Supporting information for:

Diazo group as a new chemical reporter for bioorthogonal labelling of biomolecules

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Contents

S1 General methods and materials

All reactions were conducted in oven-dried glassware under an inert atmosphere of nitrogen and with anhydrous solvents, which were previously purified according to standard methods. Starting materials were obtained from commercial suppliers and used without further purification.

NMR spectra were recorded on the following instruments: Bruker Avance 400 QNP, Bruker Avance III 400 QNP and Bruker Avance. Chemical shifts (δ) are reported in ppm downfield from TMS. Coupling constants (*J*) are quoted in Hz. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, quint. = quintuplet and m = multiplet), coupling constant, integration and assignment. Assignments of the spectra have been made using DEPT, COSY, HMQC and HMBC.

Infrared spectra were recorded on a Perkin Elmer Spectrum One FT-IR spectrometer. The assignments refer to stretching frequencies unless "bend" is specified.

High resolution mass spectra were recorded on Waters LCT Premier TOF mass spectrometer with electrospray and modular Lockspray interface.

Flash column chromatography was performed on Merck silica gel 60 (230-400 mesh particle size). The eluents, which are specified in each case, were previously distilled.

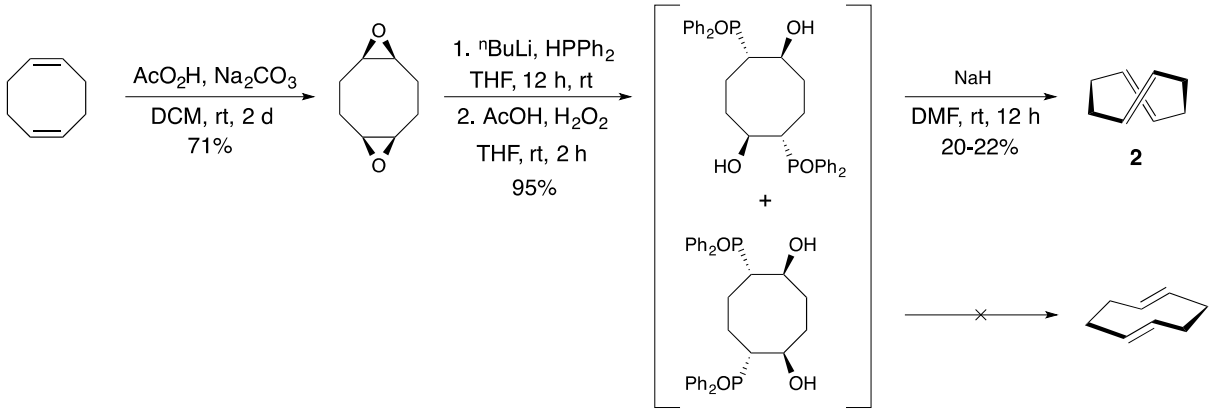
Analytical thin-layer chromatography (TLC) was carried out on Merck Silica gel 60 F254 glass plates. The eluent used is specified in each case. The spots were visualized with ultraviolet light (254 nm) and with exposure to potassium permanganate.

HPLC purification was carried out on a Varian Prostar system. The column used was a Supelco analytical C18, 25 cm x 10 mm and particle size 5 μm. UV detection was done at 254 nm.

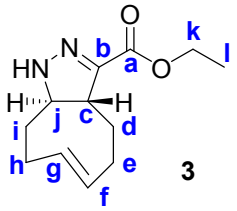
All UV kinetic measurements were made using a Varian Cary 100 Bio UV-Vis spectrophotometer.

S2 Synthetic procedures

(E,E)-Cyclooctadiene 2 was synthesised according to the literature procedure[1] and the spectroscopic data were in accordance with the literature.[2]

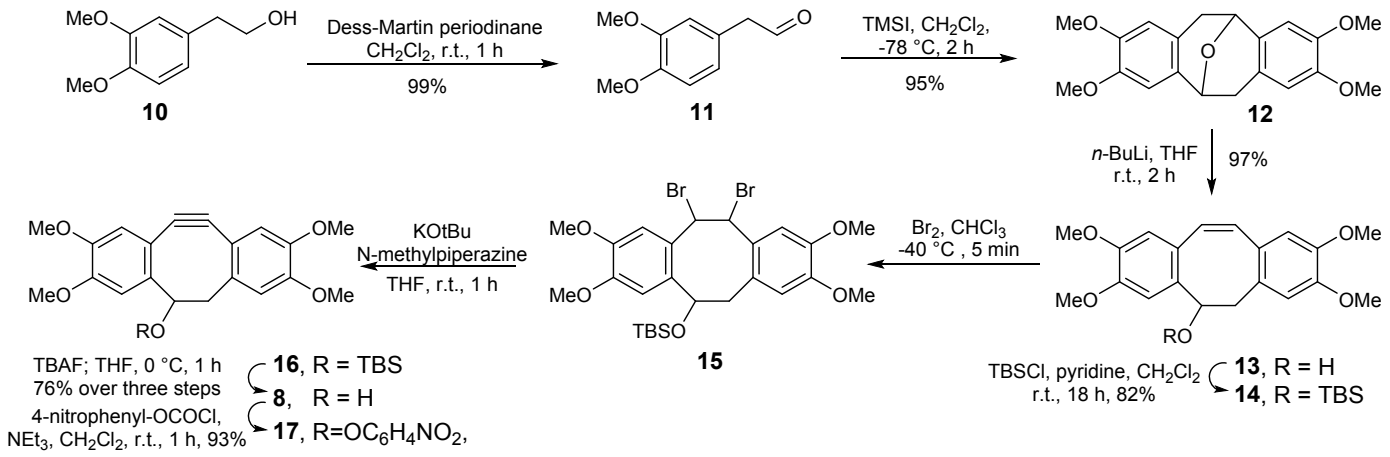


Ethyl *rel*-(3a*R*,9a*R*,*E*)-3a,4,5,8,9,9a-hexahydro-1*H*-cycloocta[*c*]pyrazole-3-carboxylate 3
To a solution of (*E,E*)-1,5-cyclooctadiene (46 mM in pentane, 2.0 mL, 92 µmol) was added ethyl diazoacetate **1** (9.7 µL, 92 µmol) with stirring at room temperature. After 2 h, evaporation of the solvent gave pyrazoline **3** (17.3 mg, 78 µmol, 85%).



