that the exo form is preferentially incorporated into sfGFP by BCNRS/$tRNA_{CUA}$.



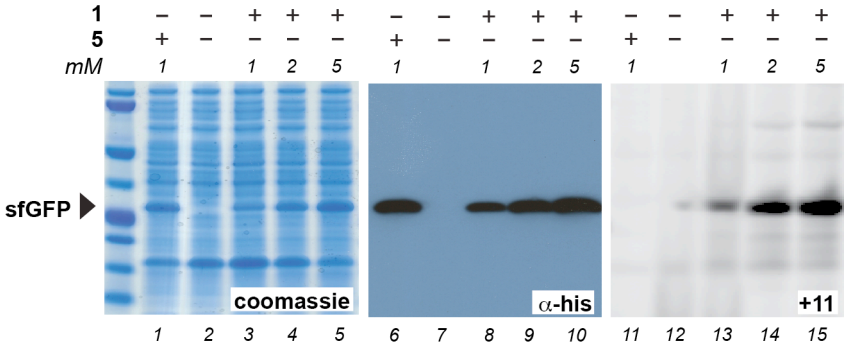
Supplementary Figure S7. LC-MS characterization of the labelling reaction of sfGFP-**1** with various tetrazines. Black peaks denote the found mass of sfGFP-**1** before labelling, colored peaks the found masses after reaction of sfGFP-**1** with **6**, **7**, **9** and **8**. All masses are given in Daltons. Labelling with all tetrazines is specific and quantitative. Reaction conditions: to a ~ 10 μM solution of sfGFP-**1** (in 20 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, pH 7.4) 10 equivalents of the corresponding tetrazine (1 mM stock solution in methanol) were added and the reaction mixture incubated for 10 to 30 minutes at room temperature.



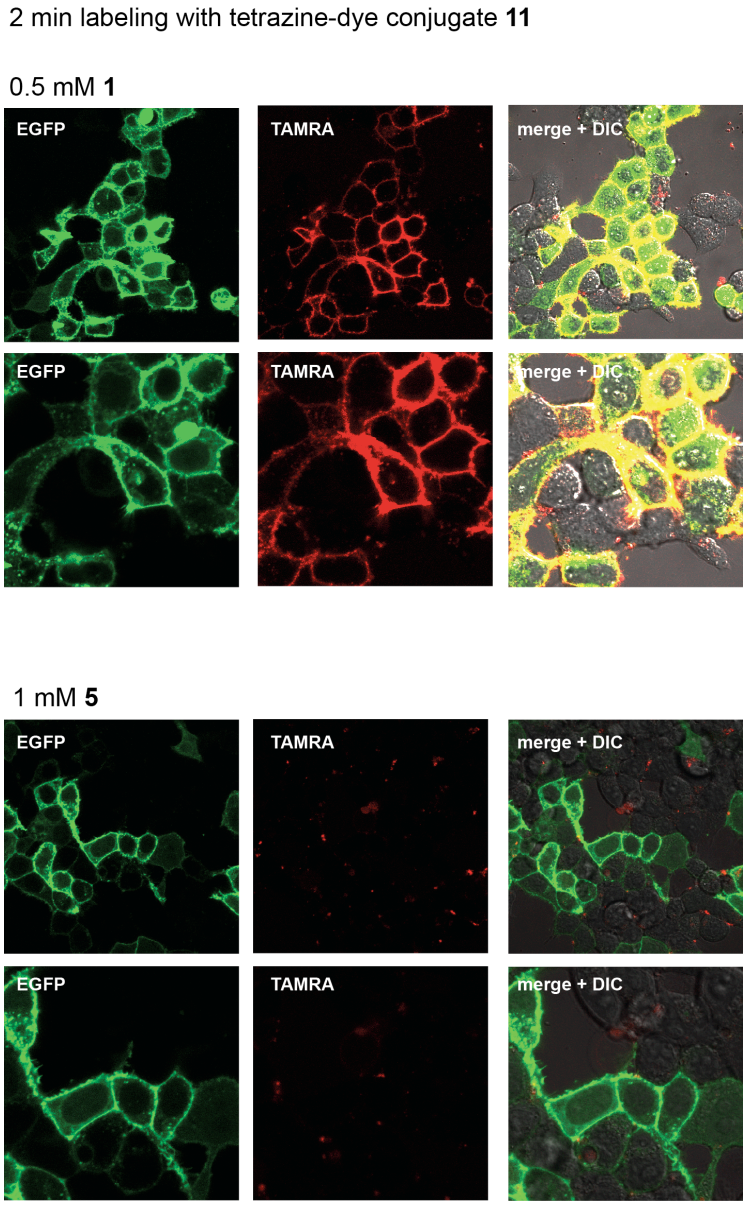
Supplementary Figure S8. LC-MS shows specific and quantitative labelling of sfGFP-**1** with tetrazine fluorophore conjugates **12**, **16**, **13** and **14**. Red peaks denote the found mass of sfGFP-**1** before labelling, colored peaks the found masses after reaction of sfGFP-**1** with **12 (a)**, **16 (b)**, **13 (c)** and **14 (d)**. Expected and found mass values are given in Daltons. Labelling with all tetrazine-fluorophores is specific and quantitative. Reaction conditions: to a ~ 10 μM solution of sfGFP-**1** (in 20 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, pH 7.4) 10 equivalents of the corresponding tetrazine dye (2 mM stock solution in DMSO) were added and the reaction mixture incubated for 10 to 30 minutes at room temperature.



