## Supporting Information

## Precise installation of diazo-tagged side-chains on proteins to enable in vitro and in-cell site-specific labelling

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## 1. General remarks

All solvents were commercially available grade. All reactions were carried out under argon atmosphere unless otherwise mentioned. All reagents were purchased from either SigmaAldrich, Alfa-Aesar or Fluorochem and used without further purification. Reaction mixtures were analysed by analytical thin-layer chromatography and flash column chromatography were performed on Merck TLC Silica gel 60 F254 glass plates and Silica Gel high purity grade (Merck grade 9385 pore size 60A, 230-400 mesh particle size), respectively. Visualisation was accomplished with UV light ( 254 nm ), ninhydrin or $\mathrm{KMnO}_{4} .{ }^{1} \mathrm{H}$ NMR and ${ }^{13} \mathrm{C}$ NMR spectra were recorded on a Bruker 400 MHz DPX-400 Dual Spectrometer and Bruker 500 MHz AVIII HD Smart Probe in the stated solvents ( $\mathrm{DMSOd}_{6}, \mathrm{CDCl}_{3}, \mathrm{CD}_{3} \mathrm{CN}, \mathrm{D}_{2} \mathrm{O}, \mathrm{MeOD}$ ) using tetramethylsilane as an internal standard. Chemical shifts were reported in parts per million (ppm) on the $\delta$ scale from an internal standard (NMR descriptions: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad). Coupling constants, J, are reported in Hertz. FTIR analysis were recorded using a Pelkin-Elmer spectrum one FT-IR universal ATR sampling accessories. Mass spectroscopy was performed using a Waters micromass ZQ (LCMS) with Waters 2795 HPLC and a Waters 2996 photodiode array detector. This system is an automated service utilising electrospray (ESI) ionisation. The mobile phases are 95\% aqueous acetonitrile with $0.05 \%$ formic acid and 10 mM ammonium acetate with $0.1 \%$ formic acid. The separation technology is based on a $50 \times 4.6 \mathrm{~mm}$ C18 column (currently a Phenomenex Kinetix solid core column). There are several methods available enabling the user to produce mass spectra for compounds up to 2 kDa in positive and negative modes of ionisation. In some cases, a Waters LCT Premier combined with an Agilent 1100 autosampler was also used. The system runs using $50 \%$ aqueous acetonitrile with $0.25 \%$ formic acid as mobile phase and can measure accurate masses from 150Da to 1500Da.

## 2. Optimisation of the HWE olefination reaction

Table S1. Conditions evaluated for the formation of 3


| Entry | 4 | 5 | Base | Base | Solvent | T ( ${ }^{\circ} \mathrm{C}$ )/time | (\%) E+Z | $E / Z$ |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| 1 | 1 | $1^{\text {a }}$ | NaOH | 1 | $\mathrm{H}_{2} \mathrm{O}$ | RT (0.5 h) | - | - |
| 2 | 1 | $1^{\text {a }}$ | NaH | 1.1 | THF | -78 (1 h) to 0 (1 h) | 27 | 2:1 |
| 3 | 2 | $1^{\text {a }}$ | NaH | 2.2 | THF | -78 (1 h) to 0 (1 h) | 40 | 1:1 |
| 4 | 1 | $2^{\text {a }}$ | NaOH | 1 | $\mathrm{H}_{2} \mathrm{O}$ | RT (0.5 h) | 0 | - |
| 5 | 1 | $2{ }^{\text {b }}$ | NaOH | 1 | $\mathrm{H}_{2} \mathrm{O}$ | RT (0.5 h) | 0 | - |
| 6 | 1 | $1^{\text {a }}$ | NaH | 1.2 | THF | 0 to r.t. (1h) | 5 | 2:1 |
| 7 | 1 | $2^{\text {a }}$ | NaH | 1 | THF | -78 (1 h) to -20 (2 h) | 15 | 2:1 |
| 8 | 1 | $1^{\text {a }}$ | EtONa | 1 | EtOH | 0 to RT (0.5 h) | 0 | - |
| 9 | 1 | $1^{\text {a }}$ | $\mathrm{Ba}(\mathrm{OH})_{2}$ | 1.25 | THF | 0 to RT (24 h) | 47 | 7:2 |
| 10 | 1 | $1^{\text {a }}$ | DIPEA ${ }^{\text {c }}$ | 1 | MeCN | 0 to RT (24 h) | 36 | 3:1 |
| 11 | 2 | $1^{\text {a }}$ | $\mathrm{Ba}(\mathrm{OH})_{2}$ | 2 | MeCN | 0 to RT (24 h) | 55 | 2:1 |
| 12 | 1 | $1^{\text {a }}$ | $t$-BuOK | 1 | THF | 0 to RT (1 h) | 35 | - |
| 13 | 1 | 1.5 | $\mathrm{Ba}(\mathrm{OH})_{2}$ | 0.8 | THF | 0 to RT (24 h) | 11 | - |
| 14 | 1 | 1.2 | DIPEA ${ }^{\text {c }}$ | 1 | MeCN | 0 to RT (24 h) | 25 | - |
| 15 | 1 | 1.2 | DBU ${ }^{\text {c }}$ | 1 | MeCN | 0 to RT (24 h) | 33 | - |
| 16 | 1 | $1{ }^{\text {e }}$ | NaH | 1 | THF | -78 (1 h) to -20 (2h) | 13 | 1:1 |
| 17 | 1 | 1.2 | DIPEA $^{\text {c }}$ | 1 | MeCN | 0 (24 h) | 78 | 3:1 |
| 18 | 1 | $2{ }^{\text {e }}$ | DIPEA $^{\text {c }}$ | 1 | MeCN | 0 (72 h) | 54 | 1:1 |
| 19 | 2 | $1^{\text {fou }}$ | LiHMDS | 2 | THF | -78 (1 h) to -20 | 6 | - |

${ }^{\text {a }}$ phenyIglyoxal after flash purification and also distillation, stored at $-20^{\circ} \mathrm{C} .{ }^{\mathrm{b}}$ commercially available phenylglyoxal hydrate 5 ( $x . \mathrm{H}_{2} \mathrm{O}$ ). ${ }^{\mathrm{c}} \mathrm{LiCl}$ as additive ( 1 equiv.). ${ }^{d}$ addition of 4 A molecular sieves. ${ }^{e}$ distilled and diluted as a solution in toluene ( 1.18 M ), stored at $-20^{\circ} \mathrm{C} .{ }^{f}$ commercially available monohydrate phenylglyoxal ( $5 . \mathrm{H}_{2} \mathrm{O}$ ) dried for 1 h using $4 \AA$ molecular sieves. ${ }^{g}$ commercially available monohydrate phenylglyoxal $\left(5.1 \mathrm{H}_{2} \mathrm{O}\right)$ under azeotropic removal of water (Dean-Stark).