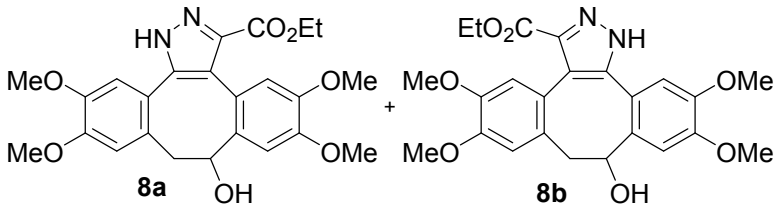
R_f (10% MeOH in DCM) = 0.48; ν_{max}/cm^{-1} 3344, 2926, 2857, 1723, 1558, 1444, 1376, 1250; δ_H (400 MHz, $CDCl_3$) 5.65 (1H, ddd, *J* 16.0, 10.6, 3.6, **g**), 5.55 (1H, ddd, *J* 16.0, 11.0, 3.4, **f**), 4.26-4.19 (2H, m, **k**), 3.71 (1H, td, *J* 10.0, 2.4, **j**), 3.01 (1H, td, *J* 10.2, 1.6, **c**), 2.58-2.51 (1H, m, $\mathbf{d_A}$), 2.50-2.41 (2H, m, $\mathbf{e_A}$ + $\mathbf{h_A}$), 2.39-2.31 (1H, m, $\mathbf{h_B}$), 2.31-2.21 (1H, m, $\mathbf{e_B}$), 2.05-1.89 (2H, m, **i**), 1.67-1.55 (1H, m, $\mathbf{d_B}$), 1.30 (3H, t, *J* 7.2, **l**); δ_C (125 MHz, $CDCl_3$) 14.6 (**l**), 34.0, 35.3, 36.8, 40.1 (**d**,**e**,**h**,**i**), 54.1 (**c**), 61.4 (**k**), 72.3 (**j**), 135.9, 136.7 (**f**,**g**), 140.7 (**b**), 164.8 (**a**); *m/z* (ESI) 223.1435 ($[M+H]^+$. $C_{12}H_{19}N_2O_2$ requires 223.1441).

3-Hydroxy-2',3',2",3"-tetramethoxy-7,8-didehydro-1,2:5,6-dibenzocyclocta-1,5,7-triene (TMDIBO) **8**
TMDIBO and its p-nitrophenyl carbonate ester were synthesised according to the literature procedure and the spectroscopic data were in accordance with the literature.[3]



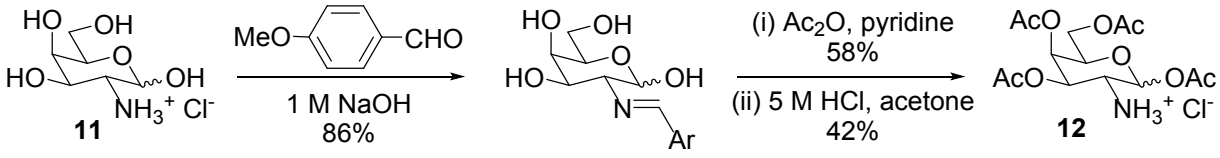
Ethyl 8- and 9-hydroxy-5,6,11,12-tetramethoxy-8,9-dihydro-1*H*-dibenzo[3,4:7,8]cycloocta[1,2-*c*]pyrazole-3-carboxylate 7a and **7b**

TMDIBO **6** (10 mg, 29 µmol) was dissolved in $CHCl_3$ (500 µL) and ethyl diazoacetate (3 µL, 29 µmol) was added. After 2 h, evaporation of the solvent gave the mixture of regioisomeric pyrazolines **7a** and **7b** (13 mg, 29 µmol, 99%) as a white solid.

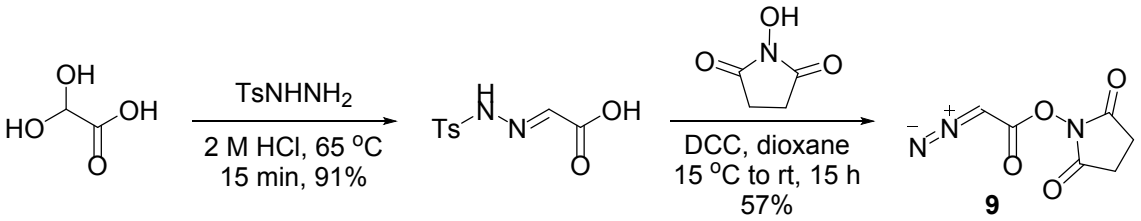


R_f (15% MeOH in DCM) = 0.54 + 0.58; ν_{max}/cm^{-1} 3288, 2935, 2844, 1711, 1666, 1609, 1515, 1464, 1248, 1199, 1159, 1053; δ_H (400 MHz, $CDCl_3$) 1.27-1.37 (3H, m, $CO_2CH_2CH_3$), 2.95-3.20 (2H, m, CH_2CHOH), 3.75-3.96 (12H, m, OCH_3), 4.22-4.47 (2H, m, $CO_2CH_2CH_3$), 4.98-5.31 (1H, m, CHOH), 6.59-7.35 (4H, m, Ar); δ_C (125 MHz, $CDCl_3$) 14.2, 40.0, 45.4, 55.8, 61.2, 68.6, 106.8, 111.2, 113.3, 113.7, 114.1, 114.3, 115.5, 115.6, 117.5, 120.4, 120.7, 121.1, 121.5, 122.2, 123.1, 128.5, 129.2, 129.5, 132.8, 135.8, 146.8, 147.1, 147.5, 148.2, 148.4, 148.9, 149.2, 149.3, 149.8, 160.8, 160.9; *m/z* (ESI) 455.1834 ($[M+H]^+$. $C_{24}H_{27}N_2O_7$ requires 455.1813).