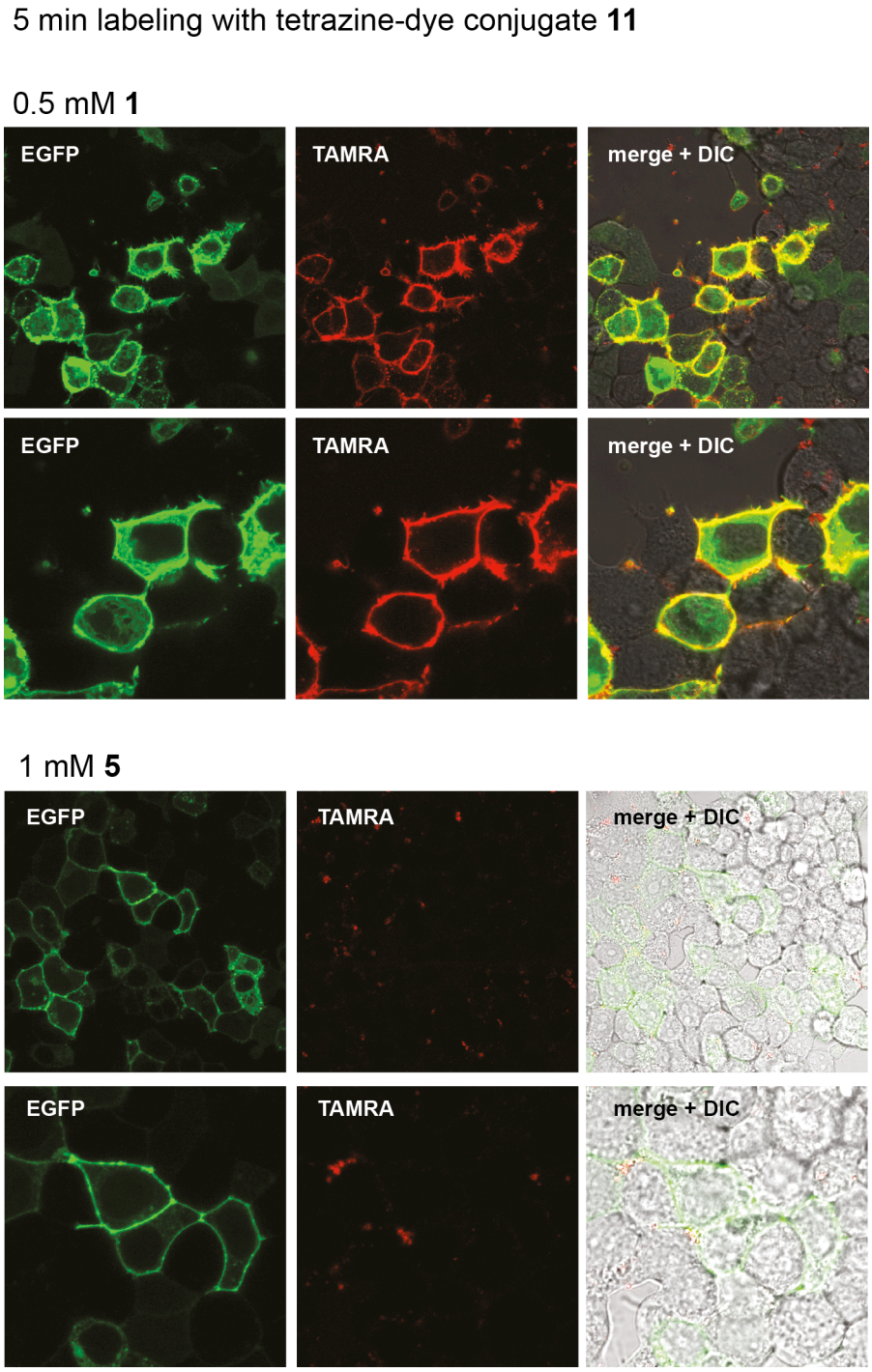
Supplementary Figure S9. Specificity of labeling **1** and **2** in sfGFP versus the *E. coli* proteome. The coomassie stained gel shows proteins from *E. coli* producing sfGFP in the presence of the indicated concentration of unnatural amino acids **1**, **2**, **3** (both exo and endo diastereomers) and **5**. In gel fluorescence gels show specific labeling with tetrazine-dye conjugate **11**. Though amino acids **1**, **2** and **3-exo** are incorporated at a similar level (as judged from coomassie stained gels and western blots), we observe only very faint, sub-stoichiometric labeling of sfGFP produced in the presence of **3-exo** and **3-endo**. These observations are consistent with the in vivo conversion of a fraction of the trans-alkene in **3** to its cis-isomer.