## 3. Synthesis of the diazocarbonylacrylic reagent 3



In a $0^{\circ} \mathrm{C}$ suspension of LiCl (preheated in oven and under high vacuum, $19 \mathrm{mg}, 0.45 \mathrm{mmol}$, 1.0 equiv.) in dry acetonitrile ( 3.8 mL ) under argon atmosphere was slowly added a 0.1 M solution of diazophosphonate ${ }^{[1]} 4 \mathrm{in} \mathrm{MeCN}$ ( $100 \mathrm{mg}, 0.45 \mathrm{mmol}, 1.0$ equiv.). Then, $80 \mu \mathrm{l}$ of DIPEA ( $0.45 \mathrm{mmol}, 1$ equiv.) was added. The solution changed from brown to light orange. After 5 minutes stirred at the same temperature, a 0.1 M solution of a freshly distilled phenylglyoxal 5 ( $73 \mathrm{mg}, 0.54 \mathrm{mmol}, 1.2$ equiv.) was added dropwise to the reaction mixture. The reaction was stirred at $2-8{ }^{\circ} \mathrm{C}$ for 24 h (in a magnetic stirrer placed inside of the fridge) and then quenched by the addition of 2 mL of $\mathrm{NH}_{4} \mathrm{Cl}$. The residue was extracted with DCM $(2 \times 15 \mathrm{~mL})$ and dried with $\mathrm{MgSO}_{4}$. Purification in column chromatography using $5-50 \%$ EtOAc /Hexanes provided the (E)-5-diazo-1-phenylpent-2-ene-1,4-dione 3 in $60 \%$ yield. ( $E+Z=78 \%$ ). Caution: phenylglyoxal is a very reactive aldehyde that can polymerise and hydrate quickly. The obtained yield can vary depending on the quality of phenylglyoxal. Best results were obtained when a freshly distilled solution was used. Storage of 5 in approx. 1 M toluene solution at $-20^{\circ} \mathrm{C}$. Isomer E (3): ${ }^{1} \mathrm{H}$ NMR ( $500 \mathrm{MHz}, \mathrm{CDCl}_{3}$ ) $\delta 8.05-8.01(\mathrm{~m}, 2 \mathrm{H})$, 7.90 (d, J = $15.2 \mathrm{~Hz}, 1 \mathrm{H}), 7.66-7.61$ (m, 1H), $7.55-7.50(\mathrm{~m}, 2 \mathrm{H}), 7.03$ (d, J = $14.9 \mathrm{~Hz}, 1 \mathrm{H}$ ), 5.58 (s, 1H); ${ }^{13} \mathrm{C}$ NMR ( $125 \mathrm{MHz}, \mathrm{CDCl}_{3}$ ) $\delta$ 189.6, 182.8, 136.9, 136.8, 133.8, 132.0, 128.9, 128.8, 58.3; FT-IR (neat, $\mathrm{cm}^{-1}$ ): 3088, 2959, 2926, 2855, 2156, 2108, 1690, 1666, 1624, 1599, 1449, 1379, 1360, 1290, 1217, 1146, 1007, 976, 712; HRMS ESI ( $\mathrm{m} / \mathrm{z}$ ): Calcd. For $\mathrm{C}_{11} \mathrm{H}_{9} \mathrm{~N}_{2} \mathrm{O}_{2}{ }^{+}[\mathrm{M}+\mathrm{H}]^{+}$201.0659, found: 201.0599; $\mathrm{R}_{\mathrm{f}} 0.30$ (25\% EtOAc/Hexanes). This diazo compound 3 can be stored at $-20^{\circ} \mathrm{C}$ for 12 months without degradation (covering from light).

## 4. Synthesis of the diazoacetophenone 10


15.6 mL of (Trimethylsilyl)diazomethane solution in ether ( $2 \mathrm{M}, 31.3 \mathrm{mmol}, 2.2$ equiv.) was added to a round bottom flask containing 17.9 mL of diethyl ether under argon at $-10^{\circ} \mathrm{C}$. Afterwards, 2.0 mL ( 14.2 mmol , 1 equiv.) of triethylamine was added followed by the addition of 1.66 mL of benzoyl chloride drop wise ( $14.2 \mathrm{mmol}, 1$ equiv.). The reaction mixture was kept at $-10^{\circ} \mathrm{C}$ for 1 h , allowed to reach room temperature and stirred for an additional period of 12 h . The white precipitate formed was filtered and washed with diethyl ether. The organic solution was concentrated in vacuo to give a yellow oil that was purified in silica gel (5-20\% EtOAc/pet. ether) to give the diazoacetophenone 10 as a yellow solid ( $1.2 \mathrm{~g}, 8.2 \mathrm{mmol}$, $58 \%$ yield): ${ }^{1} \mathrm{H}$ NMR ( $400 \mathrm{MHz}, \mathrm{CDCl}_{3}$ ) $\delta 5.91$ (s, 1H), 7.44 (t, J = $7.5 \mathrm{~Hz}, 2 \mathrm{H}$ ), $7.50-5.56$ (m, 1H), 7.75 (dd, J = 8.3, $1.3 \mathrm{~Hz}, 2 \mathrm{H}$ ); ${ }^{13} \mathrm{C}$ NMR NMR ( $100 \mathrm{MHz}, \mathrm{CDCl}_{3}$ ) $\delta 186.3,136.6$, 132.6, 128.6, 126.6, 54.1; IR (neat, $\mathrm{cm}^{-1}$ ) 3088, 2108, 1728, 1622, 1575, 1448, 1367, 1228, 1145, 702; mp $48-50^{\circ} \mathrm{C}, \mathrm{R}_{\mathrm{f}} 0.30$ ( $25 \%$ EtOAc/pet. ether). All the obtained data are in accordance with previously described in the literature. ${ }^{[2]}$