Tetra-*O*-acetyl-D-galactosamine hydrochloride 11 was synthesised according to the literature procedure and the spectroscopic data were in accordance with the literature.[7]

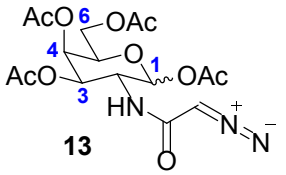


2,5-Dioxopyrrolidin-1-yl diazoacetate 9 was synthesised according to the literature procedure[4,5] and the spectroscopic data were in accordance with the literature.[6]



Tetra-O-acetyl-N-diazoacetyl-D-galactosamine 13

Triethylamine (40 µL, 290 µmol) was added to a solution of tetra-*O*-acetyl-galactosamine hydrochloride **11** (50 mg, 120 µmol) in dry DMF (500 µL) at 0 °C. A solution of NHS diazoacetate **9** (21 mg, 114 µmol) in DMF (300 µL) was then added dropwise and the mixture was stirred 30 min at 0 °C and 22 h at room temperature. The solvent was evaporated under reduced pressure and the crude product was purified by HPLC (20 min gradient (5-95% MeCN in H_2O), r.t. 13.20 min) to give the amide **12** (22 mg, 53 µmol, 45%) as a white solid.

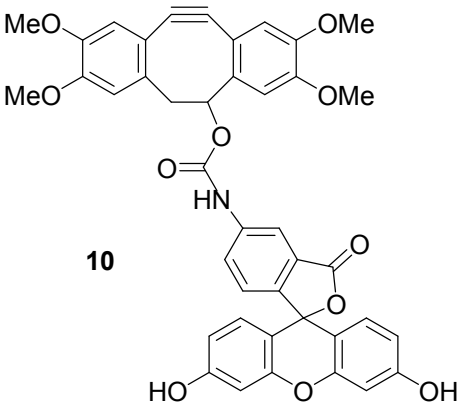


m.p. above 200 °C; ν_{max}/cm^{-1} 3317, 1747, 1673, 1544, 1410, 1367 1211; δ_H (500 MHz, DMSO-d_6) 1.88, 1.98, 2.02 and 2.12 (4 x 3H, s, Ac), 3.96-4.10 (3H, m, **5,6**), 4.20 (1H, q, *J* = 9.5, **2**), 5.23-5.31 (2H, m, **3,4**), 5.79 (1H, d, *J* = 11.0, **1**), 8.48 (1H, d, *J* = 12.0, NH), 8.81 (1H, s, $CH{=}N_2$); δ_C (125 MHz, DMSO-d_6) 20.8,

20.9, 48.9, 49.0, 61.8, 66.7, 70.3, 71.3, 92.5, 141.5, 159.0, 169.3, 169.9, 170.5; *m/z* (ESI) 853.2340 ($[2M+Na]^+$. $C_{32}H_{42}N_6O_{20}Na$ requires 853.2346).

TMDIBO-fluorescein 10

TMDIBO *p*-nitrophenyl carbonate (25 mg, 51 μmol) and fluoresceinamine isomer I (17 mg, 49 μmol) were dissolved in DMF (500 μL) with iPr_2NEt (24 μL, 140 μmol). The reaction mixture was left to stir at room temperature overnight and concentrated *in vacuo*. The residue was purified by *flash* column chromatography (5% MeOH in DCM) to give the product **10** as a bright yellow solid (7 mg, 10 μmol, 20%), which was kept in the dark as far as possible.



10

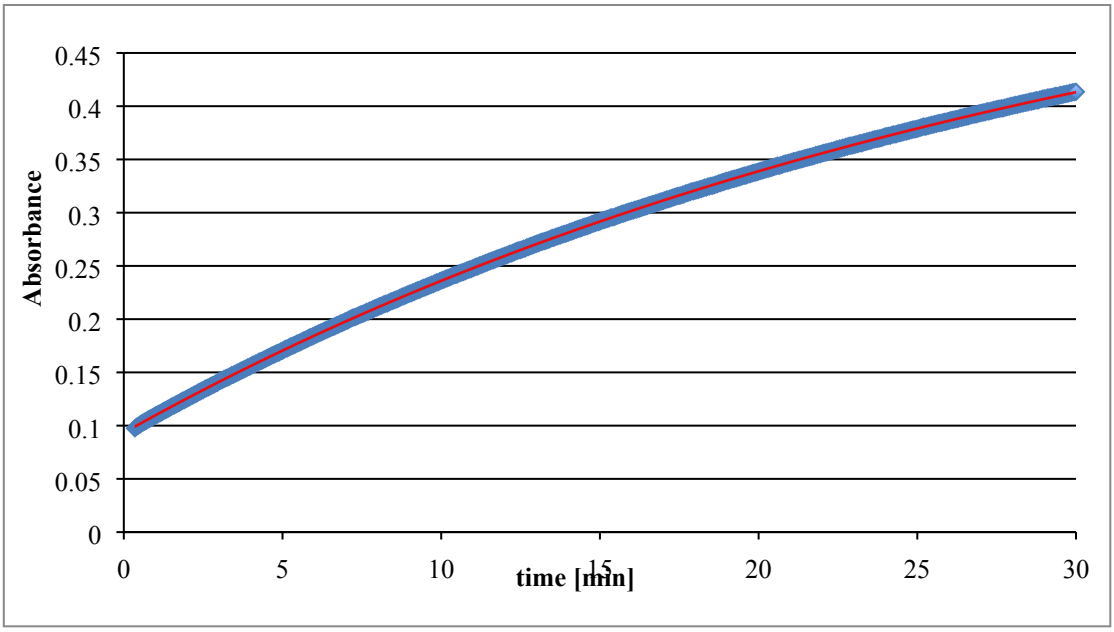
m.p. 182-184 °C; ν_{max}/cm^{-1} 3366, 2920, 1750, 1604; δ_H (500 MHz, DMSO-d_6) 2.67-2.78 and 3.37-3.44 (2 x 1H, m, CH_2), 3.74-3.84 (12H, m, 4 x OMe), 5.29-5.33 (1H, m, C*H*-O), 6.57-6.70 (3H, m, Ar), 6.88-6.93 (2H, m, Ar), 6.93-7.04 (5H, m, Ar), 7.08-7.16 (1H, m, Ar), 7.21-7.24 (1H, m, Ar), 8.09-8.13 (1H, m, Ar), 10.04 (1H, br s, N*H*), 10.15 (2H, br s, O*H*); δ_C (125 MHz, DMSO-d_6) 45.7, 56.1, 56.2, 56.3, 80.3, 81.6, 81.9, 82.0, 102.53, 106.6, 108.1, 109.2, 109.4, 110.0, 110.6, 112.5, 113.0, 113.4, 114.8, 115.1, 115.4, 116.3, 122.2, 124.6, 126.7, 129.6, 139.7, 143.5, 144.3, 148.2, 148.3, 148.4, 149.2, 149.4, 151.2, 151.7, 152.2, 152.4, 160.0, 169.7; *m/z* (ESI) 714.2006 ($[M+H]^+$. $C_{41}H_{32}NO_{11}$ requires 714.1975).