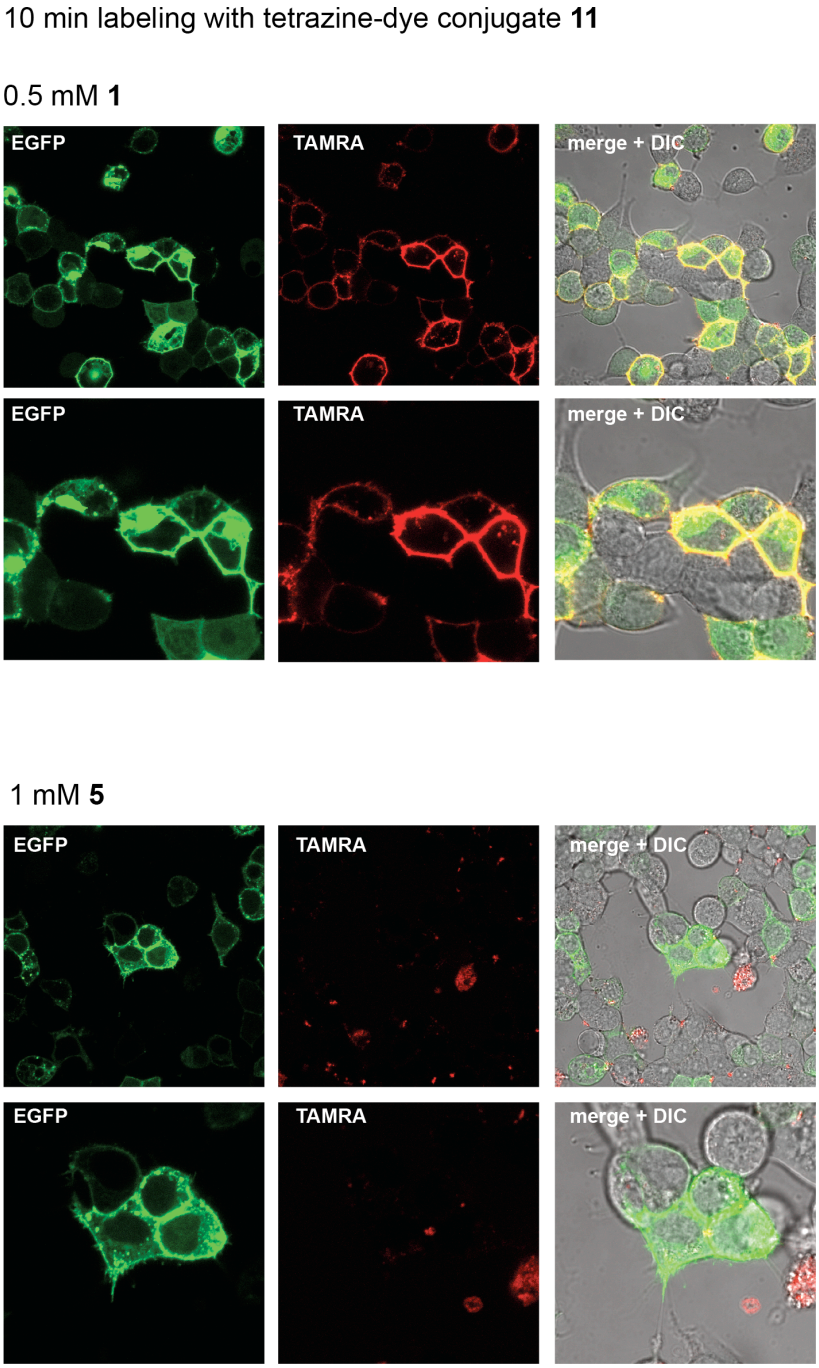
Supplementary Figure S10. Specificity of labeling **1** in sfGFP versus the *E. coli* proteome. Lanes 1-5: Coomassie stained gel showing proteins from *E. coli* producing sfGFP in the presence of the indicated concentration of unnatural amino acids **1** and **5**. Lanes 6-10: The expressed protein was detected in lysates using an anti-His6 antibody. Lanes 11-15: fluorescence images of protein labeled with the indicated fluorophore **11**.