## 5. General procedure for thiol-Michael addition



To a solution of $N$-(tert-Butoxycarbonyl)-L-cysteine methyl ester $\mathbf{6 a}(4.7 \mathrm{mg}, 0.020 \mathrm{mmol}$, 1 equiv.) in 0.4 mL of $\mathrm{NaP}_{\mathrm{i}}$ buffer ( $\mathrm{pH} 8.0,50 \mathrm{mM}$ ) containing $30 \%$ of DMF was added a solution of commercially available ethyl ( $E$ )-5-diazo-1-phenylpent-2-ene-1,4-dione 3 $(4.0 \mathrm{~g}, 0.020 \mathrm{mmol}, 1$ equiv.) in DMF ( 0.1 mL ) at room temperature. The completion of reaction was monitored by TLC using short UV and ninhydrin staining solution. After 10 min , the reaction mixture was extracted with DCM ( $3 \times 30 \mathrm{~mL}$ ), dried with $\mathrm{MgSO}_{4}$ and concentrated in vacuo to give the crude product $7 \mathbf{a}$ in $90 \%$ yield ( $7.8 \mathrm{mg}, 0.018 \mathrm{mmol}$ ) as a pale-yellow oil: $\mathrm{R}_{\mathrm{f}}=0.40$ (40\% EtOAc/pet. ether); (mixture of rotamers/isomers) ${ }^{1} \mathrm{H}$ NMR ( $400 \mathrm{MHz}, \mathrm{CDCl}_{3}$ $\delta 7.96(\mathrm{~d}, J=8.2 \mathrm{~Hz}, 2 \mathrm{H}), 7.57(\mathrm{t}, J=7.4 \mathrm{~Hz}, 1 \mathrm{H}), 7.46(\mathrm{t}, J=7.6 \mathrm{~Hz}, 2 \mathrm{H}), 5.69(\mathrm{~s}, 1 \mathrm{H}), 5.38$ (d, $J=8.0 \mathrm{~Hz}, 1 \mathrm{H}), 4.64-4.42(\mathrm{~m}, 1 \mathrm{H}), 3.97-3.80(\mathrm{~m}, 2 \mathrm{H}), 3.76(\mathrm{~s}, 3 \mathrm{H}), 3.35-3.21(\mathrm{~m}$, 1H), $3.19-2.90(\mathrm{~m}, 2 \mathrm{H}), 1.44(\mathrm{~s}, 9 \mathrm{H})$; ${ }^{13} \mathrm{C}$ NMR ( $100 \mathrm{MHz}, \mathrm{CDCl}_{3}$, major isomer) $\delta 196.9$, 191.1, 171.1, 155.1, 136.1, 133.6, 128.7, 128.2, 80.4, 55.3, 53.5, 52.8, 40.9, 32.9, 29.7, 28.3; HRMS ESI ${ }^{+}(\mathrm{m} / \mathrm{z})$ : Calcd. for $\mathrm{C}_{20} \mathrm{H}_{25} \mathrm{~N}_{3} \mathrm{NaO}_{6} \mathrm{~S}^{+}[\mathrm{M}+\mathrm{Na}]^{+} 458.1356$, found: 458.1368; FTIR ( $\mathrm{cm}^{-1}$ ): 3368, 2921, 2105, 1710, 1684, 1634, 1597, 1509, 1366, 1347, 1216, 1065, 985, 759; Cbz-protected 7b prepared in $88 \%$ as a yellow solid from 3 and $\mathbf{6 b}$ using the same procedure: $\mathrm{R}_{\mathrm{f}}=0.57$ ( $50 \%$ EtOAc/pet. ether); (mixture of isomers/rotamers) ${ }^{1} \mathrm{H}$ NMR ( 400 $\left.\mathrm{MHz}, \mathrm{CDCl}_{3}\right) \delta 7.98-7.92(\mathrm{~m}, 2 \mathrm{H}), 7.60-7.54(\mathrm{~m}, 1 \mathrm{H}), 7.50-7.40(\mathrm{~m}, 2 \mathrm{H}), 7.37-7.29(\mathrm{~m}$, $5 \mathrm{H}), 5.75-5.55(\mathrm{~m}, 2 \mathrm{H}), 5.18-5.09(\mathrm{~m}, 2 \mathrm{H}), 4.65(\mathrm{~d}, \mathrm{~J}=6.9 \mathrm{~Hz}, 1 \mathrm{H}), 3.93-3.80(\mathrm{~m}, 2 \mathrm{H})$, 3.77 ( $\mathrm{d}, \mathrm{J}=2.8 \mathrm{~Hz}, 3 \mathrm{H}$ ), $3.32-2.97(\mathrm{~m}, 3 \mathrm{H}) ;{ }^{13} \mathrm{C} \mathrm{NMR}\left(100 \mathrm{MHz}, \mathrm{CDCl}_{3}\right.$, major isomer) $\delta$ 196.9, 191.1, 170.8, 155.7, 136.0, 133.6, 128.7, 128.6, 128.3, 128.2, 128.1, 125.1, 67.3, 55.4, 53.8, 53.6, 52.9, 40.8, 32.8; HRMS ESI ${ }^{+}(\mathrm{m} / \mathrm{z})$ : Calcd. for $\mathrm{C}_{23} \mathrm{H}_{23} \mathrm{O}_{6} \mathrm{~N}_{3} \mathrm{NaS}^{+}[\mathrm{M}+\mathrm{Na}]^{+}$ 492.1200, found: 492.1195; FTIR (cm¹): 3329, 3093, 2105, 1731, 1684, 1625, 1537, 1379, 1349, 1268, 1227, 1021, 983, 764.

## 6. Diazo stability studies in the presence of GSH

Reactions were performed by treating reduced glutathione - GSH ( $2.1 \mathrm{mg}, 0.0068 \mathrm{mmol}, 1.5$ equiv.) with compound $\mathbf{7 b}$ ( $2.0 \mathrm{mg}, 0.0046 \mathrm{mmol}, 1$ equiv.) in a solution of $\mathrm{NaP}_{\mathrm{i}}$ buffer $(50 \mathrm{mM})$ at pH 7.4 (Figure S1b) and pH 8.0 (Figure S1c) containing 50\% of DMF at room temperature ( 120 mL buffer +120 mL of DMF). After 24 h at RT, an aliquot was analysed by liquid-chromatography mass-spectrometry (LC-MS). The diazogroup did not show reactivity with the carboxylic acid present at the GSH structure.


Figure S1 Stability studies of $\mathbf{7 b}(\mathbf{a})$ in the presence of reduced GSH (1.5 equiv.) in $\mathrm{NaP}_{\mathrm{i}}$ ( 50 $\mathrm{mM}), \mathrm{pH} 7.4$ (b), pH 8.0 (c) and the mass spectra of the peak at $2.00 \mathrm{~min}(3-28 \mathrm{~m} / \mathrm{z})$.