S3 Kinetic evaluation by UV-Vis spectroscopy

Kinetic experiments for the [3+2] cycloaddition and inverse-electron-demand Diels-Alder reactions were performed under pseudo first order conditions by UV-Vis spectroscopy.

[3+2] Cycloaddition of (*E,E*)-1,5-cyclooctadiene with ethyl diazoacetate

The reaction between (*E,E*)-1,5-cyclooctadiene **2** and ethyl diazoacetate **1** was monitored by UV-Vis spectroscopy at 310 nm (product absorption) using them in a 11:1 molar ratio (**2** in excess) in pentane at room temperature.

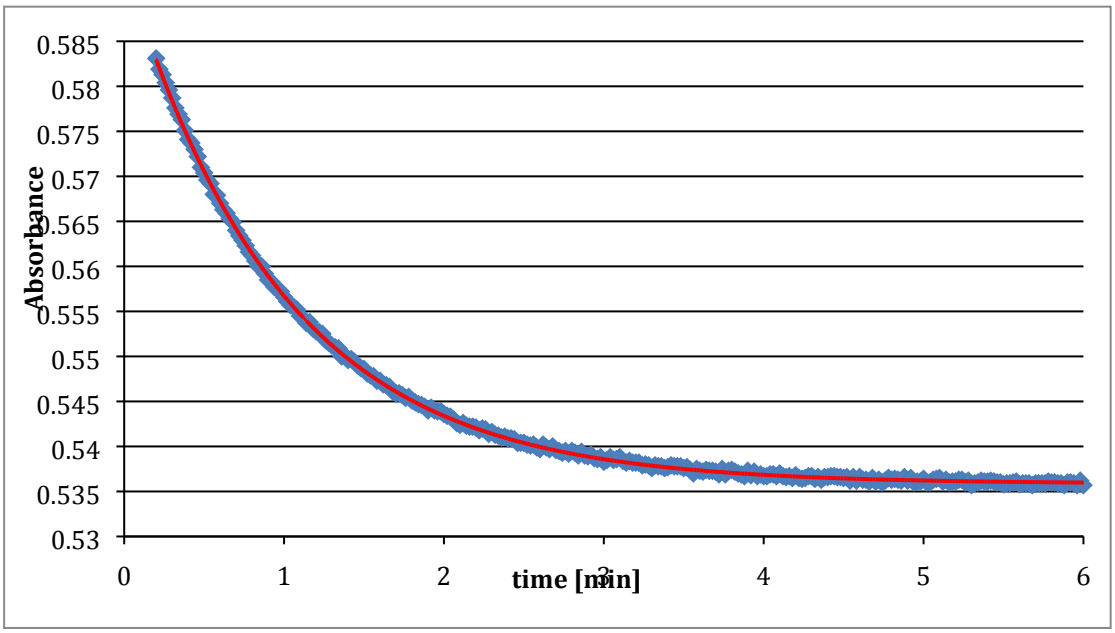


Entry	k' [min^{-1}]	A_∞	C_o [M]	k [M^{-1} s^{-1}]
1	0.0294	0.6085	0.0115	0.0426
2	0.0327	0.6053	0.0115	0.0473
3	0.0316	0.5711	0.0115	0.0459
			average	**0.0453**
			standard deviation	**0.0024**

Figure S3a Kinetic evaluation of the [3+2] cycloaddition of (*E,E*)-1,5-cyclooctadiene and ethyl diazoacetate. Top: Example experimental data (blue) and fitted data (red line) from one of the repetitions. Bottom: Summary of data from the three repetitions; data given: calculated pseudo first order rate constant and absorbance at infinite time from curve fitting, concentration of excess substrate **2** and second order rate constant.

Inverse-electron-demand Diels-Alder of pyrazoline 3 with 3,6-di-(d-pyridyl)-*s*-tetrazine

The reaction between pyrazoline **3** and 3,6-di-(2-pyridyl)-s-tetrazine **5** was monitored by UV-Vis spectroscopy at 290 nm (tetrazine absorption) using them in a 10:1 molar ratio (**3** in excess) in methanol at room temperature.



Entry	k' (min^{-1})	A_∞	C_o [M]	k [$M^{-1}s^{-1}$]
1	0.9672	0.5278	8.1×10^{-5}	199.0
2	1.0199	0.5330	8.1×10^{-5}	209.8
3	1.0181	0.5359	8.1×10^{-5}	209.5
			average	**206.1**
			error	**6.1**