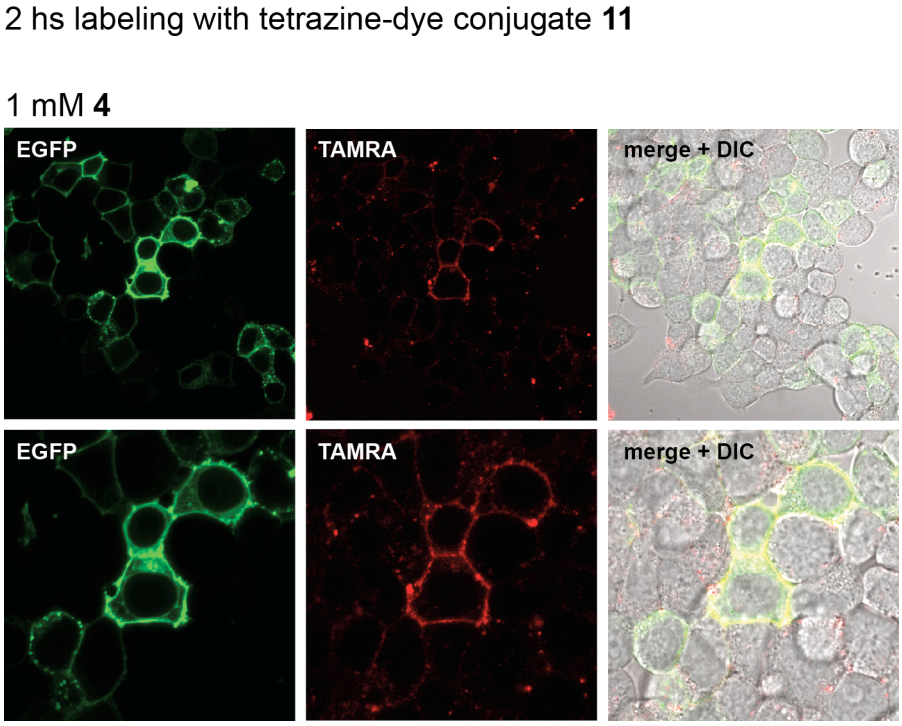
Supplementary Figure S11. Specific and ultra-rapid labelling of EGFR-GFP with tetrazine-fluorophore conjugate **11** for 2 minutes. EGFR-GFP bearing **1** at position 128 is visible as green fluorescence at the membrane of transfected cells (left panels). Treatments of cells with **11** (400 nM) leads to selective labelling of EGFR-GFP containing **1** (middle panels). Right panels show merged green and red fluorescence images, DIC = differential interference contrast. Cells were imaged 2 minutes after addition of **11**. No labelling was observed for cells in the same sample that did not express EGFR-GFP, and cells bearing EGFR-**5**-GFP were not labeled with **11**.