## 7. General procedure for 1,3-dipolar cycloaddition on small molecules



In an eppendorf tube, diazo-Cyz-Cbz 7b ( $30 \mathrm{mg}, 0.064 \mathrm{mmol}$ ) was dissolved in 0.6 mL of DMF at room temperature. A suspension of (1R,8S,9s)-Bicyclo[6.1.0]non-4-yn-9ylmethanol 8 ( $9.6 \mathrm{mg}, 0.064 \mathrm{mmol}$ ) in $40 \% \mathrm{DMF} / \mathrm{NaP}_{i}$ buffer ( $\mathrm{pH} 8.0,50 \mathrm{mM}$ ) was then added under stirring and the reaction placed in a shaker at room temperature. The reaction progress was monitored by thin-layer chromatography (50\% EtOAc/pet. ether) and LC-MS. After the reaction was judged to be complete ( 24 h ), the mixture was extracted with DCM ( $3 \times 30 \mathrm{~mL}$ ), dried with $\mathrm{MgSO}_{4}$ and concentrated under reduced pressure. Purification by silica gel chromatography afforded the pyrazole 9 as a clear oil ( $27.8 \mathrm{mg}, 0.045 \mathrm{mmol}, 70 \%$ ): $\mathrm{R}_{\mathrm{f}}=0.29$ ( $80 \% \mathrm{EtOAc} /$ pet. ether); ${ }^{1} \mathrm{H}$ NMR ( 500 MHz , DMSO- $d_{6}, 9{ }^{\circ} \mathrm{C}$, mixture of rotamers/isomers) $\delta 12.76$ (s, 1H), $7.97-7.92(\mathrm{~m}, 2 \mathrm{H}), 7.63(\mathrm{td}, J=7.4,1.3 \mathrm{~Hz}, 1 \mathrm{H}), 7.52(\mathrm{td}, J=7.8,2.5 \mathrm{~Hz}$, 2H), 7.41 (s, 1H), $7.36-7.26(m, 5 H), 5.10-4.98(m, 3 H), 4.34-4.22(m, 1 H), 3.89-3.78$ (m, 1H), 3.63 (d, J = 6.0 Hz, 3H), $3.55-3.37$ (m, 3H), 2.97 - 2.67 (m, 6H), 2.16 - 2.02 (m, 2 H ), $1.70-1.56(\mathrm{~m}, 1 \mathrm{H}), 1.55-1.42(\mathrm{~m}, 1 \mathrm{H}), 1.00(\mathrm{dd}, J=9.2,7.2 \mathrm{~Hz}, 1 \mathrm{H}), 0.90-0.76(\mathrm{~m}$, 3 H ); ${ }^{13} \mathrm{C}$ NMR ( $125 \mathrm{MHz}, \mathrm{DMSO}, 9{ }^{\circ} \mathrm{C}$, mixture of rotamers/isomers) $\delta 196.9,191.9,170.5$, $155.2,136.5,136.4,136.1,132.7,128.2,127.8,127.4,127.2,127.0,127.0,124.8,119.8$, $65.3,59.2,57.2,54.2,51.5,43.1,42.4,31.9,23.9,23.8,22.6,22.5,21.9,21.7,21.6,20.5$, 20.3, 20.1, 18.5, 18.3, 18.2, 13.6 ; $\mathrm{HRMS} \mathrm{ESI}^{+}(\mathrm{m} / \mathrm{z})$ : Calcd. for $\mathrm{C}_{33} \mathrm{H}_{38} \mathrm{~N}_{3} \mathrm{O}_{7} \mathrm{~S}[\mathrm{M}+\mathrm{H}]^{+}$ 620.2430, found: 620.2415; FTIR (cm¹): 3307, 2922, 1715, 1673, 1597, 1513, 1343, 1264, 1219, 1015, 937, 732.

The same procedure was used for converting $10(0.032 \mathrm{mmol})$ and $8(0.032 \mathrm{mmol})$ to 11 ( $0.031 \mathrm{mmol}, 99 \%$ yield, white solid). The reaction was monitored by LC-MS (Figure 2) and was complete after 24 h , no need for purification in silica gel: $\mathrm{R}_{\mathrm{f}}=0.30(80 \% \mathrm{EtOAc} / \mathrm{pet}$. ether); ${ }^{1} \mathrm{H}$ NMR ( $400 \mathrm{MHz}, \mathrm{MeOD}$ ) $\delta 7.93(\mathrm{~s}, 2 \mathrm{H}), 7.58(\mathrm{t}, \mathrm{J}=7.4 \mathrm{~Hz}, 1 \mathrm{H}), 7.46(\mathrm{t}, \mathrm{J}=7.6$ $\mathrm{Hz}, 2 \mathrm{H}), 4.58(\mathrm{~s}, 1 \mathrm{H}), 3.71-3.57(\mathrm{~m}, 2 \mathrm{H}), 3.13-3.05(\mathrm{~m}, 1 \mathrm{H}), 3.01$ (ddt, $J=15.0,7.8,3.9$ $\mathrm{Hz}, 1 \mathrm{H}$ ), 2.80 (ddt, $J=15.0,9.2,3.9 \mathrm{~Hz}, 2 \mathrm{H}$ ), $2.29-2.16(\mathrm{~m}, 2 \mathrm{H}), 1.66(\mathrm{~s}, 1 \mathrm{H}), 1.58-1.48$ (m, 1H), 1.11 (m, 1H), 1.06-0.93 (m, 2H); ${ }^{13} \mathrm{C}$ NMR ( $100 \mathrm{MHz}, \mathrm{MeOD}$ ) $\delta 191.6,142.9,138.4$, 132.3, 132.3, 130.0, 127.8, 121.0, 58.4, 28.6, 24.0, 23.5, 22.6, 21.9, 20.6, 19.3; HRMS ESI ${ }^{+}$ $(\mathrm{m} / \mathrm{z})$ : Calcd. for $\mathrm{C}_{18} \mathrm{H}_{21} \mathrm{~N}_{2} \mathrm{O}_{2}[\mathrm{M}+\mathrm{H}]^{+}$297.1603, found: 297.1603; FTIR ( $\mathrm{cm}^{-1}$ ): 3234, 2933, 1645, 1597, 1577, 1450, 1399, 1257, 1173,1019, 909, 697.



Figure S2 Progress of the 1,3-cycloaddition reaction between 10 and $\mathbf{8}$ to give $\mathbf{1 1}$ by LCMS . The reaction was complete after 24 h .