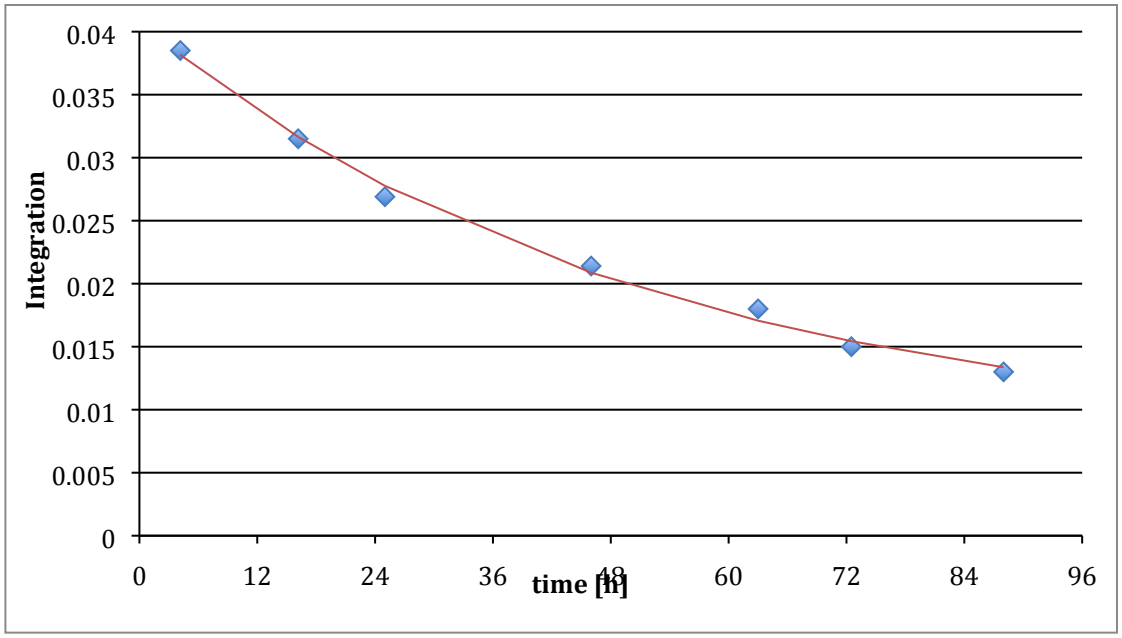
Figure S3b Kinetic evaluation of the inverse-electron-demand Diels-Alder of pyrazoline **3** and 3,6-di-(2-pyridyl)-*s*-tetrazine **5**. Top: Example experimental data (blue) and fitted data (red line) from one of the repetitions. Bottom: Summary of data from the three repetitions; data given: calculated pseudo first order rate constant and absorbance at infinite time from curve fitting, concentration of excess substrate **3** and second order rate constant.

S4 Kinetic evaluation by ^{1}H NMR

Kinetic experiments for the [3+2] cycloaddition were performed under pseudo first order conditions and monitored by ^{1}H NMR (400 MHz).

Second [3+2] cycloaddition of (*E,E*)-1,5-cyclooctadiene with ethyl diazoacetate

Pyrazoline **3** and ethyl diazoacetate **1** were mixed in a 1:10 ratio (3 mM and 30 mM final concentrations respectively) in CD_3OD and monitored by ^{1}H NMR at room temperature, monitoring the peak for pyrazoline **3** at 5.65 ppm.



k' (min^{-1})	C_o [M]	k [M^{-1}s^{-1}]
3.3 x 10^{-4}	0.03	1.8 x 10^{-4}

Figure S4a Kinetic evaluation of the [3+2] cycloaddition of pyrazoline **3** and ethyl diazoacetate **1**. Top: Experimental data (blue) and fitted data (red line). Bottom: Summary of data; data given: calculated pseudo first order rate constant from curve fitting, concentration of excess substrate **3** and second order rate constant.

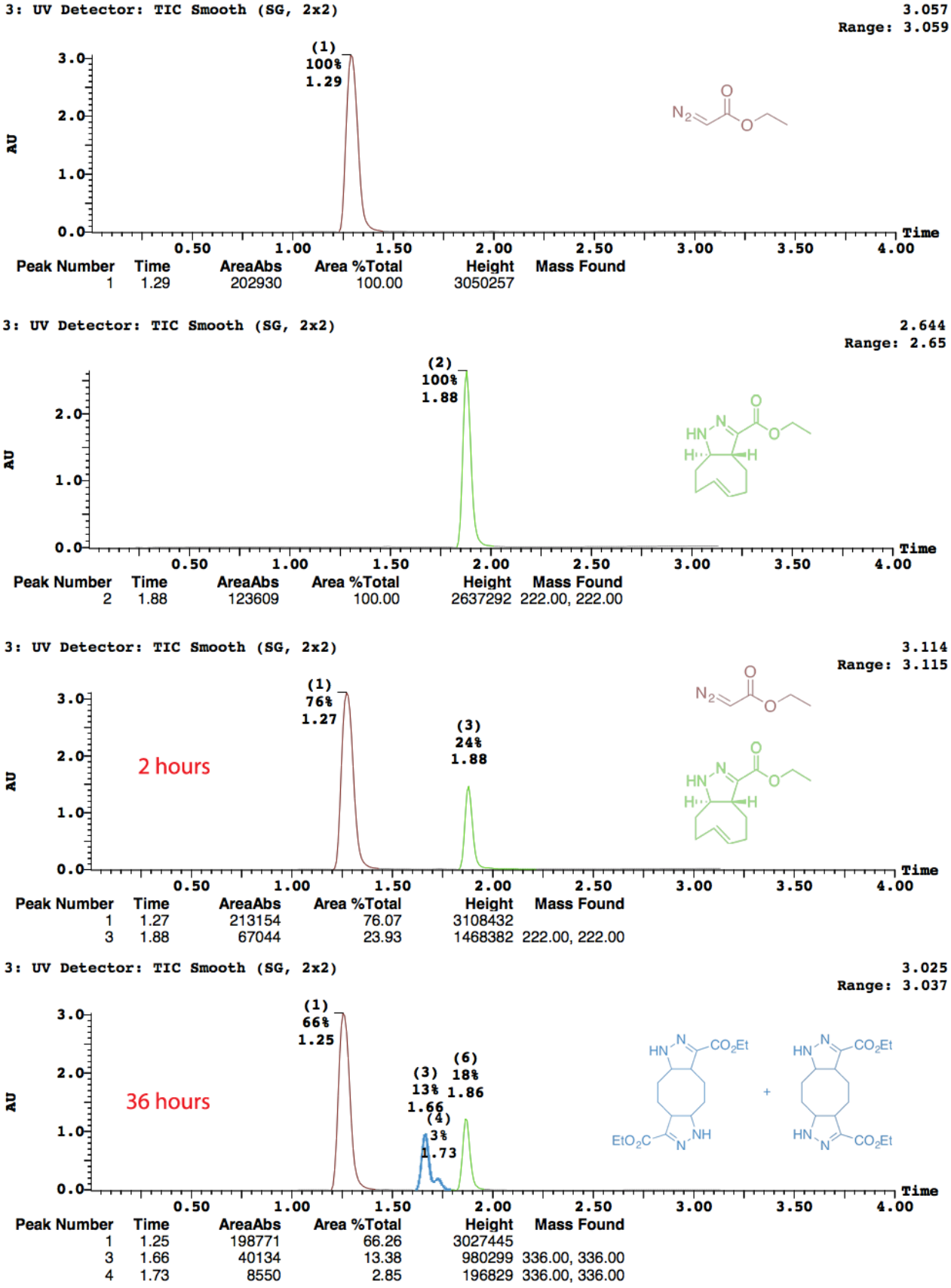
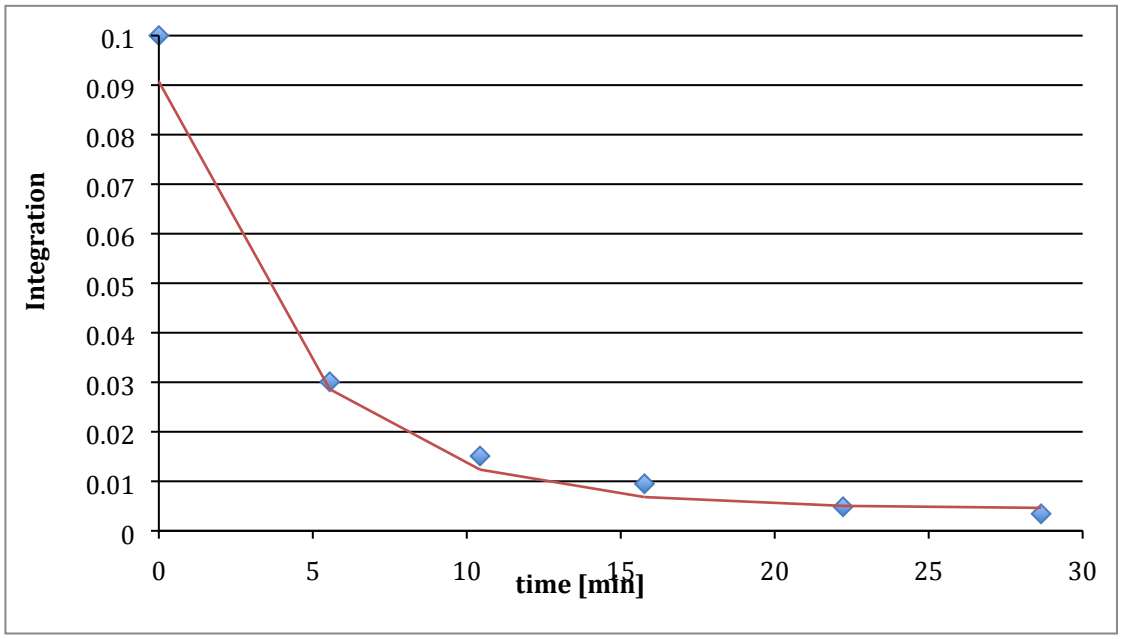