Supplementary Figure S12. Specific and ultra-rapid labelling of EGFR-GFP with tetrazine-fluorophore conjugate **11** for 5 minutes. EGFR-GFP bearing **1** at position 128 is visible as green fluorescence at the membrane of transfected cells (left panels). Treatments of cells with **11** (400 nM) leads to selective labelling of EGFR-GFP containing **1** (middle panels). Right panels show merged green and red fluorescence images, DIC = differential interference contrast. Cells were imaged 5 minutes after addition of **11**. No labelling was observed for cells in the same sample that did not express EGFR-GFP, and cells bearing EGFR-**5**-GFP were not labeled with **11**.

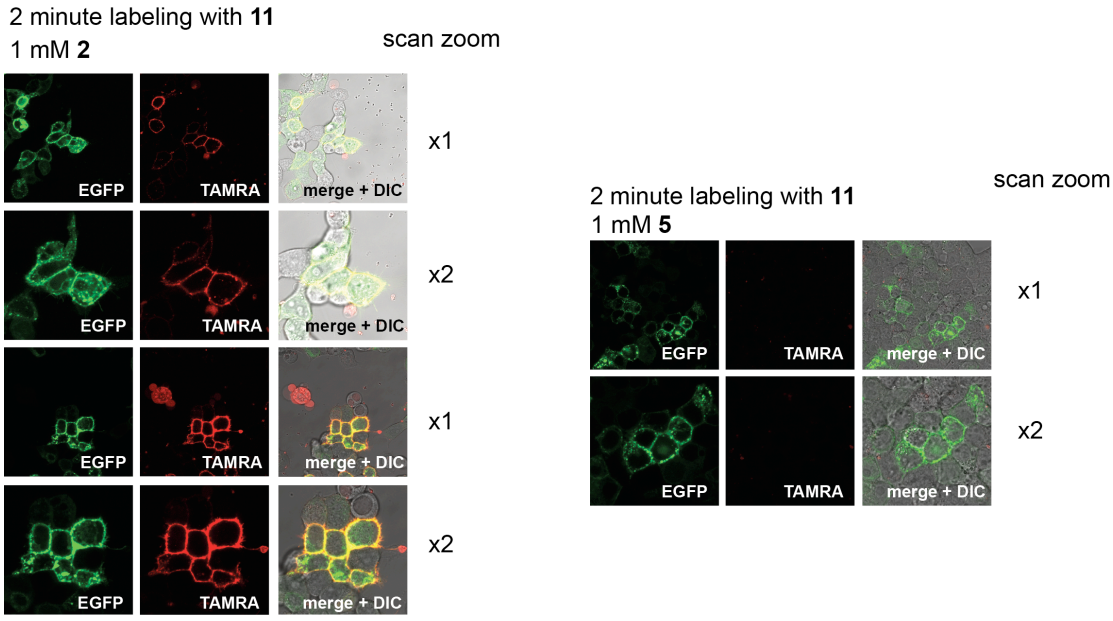


Supplementary Figure S13. Specific and ultra-rapid labelling of EGFR-GFP with tetrazine-fluorophore conjugate **11** for 10 minutes. EGFR-GFP bearing **1** at position 128 is visible as green fluorescence at the membrane of transfected cells (left panels). Treatments of cells with **11** (400 nM) leads to selective labelling of EGFR-GFP containing **1** (middle panels). Right panels show merged green and red fluorescence images, DIC = differential interference contrast. Cells were imaged 10 minutes after addition of **11**. No labelling was observed for cells in the same sample that did not express EGFR-GFP, and cells bearing EGFR-**5**-GFP were not labeled with **11**.