## 8. Preparation of DIBONE




DIBONE was prepared in five steps following a procedure described in the literature from commercially available 5-Dibenzosuberenone and spectroscopic data is in accordance as previously reported. ${ }^{[3]}{ }^{1} \mathrm{H} \mathrm{NMR}\left(400 \mathrm{MHz}, \mathrm{CDCl}_{3}\right) \delta 7.55$ (ddd, $\left.J=7.6,1.4,0.6 \mathrm{~Hz}, 1 \mathrm{H}\right), 7.52$ $-7.46(\mathrm{~m}, 2 \mathrm{H}), 7.42-7.30(\mathrm{~m}, 5 \mathrm{H}), 4.18(\mathrm{~d}, J=12.3 \mathrm{~Hz}, 1 \mathrm{H}), 3.65(\mathrm{~d}, J=12.4 \mathrm{~Hz}, 1 \mathrm{H})$.

## 9. Preparation of diazoester 13



Modified procedure from ref. ${ }^{[4]}$. Over a period of 30 min , 9-fluorenylmethyl chloroformate ( $2.04 \mathrm{mmol}, 528 \mathrm{mg}$ ) in THF ( 2 mL ), and diisopropylethylamine ( $2.04 \mathrm{mmol}, 356 \mu \mathrm{~L}$ ) were simultaneously added to a stirred solution of p-iodobenzylamine hydrochloride ( 1.86 mmol , $500 \mathrm{mg})$ and $\mathrm{Na}_{2} \mathrm{CO}_{3}(1.86 \mathrm{mmol}, 197 \mathrm{mg})$ in $\mathrm{THF} / \mathrm{H}_{2} \mathrm{O}(2 \mathrm{~mL} / 4 \mathrm{~mL})$ at $0^{\circ} \mathrm{C}$. The resulting mixture was stirred for an additional 3 h at RT . The reaction mixture was extracted with DCM $(25 \mathrm{~mL})$ and the aqueous layer was acidified with $\mathrm{HCl}(10 \mathrm{~mL}, 3 \mathrm{M})$ and extracted with DCM $(4 \times 10 \mathrm{~mL})$. The organic phase was dried over $\mathrm{MgSO}_{4}$ and the solvent was removed in vacuo. The dry crude product was recrystallised from hot absolute ethanol, and the pure product, N-Fmoc p-lodobenzylamine, was obtained as white crystals ( $568 \mathrm{mg}, 1.25 \mathrm{mmol}, 67$ \%). ${ }^{1} \mathrm{H}$ NMR ( $400 \mathrm{MHz}, \mathrm{CDCl}_{3}$ ) $\delta 7.79(2 \mathrm{H}, \mathrm{d}, J=7.44 \mathrm{~Hz}), 7.68(2 \mathrm{H}, \mathrm{d}, J=7.90 \mathrm{~Hz}), 7.61$ $(2 \mathrm{H}, \mathrm{d}, J=7.36 \mathrm{~Hz}), 7.43(2 \mathrm{H}, \mathrm{t}, J=7.44 \mathrm{~Hz}), 7.34(2 \mathrm{H}, \mathrm{t}, J=7.36 \mathrm{~Hz}), 7.02(2 \mathrm{H}, \mathrm{d}, J=7.90$ $\mathrm{Hz}), 5.10(1 \mathrm{H}, \mathrm{brs}), 4.51(2 \mathrm{H}, \mathrm{d}, J=6.65 \mathrm{~Hz}), 4.33(2 \mathrm{H}, \mathrm{d}, \mathrm{J}=5.9 \mathrm{~Hz}), 4.24(1 \mathrm{H}, \mathrm{t}, J=6.65$ $\mathrm{Hz}) .{ }^{13} \mathrm{C}$ NMR ( $400 \mathrm{MHz}, \mathrm{CDCl}_{3}$ ) $\delta 156.4,143.8,141.6,138.2,137.8,129.4,127.7,127.1$, 125.0, 122.6, 120.0, 66.7, 47.3, 44.5. IR (neat) 3342, 2935, 1687, 1532, 1264, $1205 \mathrm{~cm}^{-1}$. HRMS(ESI) calcd for $\mathrm{C}_{22} \mathrm{H}_{19} \mathrm{NO}_{2}{ }^{[ }[\mathrm{M}+\mathrm{H}]^{+} 456.0460$, found 456.0463 .


Procedure modified from ref. ${ }^{[5]}$. $\mathrm{Pd}\left(\mathrm{PPh}_{3}\right)_{4}(5 \mathrm{~mol} \%, 50.8 \mathrm{mg})$, N-Fmoc p-lodobenzylamine $(0.879 \mathrm{mmol}, 400 \mathrm{mg}), \mathrm{Ag}_{2} \mathrm{CO}_{3}(0.435 \mathrm{mmol}, 121 \mathrm{mg})$ and triethylamine $(1.16,163 \mu \mathrm{~L})$ were suspended in anhydrous toluene ( 4.10 mL ) under nitrogen. Then a $15 \%$ soln. of ethyl diazoacetate in toluene ( $869 \mu \mathrm{~L}, 1.143 \mathrm{mmol}$ ) was added. The resulting solution was stirred at room temperature for 6 h and then filtered through a short path of silica gel, eluting with ethyl acetate. The volatile compounds were removed in vacuo and the residue purified by column chromatography (silica gel, Toluene: ethyl acetate $=12: 1, R_{f}=0.24$ ) to give the Fmoc diazoacetate as an orange, crystalline solid ( $86 \mathrm{mg}, 20 \%$ ). ${ }^{1} \mathrm{H} \mathrm{NMR} \mathrm{( } 400 \mathrm{MHz}, \mathrm{CDCl}_{3}$ ) $\delta 7.79$
(d, $J=7.6 \mathrm{~Hz}, 2 \mathrm{H}), 7.62(\mathrm{~d}, \mathrm{~J}=7.5 \mathrm{~Hz}, 2 \mathrm{H}), 7.48-7.41(\mathrm{~m}, 4 \mathrm{H}), 7.36-7.27(\mathrm{~m}, 4 \mathrm{H}), 5.14$ (d, J = 6.2 Hz, 1H), 4.49 (d, J = 6.8 Hz, 2H), 4.41-4.30 (m, 4H), 4.25 (t, J = 6.8 Hz, 1H), 1.38 (t, J = 7.1 Hz, 3H); ${ }^{13} \mathrm{C}$ NMR ( $400 \mathrm{MHz}, \mathrm{CDCl}_{3}$ ) $\delta 165.2,156.5,143.9,141.4,136.0$, 128.2, 127.7, 127.1, 126.9, 125.0, 124.9, 124.3, 120.0, 66.7, 61.1, 47.3, 44.7, 14.5. IR (neat) 3343, 2962, 2079, 1687, 1540, 1516, 1266, $1243 \mathrm{~cm}^{-1}$. HRMS(ESI) calcd for $\mathrm{C}_{26} \mathrm{H}_{24} \mathrm{~N}_{3} \mathrm{O}_{4}$ $[\mathrm{M}+\mathrm{H}]^{+} 442.1767$, found 442.1764 .