Figure S4b LC/MS spectra of ethyl diazoacetate (top), pyrazoline **3** (second), mixture of ethyl diazoacetate and pyrazoline **3** after 2 h (third) and after 36 h (bottom)

[3+2] Cycloaddition of TMDIBO with ethyl diazoacetate

TMDIBO **7** and ethyl diazoacetate **1** were mixed in a 1:10 ratio (10 mM and 100 mM final concentrations) in $CDCl_3$ and monitored by ^1H NMR at room temperature, monitoring the TMDIBO **7** peak at 7.35 ppm.



Entry	k' (min^{-1})	C_o [M]	k [$M^{-1}s^{-1}$]
1	0.2571	0.1	0.0429
2	0.2298	0.1	0.0383
3	0.2774	0.1	0.0462
		average	**0.0426**
		error	**0.0040**

Figure S4c Kinetic evaluation of the [3+2] cycloaddition of TMDIBO and ethyl diazoacetate. Top: Example experimental data and fitted data from one of the repetitions. Bottom: Summary of data from the three repetitions; data given: calculated pseudo first order rate constant from curve fitting, concentration of excess substrate **3** and second order rate constant.

S5 Stability of diazoesters in the presence of biologically relevant functional groups

Stock solutions of glutathione and ethyl diazoacetate were prepared in 1:1 CD_3CN/D_2O at 20 mM. The glutathione solution was basified to pH 7.4. The following solutions were prepared:

- 700 µL of 1:1 glutathione/ethyl diazoacetate, each at 10 mM
- 700 µL of individual controls (glutathione and ethyl diazoacetate) diluted to 10 mM

The NMR tubes were incubated at 37 °C for 24 h. ^1H NMR spectra were run at 1, 2 and 24 h.