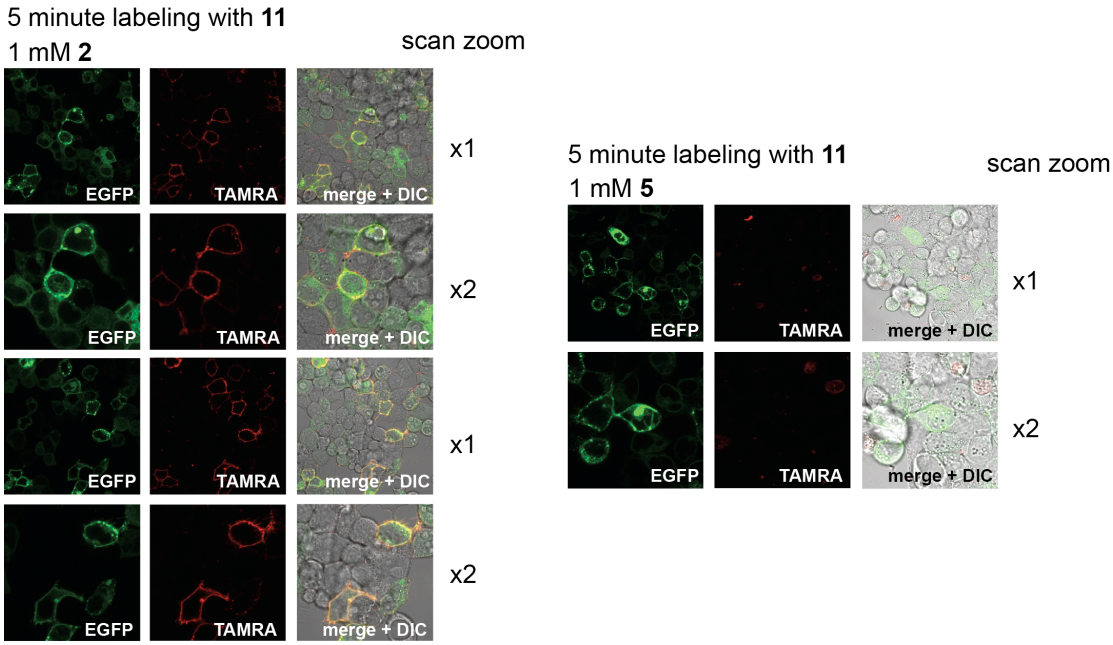


Supplementary Figure S14. In contrast to the ultra-rapid labelling of EGFR-GFP containing amino acid **1**, it took 2 hours to specifically label cells bearing EGFR-**4**-GFP with tetrazine-fluorophore conjugate **11**.[2]

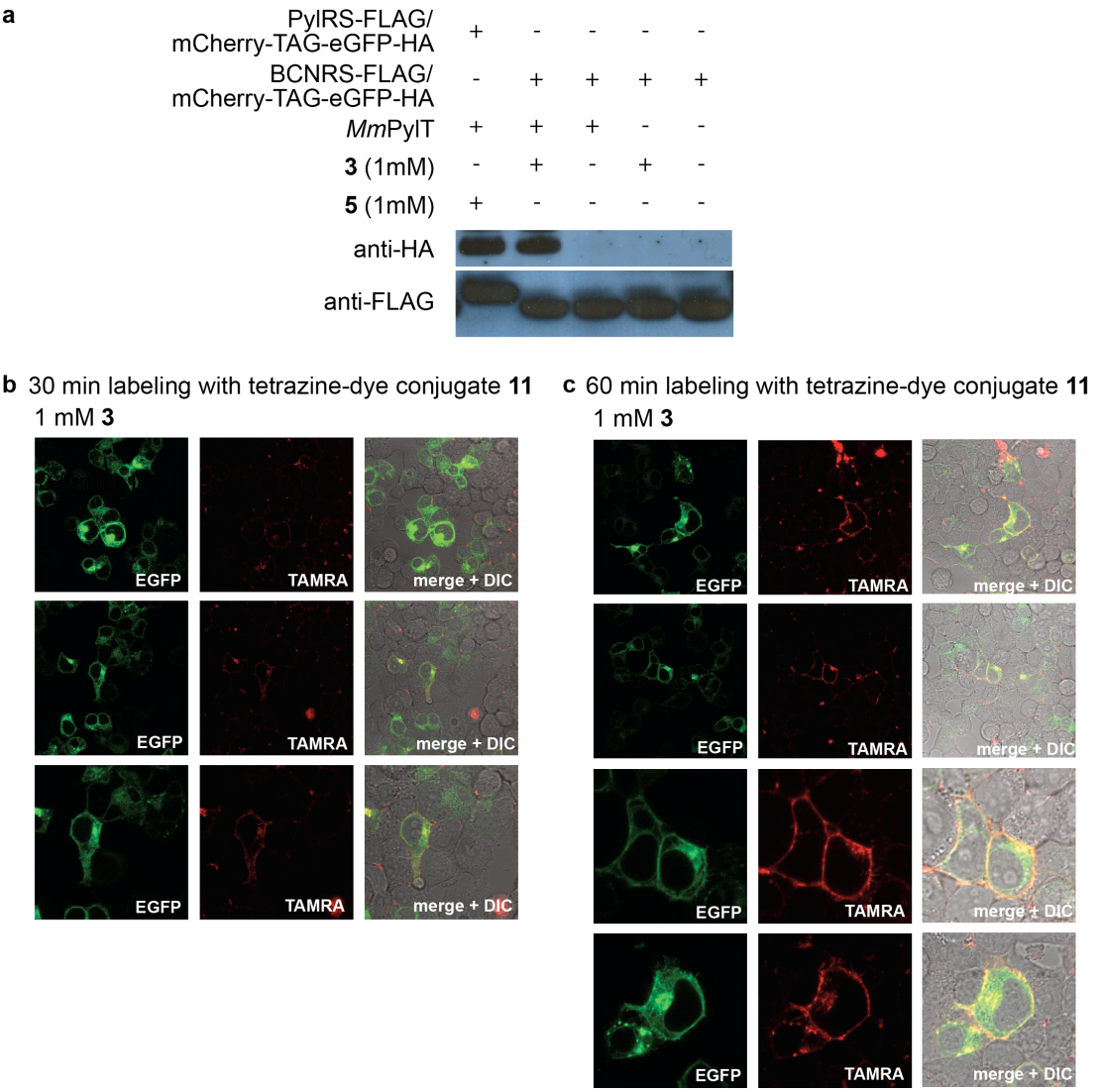
EGFR-GFP bearing **4** at position 128 is visible as green fluorescence at the membrane of transfected cells (left panels). Treatments of cells with **11** (200 nM) leads to labelling of EGFR-GFP containing **4** (middle panels). Right panels show merged green and red fluorescence images, DIC = differential interference contrast. Cells were imaged 2 hours after addition of **11**.