Fmoc-diazoacetate was dissolved in DMF containing 20\% of piperidine and reacted at room temperature for 30 min . The orange solution was diluted with DCM and washed with $\mathrm{H}_{2} \mathrm{O}(4$ x). The organic phase was dried with $\mathrm{MgSO}_{4}$, filtered and concentrated in vacuo to give a yellow solid. The solid was filtered using a short pad of silica using EtAcO/Hex 1:1, EtAcO and EtAcO/MeOH 9:1 as eluent. The solvents were removed in vacuo to give a yellow oil that was used directly in the next step. To a solution of the free amine ( $25 \mathrm{mg}, 0.011 \mathrm{mmol}, 1.1$ equiv.) in DCM ( 2 mL ) at room temperature was added perfluorophenyl ( E )-4-oxo-4-phenylbut-2-enoate ( $35 \mathrm{mg}, 0.102 \mathrm{mmol}$, 1 equiv.) as a solid followed by DIPEA ( $53 \mu \mathrm{~L}, 0.306$ mmol, 3 equiv.). The yellow solution slowly turned read and was reacted at the same temperature for 1 h . The solvents were removed in vacuo and purified by column chromatography to afford 13 as a yellow solid in $52 \%$ yield ( $20 \mathrm{mg}, 0.053 \mathrm{mmol}$ ). ${ }^{1} \mathrm{H}$ NMR ( $400 \mathrm{MHz}, \mathrm{CDCl}_{3}$ ) ס 8.01 (d, $J=7.8 \mathrm{~Hz}, 3 \mathrm{H}$ ), 7.62 (t, $J=7.4 \mathrm{~Hz}, 1 \mathrm{H}$ ), $7.55-7.43$ (m, 4H), 7.34 (d, J = 8.0 Hz, 2H), 6.99 (d, J = 15.0 Hz, 1H), 6.28 (s, 1H), 4.57 (d, J = $5.7 \mathrm{~Hz}, 2 \mathrm{H}$ ), 4.33 ( $q, J=7.1 \mathrm{~Hz}, 2 \mathrm{H}$ ), $1.34(\mathrm{t}, \mathrm{J}=7.1 \mathrm{~Hz}, 3 \mathrm{H}) ;{ }^{13} \mathrm{C} \operatorname{NMR}\left(100 \mathrm{MHz}, \mathrm{CDCl}_{3}\right) \delta 189.6,165.1,163.9$, 137.90, 136.8, 134.9, 134.8, 133.8, 133.7, 128.9, 128.6, 125.4, 124.3, 61.1, 43.7, 29.7, 14.5. FT-IR (neat) 3307, 3065, 2924, 2853, 2085, 1700, 1643, 1516, 1326, 1247, 1154, 1044.

## 10. General method for protein LC-MS

Protein liquid chromatography-mass spectrometry (LC-MS) was performed on a Xevo G2S TOF mass spectrometer coupled to an Acquity UPLC system using an Acquity UPLC BEH300 C4 column ( $1.7 \mu \mathrm{~m}, 2.1 \times 50 \mathrm{~mm}$ ). Solvents A, a water with $0.1 \%$ formic acid and B, $71 \%$ acetonitrile, $29 \%$ water and $0.075 \%$ formic acid were used as the mobile phase at a flow rate of $0.2 \mathrm{~mL} \mathrm{~min}{ }^{-1}$. The gradient was programmed as follows: $72 \%$ A to $100 \%$ B after 25 min then $100 \%$ B for 2 min and after that $72 \%$ A for 18 min . The electrospray source was operated with a capillary voltage of 2.0 kV and a cone voltage of 40 V . Nitrogen was used as the desolvation gas at a total flow of $700 \mathrm{~L} \mathrm{~h}^{-1}$. Total mass spectra were reconstructed from the ion series using the MaxEnt algorithm preinstalled on MassLynx software (v. 4.1 from Waters) according to the manufacturer's instructions.

## 11. Analysis of protein conjugation by LC-MS

A typical analysis of a conjugation reaction by LC-MS is described in Figure S3. The total ion chromatogram, combined ion series and deconvoluted spectra are shown for the starting material and the product of the reaction. Identical analyses were carried out for all the conjugation reactions performed in this work.




Figure S3 The total ion chromatogram, combined ion series and deconvoluted spectra are shown for the starting mateiral (reduced C2Am) and product (diazo-C2Am).

## 12. Protein and antibodies used in this study

Human serum albumin was kindly provided by Albumedix Limited; C2Am was provided by Dr. A. Neves and Prof. K. Brindle; ${ }^{[6]}$ Ubiquitin-K63C and Annexin V was expressed and purified as previously described; ${ }^{[7,8]}$ Her2-targeting nanobody 2Rb17c was provided by S . Massa and N. Devoogdt (Vrije Universiteit Brussel (VUM), Brussels). ${ }^{[9]}$

## Ubiquitin-K63C

Sequence:
SAQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSD YNIQㅡESTLHLVLRLRGG

Calculated Isotopically Averaged Molecular Weight $=8565$ Da (Figure S4)


Figure S4 ESI-MS of Ubiquitin-K63C.

C2Am-Cys95
Sequence:
GSPGISGGG GGILDSMVE KLGKLQYSLD YDFQNNQLLV GIIQAAELPA LDMGGTSDPY VKVFLLPDKK KKFETKVHRK TLNPVFNEQF TFKVPYㅡELG GKTLVMAVYD FDRFSKHDII GEFKVPMNTV DFGHVTEEWR DLQSAEK

Calculated Isotopically Averaged Molecular Weight = 16222 Da (Figure S5)


Figure S5 ESI-MS of C2Am-Cys95 after reduction with TCEP.

## Annexin V-Cys316

Sequence:
AQVLRGTVTDFPGFDERADAETLRKAMKGLGTDEESILTLLTSRSNAQRQEISAAFKTLFG RDLLDDLKSELTGKFEKLIVALMKPSRLYDAYELKHALKGAGTNEKVLTEIIASRTPEELRAI KQVYEEEYGSSLEDDVVGDTSGYYQRMLVVLLQANRDPDAGIDEAQVEQDAQALFQAGE LKWGTDEEKFITIFGTRSVSHLRKVFDKYMTISGFQIEETIDRETSGNLEQLLLAVVKSIRSIP AYLAETLYYAMKGAGTDDHTLIRVMVSRSEIDLFNIRKEFRKNFATSLYSMIKGDTSGDYKK ALLLLCGGEDD

Calculated Isotopically Averaged Molecular Weight = 35805 Da (Figure S6).