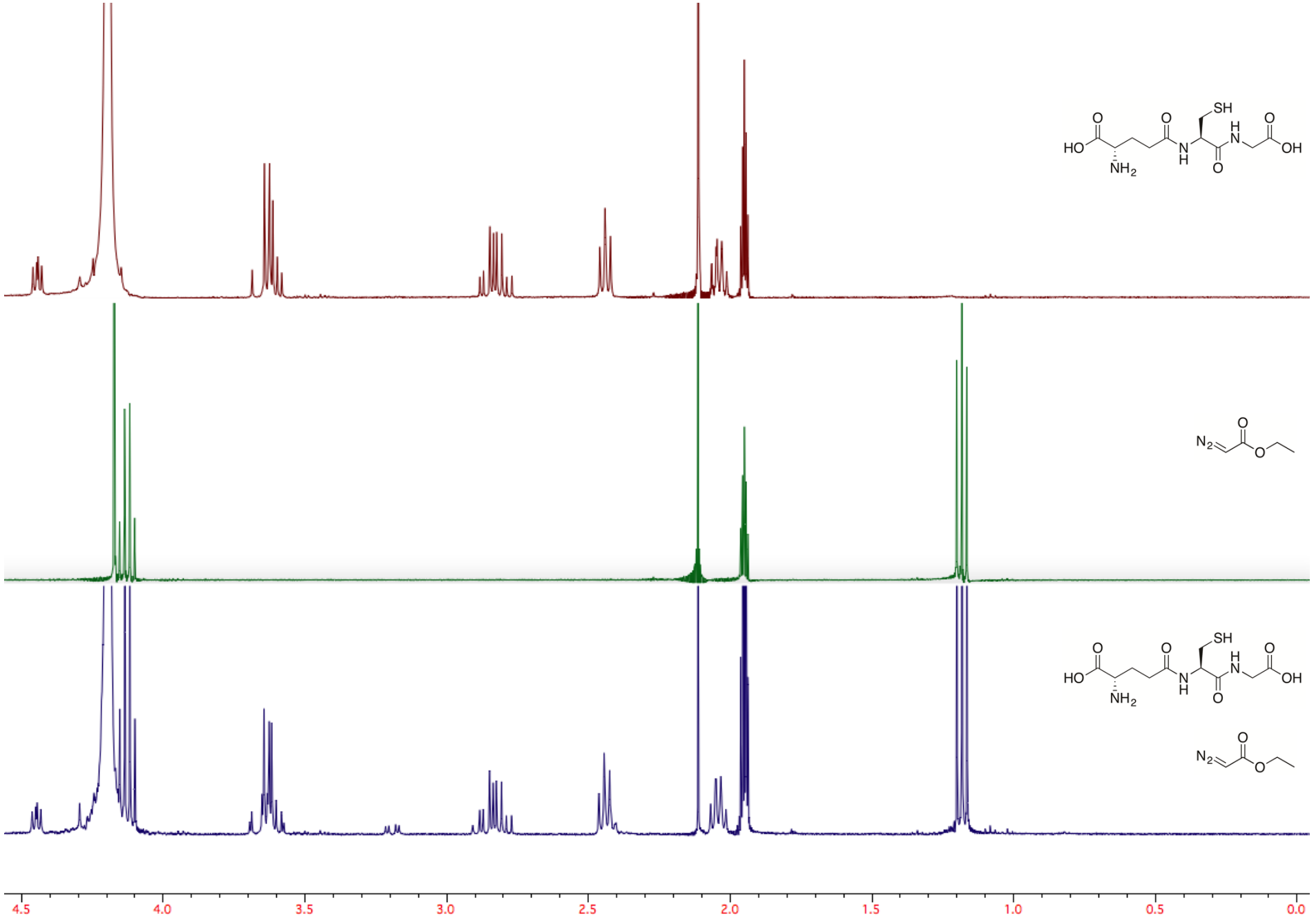


Figure S5 ^{1}H NMR spectra of glutathione (top), ethyl diazoacetate (middle) and a 1:1 mixture of both (bottom) after 24 h in 1:1 CD_3CN/D_2O

S6 Cell surface labeling

LL2 cells were grown in Nunc 6-well dishes in 90% DMEM (Dulbecco's Modified Eagle's Medium, Invitrogen, Paisley, UK) supplemented with 4.5 $g.L^{-1}$ glucose, 4 mM L-glutamine, 1 mM pyruvate and 10% FBS (fetal bovine serum, Gibco, UK) and maintained in a 5% CO_2, water-saturated atmosphere at 37 °C. Cells were incubated for 24 h in medium containing 200 µM diazo sugar and vehicle (DMSO ≤ 0.25% *v/v* in buffer) or vehicle alone. The medium was gently removed from the wells and cells were washed with warm PBS (phosphate buffered saline; water, NaCl, KCl, Na_2HPO_4, KH_2PO_4; Fisher Scientific, Loughborough, UK). Trypsin-EDTA (0.25% trypsin and 1 mM EDTA in Hanks' Balanced Salt Solution without $CaCl_2$, $MgCl_2$ or $MgSO_4$, Invitrogen) was added to the flask (7% of the original volume) and the cells incubated at 37 °C for 4.5 min. Warm complete DMEM was then added to neutralize the trypsin. The contents of each well were transferred to eppendorf tubes, centrifuged (700 g, 4 °C, 4 min) and washed with cold FACS buffer (1% FBS in PBS).

Cells were centrifuged and resuspended in 100 µL labeling buffer A (TMDIBO-PEG-biotin) or as a control 100 µL FACS buffer. The cells were incubated in a hot block with orbital shaking (450 rpm, 37 °C, 30 min), then washed three times with 700 µL ice-cold FACS buffer and then suspended in 100 µl labeling buffer B (50 µg/ml Neutravidin-Dylight680, Invitrogen in FACS buffer containing 50 nM SYTOX Green) or 100 µl FACS buffer as a control. The cells were incubated in a hot block with orbital shaking (450 rpm, 37 °C, 15 min) and then washed two times with 700 µL ice cold FACS buffer. The cell suspensions were filtered through a 50 µm cut-off membrane into flow cytometry tubes prior to analysis by flow cytometer (model LSRII, BD Oxford, UK) using 10,000 events. Data analysis was performed using FlowJo flow cytometry analysis software (Tree Star, Ashland, OR). The viable cell population was determined by gating cells to exclude those with high intensity in the UV and the blue channels (SYTOX Green). The far-red (for NA680) mean fluorescence intensity (MFI) was recorded. Data points were collected in triplicate.