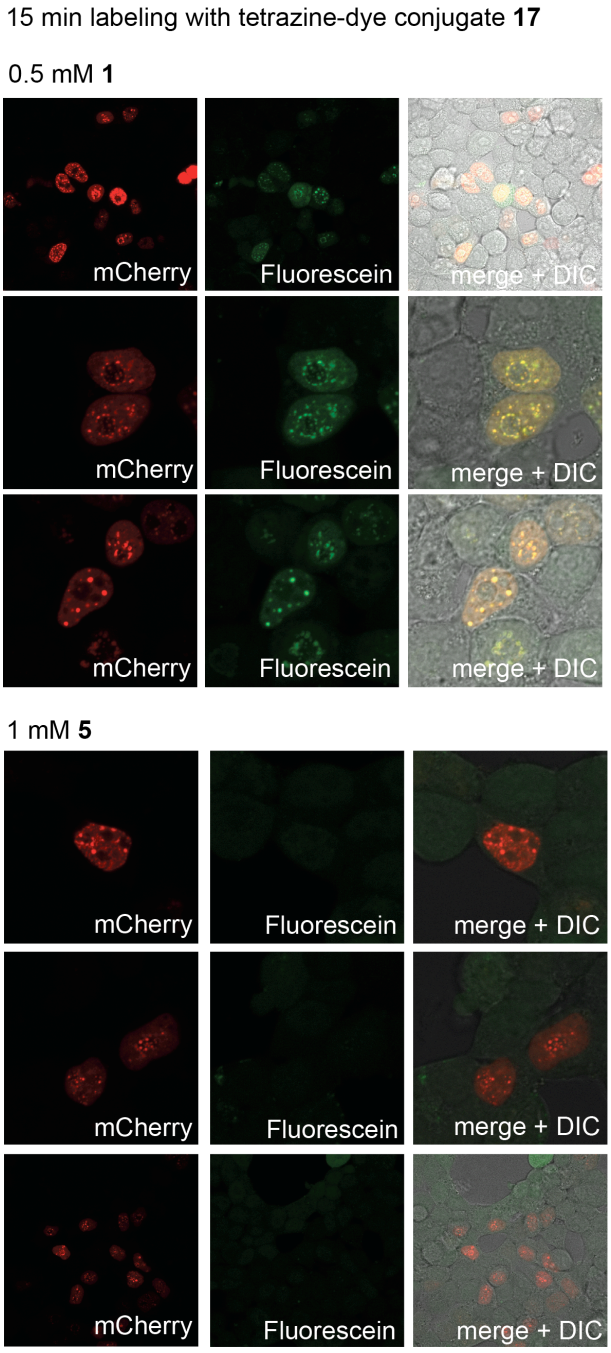
Supplementary Figure S15. Specific and ultra-rapid labelling of EGFR-GFP with tetrazine-fluorophore conjugate **11** for 2 minutes. EGFR-GFP bearing **2** at position 128 is visible as green fluorescence at the membrane of transfected cells (left panels). Treatments of cells with **11** (400 nM) leads to selective labelling of EGFR-GFP containing **2** (middle panels). Right panels show merged green and red fluorescence images, DIC = differential interference contrast. Cells were imaged 2 minutes after addition of **11**. No labelling was observed for cells in the same sample that did not express EGFR-GFP, and cells bearing EGFR-**5**-GFP were not labeled with **11**.