Figure S6 ESI-MS of Annexin V-Cys316

## Albumin

Isotopically Averaged Molecular Weight = 66345 Da (Figure S7)


Figure S7 ESI-MS of albumin.

## Nanobody 2Rb17c

Sequence:
EVQLQESGGGLVQPGGSLRLSCAASGFIFSNDAMTWVRQAPGKGLEWVSSINWS GTHTNYADSVKGRFTISRDNAKRTLYLQMNSLKDEDTALYYCVTGYGVTKTPTGQ GT QVTVSSHHHHHHSPSTPPTPSPSTPPC

Isotopically Averaged Molecular Weight $=14861$ Da (Figure S8)


Figure S8 ESI-MS of Nanobody after reduction with TCEP.

## 13. Procedure for Ubiquitin-K63C bioconjugation



Ubiquitin-K63C ( $20 \mu \mathrm{M}$ )

$\xrightarrow{3 \text { (25 equiv.) }}$
$\mathrm{NaP}_{\mathrm{i}}(\mathrm{pH} 8.0,50 \mathrm{mM})$
RT, 3 h
$28.9 \mu \mathrm{l}$ of a stock solution of Ubiquitin-K63C $(90 \mu \mathrm{M})$ was added to an eppendorf containing $99.8 \mu \mathrm{l}$ of $\mathrm{NaP}_{\mathrm{i}}$ buffer ( $\mathrm{pH} 8.0,50 \mathrm{mM}$ ). The resulting mixture was vortexed, and afterwards a 50 mM solution of (E)-5-diazo-1-phenylpent2-ene-1,4-dione 3 ( $1.3 \mu \mathrm{~L}, 25$ equiv.) in DMF was added. The reaction mixture was then shaken for 3 h at RT. After this time, a $10 \mu \mathrm{~L}$ aliquot of the reaction mixture was analysed by LC-MS. Full conversion of the Ubiquitin was observed (Calculated mass: 8767 Da; Observed mass: 8763 Da ). The peak 8736 corresponds to the loss of $\mathrm{N}_{2}$ during ESI-MS analysis ( $-28 \mathrm{~m} / \mathrm{z}$ ), Figure 59 .


Figure S9 ESI-MS of the reaction of Ubiquitin with 3 after 3 h at RT.

## 14. Procedure for C2Am-Cys95 bioconjugation



A $11.4 \mu \mathrm{~L}$ aliquot of a stock solution of C 2 A domain of synaptotagmin- $(176 \mu \mathrm{M})$ was added to an eppendorf containing $58.6 \mu \mathrm{~L}$ of $\mathrm{NaP}_{\mathrm{i}}$ buffer ( $\mathrm{pH} 8.0,50 \mathrm{mM}$ ). TCEP (20 equiv., $10 \mu \mathrm{~L}$ of a 4 mM solution) was added and the mixture reacted for 30 min at room temperature. Afterwards, in $38.4 \mu \mathrm{~L}$ of this solution was added 25 equiv. of a DMF solution of (E)-5-diazo-1-phenylpent-2-ene-1,4-dione 3 (1.60 $\mu \mathrm{L}$ of a 16.00 mM stock solution) and the reaction was stirred for another 1 h at room temperature. At the end, a $10 \mu \mathrm{~L}$ aliquot was analysed by LC-MS and complete conversion to the expected product was observed (calculated mass: 16420; observed mass: 16417), Figure S10.


Figure S10. ESI-MS of the reaction of C2Am with 3 after 1 h at RT.

## 15. Procedure for Annexin V-Cys316 bioconjugation



Annexin V-Cys316 ( $10 \mu \mathrm{M}$ )

$37^{\circ} \mathrm{C}, 1 \mathrm{~h}$


To an eppendorf with $18.3 \mu \mathrm{~L}$ of $\mathrm{NaP}_{\mathrm{i}}(\mathrm{pH} 8.0,50 \mathrm{mM})$ was added a $11.70 \mu \mathrm{~L}$ aliquot of a stock solution of Annexin $\mathrm{V}(34.30 \mu \mathrm{M})$ and the resulting mixture was vortexed for 30 seconds. Afterwards, a 2.00 mM solution of (E)-5-diazo-1-phenylpent-2-ene-1,4-dione 3 ( $10.0 \mu \mathrm{~L}, 50$ equiv.) in DMF was added and the reaction shaken for 1 h at $37^{\circ} \mathrm{C}$. At the end, a $10 \mu \mathrm{~L}$ aliquot was analysed by LC-MS and complete conversion to the expected product was observed (calculated mass: 36006; observed mass: 36006), Figure S11.


Figure S11 ESI-MS of the reaction of Annexin $V$ with 3 after 1 h at $37^{\circ} \mathrm{C}$.

## 16. Procedure for Albumin bioconjugation


$17.25 \mu \mathrm{l}$ of a stock solution of Albumin $(150.7 \mu \mathrm{M})$ was added to an Eppendorf containing $110.15 \mu \mathrm{l}$ of $\mathrm{NaP}_{\mathrm{i}}$ buffer ( $\mathrm{pH} 8.0,50 \mathrm{mM}$ ). The resulting mixture was vortexed, and afterwards a 5 mM solution of (E)-5-diazo-1-phenylpent2-ene-1,4-dione 3 ( $2.6 \mu \mathrm{~L}, 5$ equiv.) in DMF was added. The reaction mixture was then shaken for 3 h at RT. After this time, an aliquot of the reaction mixture in $\mathrm{NaP}_{\mathrm{i}}$ buffer ( $\mathrm{pH} 8.0,50 \mathrm{mM}$ ) was analysed by LC-MS. >95\% conversion of the Albumin was observed (Calculated mass: 66545 Da; Observed mass: 66545 Da), Figure S12.


Figure S12 ESI-MS of the reaction of Albumin with 3 after 3 h at RT.