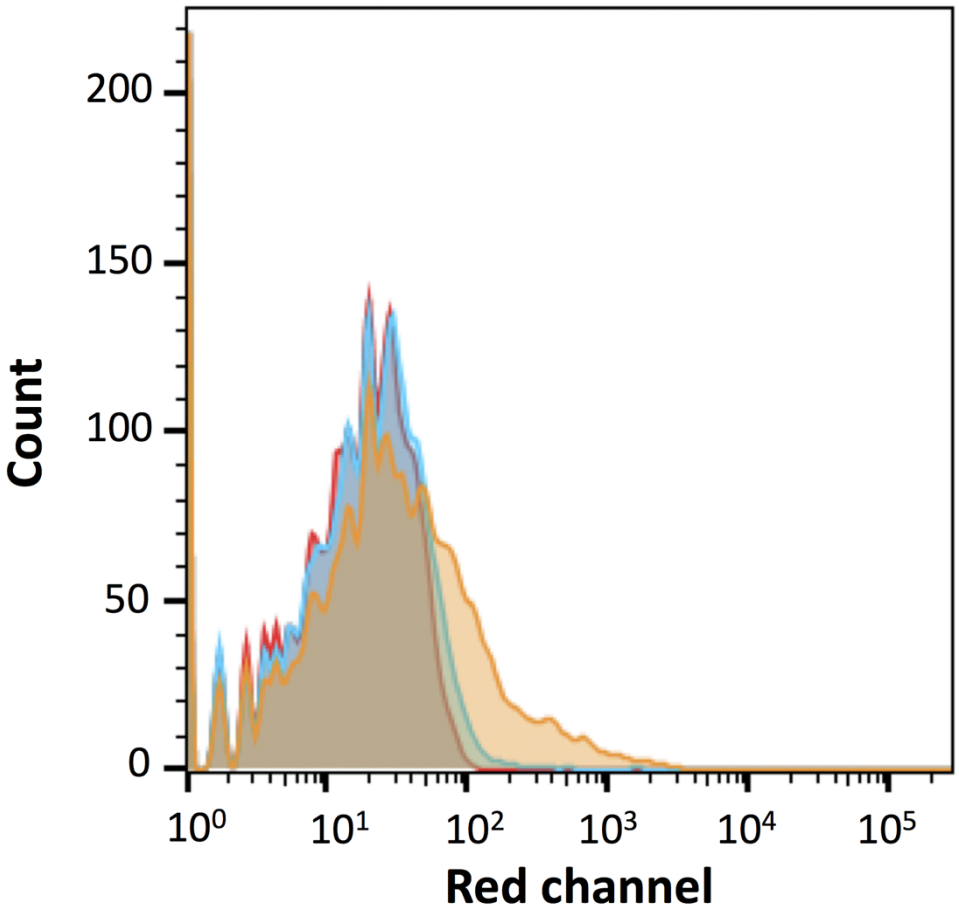


Figure S6 Overlaid histograms of the Fluorescence in the Red Channel from Flow Cytometry. Red: untreated cells. Cells incubated with, Blue, vehicle only (DMSO) or Orange, diazo sugar, were subsequently treated with TMDIBO-PEG-biotin followed by Neutravidin-Dylight680.

S7 Lysozyme labeling

Preparation of the diazo-lysozyme[8]

Hen egg white lysozyme (100 mg, Sigma) was dissolved in PBS (5 mL). Diazo NHS ester **11** (7.5 mg, 41 μmol) was dissolved in DMSO (300 μL) with a small amount of DMF (60 μL). A portion of the lysozyme solution (0.5 mL) was combined with the diazo solution (100 μL) and DMSO (200 μL). After 3 h, the protein was purified on a PD-10 column pre-equilibrated with PBS (see GE healthcare protocol). Four fractions were collected with the first fraction containing the most protein. The protein concentration was 2.3 mg/mL (assayed with PicoDrop). 50 μL of this protein solution was mixed with 50 μL of PBS containing TMDIBO-fluorescein **10** to give final concentrations of **10** of 0, 25, 50 and 100 μM (and also 0%, 0.125%, 0.25% and 0.5% v/v of DMSO). The solution was incubated at 37 °C for 1 h and then a 10 μL aliquot was mixed with 10 mL of staining buffer prior to running on the gel. The gel was a Thermo Scientific Precise Protein gel, with a gradient of 4-20% acrylamide. It was run with a buffer system of Tris-HEPES according to the manufacturers instructions. The ladder was a prestained PageRuler Plus (Thermo Scientific). The gel was fluorescence imaged on a Typhoon Trio variable mode imager for fluorescence (excitation: 488 nm, emission: 532 nm, green light). Subsequently the gel was stained using coomassie blue using standard procedures and photographed.

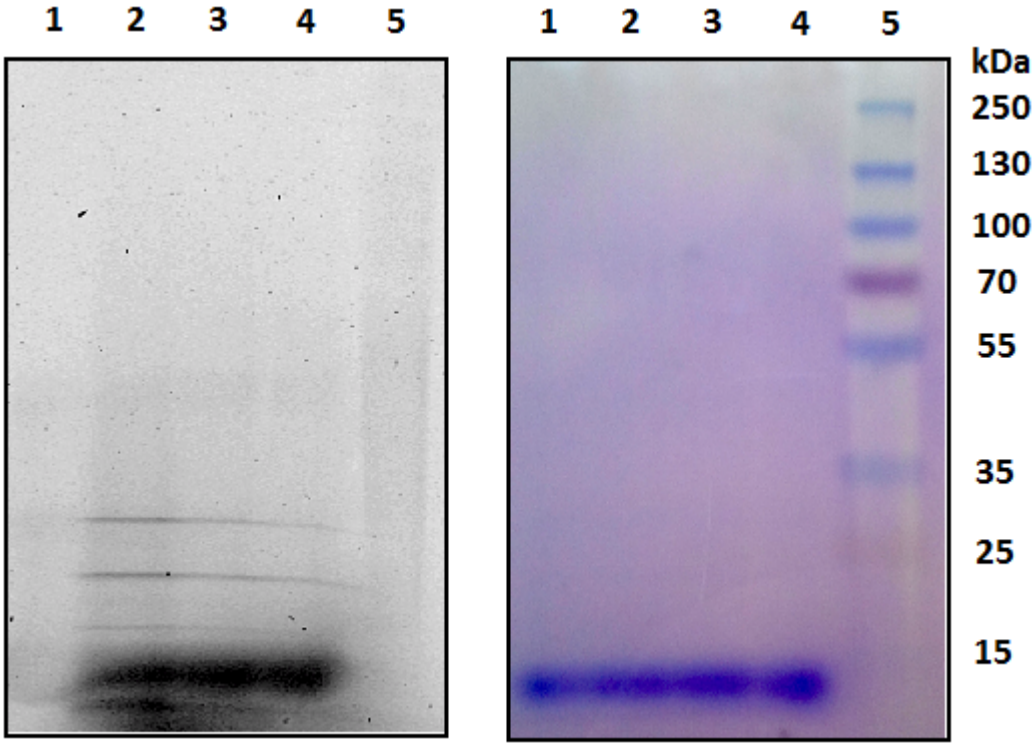


Figure S7 TMDIBO-fluorescein labelling of diazo-modified lysozyme. Left; fluorescence image. Right; visible image after coomassie blue staining. Lanes 1-4: diazo-modified lysozyme treated with 0, 25, 50 and 100 μM of TMDIBO-fluorescein respectively. Lane 5: protein molecular weight ladder. Lysozyme (M.W. 16 kDa) migrates at the expected level on the gel. The fluorescence in lanes 2-4 was quantified as follows (relative to lane 4): lane 2 81%, lane 3 95.5%, lane 4 100%.

S8 Bibliography

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