Supplementary Figure S16. Specific and ultra-rapid labelling of EGFR-GFP with tetrazine-fluorophore conjugate **11** for 5 minutes. EGFR-GFP bearing **2** at position 128 is visible as green fluorescence at the membrane of transfected cells (left panels). Treatments of cells with **11** (400 nM) leads to selective labelling of EGFR-GFP containing **2** (middle panels). Right panels show merged green and red fluorescence images, DIC = differential interference contrast. Cells were imaged 5 minutes after addition of **11**. No labelling was observed for cells in the same sample that did not express EGFR-GFP, and cells bearing EGFR-**5**-GFP were not labeled with **11**.



Supplementary Figure S17. Site specific incorporation of **3** in mammalian cells and the labeling of EGFR-GFP with tetrazine-fluorophore conjugate **11** for 30 and 60 minutes. a) Western blots demonstrate that the expression of full length mCherry(TAG)eGFP-HA is dependent on the presence of **3** or **5** and $tRNA_{CUA}$. BCNRS and PylRS are FLAG tagged. B and c) EGFR-GFP in the presence **3** at position 128 is visible as green fluorescence at the membrane of transfected cells (left panels). Treatments of cells with **11** (400 nM) leads to faint, but measurable labelling of EGFR-GFP containing **3** (middle panels) This observation is consistent with the isomerization of the trans-alkene bond to its cis form of a fraction of **3** in mammalian cells. Right panels show merged green and red fluorescence images, DIC = differential interference contrast. Cells were imaged 30 or 60 minutes after addition of **11**. No labelling was observed for cells in the same sample that did not express EGFR-GFP.



Supplementary Figure S18. Specific and ultra-rapid labelling of a nuclear protein in live mammalian cells. Jun-**1**-mCherry is visible as red fluorescence in the nuclei of transfected cells (left panels). Treatment of cells with the cell permeable tetrazine dye **17** (200 nM) leads to selective labeling of jun-**1**-mCherry (middle panel). Right panels show merged red and green fluorescence. DIC = differential interference contrast. Cells were imaged 15 minutes after addition of **11**. No labelling was observed for cells in the same sample that did not express jun-mCherry, and cells bearing jun-**5**-mCherry were not labeled with **11**.

References for Supplementary Information:

1. Gautier, A. et al. Genetically encoded photocontrol of protein localization in mammalian cells. *J Am Chem Soc* **132**, 4086-8 (2010).
2. Lang, K. et al. Genetically encoded norbornene directs site-specific cellular protein labelling via a rapid bioorthogonal reaction. *Nature chemistry* **4**, 298-304 (2012).
3. Dommerholt, J. et al. Readily Accessible Bicyclononynes for Bioorthogonal Labeling and Three-Dimensional Imaging of Living Cells. *Angewandte Chemie-International Edition* **49**, 9422-9425 (2010).
4. Yang, J., Karver, M.R., Li, W., Sahu, S. & Devaraj, N.K. Metal-catalyzed one-pot synthesis of tetrazines directly from aliphatic nitriles and hydrazine. *Angewandte Chemie* **51**, 5222-5 (2012).
5. Taylor, M.T., Blackman, M.L., Dmitrenko, O. & Fox, J.M. Design and synthesis of highly reactive dienophiles for the tetrazine-trans-cyclooctene ligation. *Journal of the American Chemical Society* **133**, 9646-9 (2011).
6. Royzen, M., Yap, G.P. & Fox, J.M. A photochemical synthesis of functionalized trans-cyclooctenes driven by metal complexation. *Journal of the American Chemical Society* **130**, 3760-1 (2008).
7. Zhang, K., Lackey, M.A., Cui, J. & Tew, G.N. Gels based on cyclic polymers. *Journal of the American Chemical Society* **133**, 4140-8 (2011).