## 17. Chemical controls

Ellman's reaction with Annexin V-Cys316


A $11.7 \mu \mathrm{~L}$ aliquot of Annexin V -Cys316 ( $34.3 \mu \mathrm{M}$ ) was transferred to a 0.5 mL eppendorf tube. A $4 \mu \mathrm{~L}$ aliquot of Ellman's reagent ( 50.5 mM , 500 equiv.) was added at room temperature and the resulting mixture vortexed for 30 seconds. After 4 h of additional shaking at $37^{\circ} \mathrm{C}$, a $10 \mu \mathrm{~L}$ aliquot was analysed by LC-MS and full conversion to the expected product (calculated mass: 36003; observed mass: 36002) was observed. Small molecules were removed from the reaction mixture by loading the sample onto a Zeba Spin Desalting Column previously equilibrated with $\mathrm{NaP}_{\mathrm{i}}$ (pH 8.0, 50 mM ). The sample was eluted via centrifugation ( $2 \mathrm{~min}, 1000 \mathrm{xg}$ ). The protein sample was used to the next reaction control (Figure S13).



Figure S13 ESI-MS of the reaction of Annexin-Cys316 with Ellman's reagent after 4 $h$ at $37^{\circ} \mathrm{C}$.

## Ellman's-Annexin V reaction with 3



A $22.5 \mu \mathrm{~L}$ aliquot of Annexin V-Cys316 (10 $\mu \mathrm{M}, 260 \mathrm{nmol}$ ) was transferred to a 0.5 mL eppendorf tube. A $7.5 \mu \mathrm{~L}$ aliquot of compound ( $E$ )-5-diazo-1-phenylpent-2-ene-1,4-dione 3 ( $2.00 \mathrm{mM}, 50$ equiv.) was added at room temperature and the resulting mixture vortexed for 30 seconds. After 1 h of additional shaking at $37^{\circ} \mathrm{C}$, a $10 \mu \mathrm{~L}$ aliquot was analysed by LC-MS and no reaction observed. The protein sample was stored at $-20^{\circ} \mathrm{C}$, Figure S14.


Figure S14 ESI-MS of the reaction of Annexin V-SS-Ellman's with 3 after 1 h at $37^{\circ} \mathrm{C}$.

## Annexin V conjugate reaction with Ellman's Reagent



A $13.5 \mu \mathrm{~L}$ aliquot of Annexin V conjugate ( $10 \mu \mathrm{M}, 135 \mathrm{nmol}$ ) was transferred to a 0.5 mL eppendorf tube. A $1.5 \mu \mathrm{~L}$ aliquot of Ellman's reagent ( $50.5 \mathrm{mM}, 500$ equiv.) was added at room temperature and the resulting mixture was vortexed for 30 seconds. After 1 h of additional shaking at $37^{\circ} \mathrm{C}$, a $10 \mu \mathrm{~L}$ aliquot was analysed by LC-MS and no conversion to the expected product was observed, Figure S15.


Figure S15 ESI-MS of the reaction of Annexin V conjugate with Ellman's reagent after 1 h at $37^{\circ} \mathrm{C}$.

## 18. Procedure for MSMS analysis of diazo-C2Am

The C2A domain of synaptotagmin-I protein solution was subjected to overnight digestion with trypsin. The peptide solution was then pipetted into an autosampler vial and placed in the Waters nanoAcquity autosampler.

All LC-MS/MS experiments were performed using a nanoAcquity UPLC (Waters Corp., Milford, MA) system and an LTQ Orbitrap Velos hybrid ion trap mass spectrometer (Thermo Scientific, Waltham, MA). Separation of peptides was performed by reverse-phase chromatography using a Waters reverse-phase nano column (BEH C18, $75 \mu \mathrm{~m}$ i.d. x $250 \mathrm{~mm}, 1.7 \mu \mathrm{~m}$ particle size) at flow rate of 300 $\mathrm{nL} / \mathrm{min}$. Peptides were initially loaded onto a pre-column (Waters UPLC Trap Symmetry C18, $180 \mu \mathrm{~m}$ i.d x 20mm, $5 \mu \mathrm{~m}$ particle size) from the nanoAcquity sample manager with $0.1 \%$ formic acid for 3 minutes at a flow rate of $10 \mu \mathrm{~L} / \mathrm{min}$. After this period, the column valve was switched to allow the elution of peptides from the precolumn onto the analytical column. Solvent A was water $+0.1 \%$ formic acid and solvent B was acetonitrile $+0.1 \%$ formic acid. The linear gradient employed was $2-$ $40 \%$ B in 30 minutes, followed by wash and equilibration steps ( 60 minute total run time).

The LC eluant was sprayed into the mass spectrometer by means of a Thermo Scientific nanospray source. All $\mathrm{m} / \mathrm{z}$ values of eluting ions were measured in the Orbitrap Velos mass analyser, set at a resolution of 30000. Data dependent scans (Top 20) were employed to automatically isolate and generate fragment ions by collision-induced dissociation in the linear ion trap, resulting in the generation of MS/MS spectra. Ions with charge states of 2+ and above were selected for fragmentation. Post-run, all MS/MS data were converted to mgf files and these were submitted to the Mascot search algorithm (Matrix Science, London UK) and searched against a custom database containing the C2A domain of synaptotagmin-I sequence and a database of common contaminant sequences ( 115 sequences, 38274 residues; http://www.thegpm.org/crap/). Variable modifications of BBC283A (C), demidation $(N, Q)$ and oxidation (M) were applied. Peptide identifications were accepted if they could be established at greater than $95.0 \%$ probability.


Figure S16 MS/MS spectrum of the $m / z 583.27$ doubly charged ion of the tryptic peptide YPYCELGGK, containing the BBC283A modification at the cysteine residue.

## 19. Circular dichroism

Circular dichroism (CD) spectroscopy was used to analyse protein secondary structure in solution. Samples were concentrated to 10 nM in $\mathrm{NaP}_{\mathrm{i}}$ buffer (pH 8.0, 50 mM ). CD measurements were recorded using a Chirascan spectrophotometer equipped with a Quantum TC125 temperature control unit ( $25^{\circ} \mathrm{C}$ ). The data was acquired in a 0.1 cm path length with a response time of 1 s , a per-point acquisition delay of 5 ms and a pre- and post-scan delay of 50 ms . Spectra were averaged over three scans, in a wavelength range from 200 nm to 260 nm , and the spectrum from a blank sample containing only buffer was subtracted from the averaged data.


Figure S17 Circular dichroism (CD) analysis of Albumin and Albumin-3 showed no alterations in secondary structural content.


Figure S18 Circular dichroism (CD) analysis of C2Am and C2Am-3 showed no alterations in secondary structural content.

## 20. Diazo-Ubiquitin 1,3-dipolar cycloaddition



A 106 mmol solution of (1R,8S,9s)-Bicyclo[6.1.0]non-4-yn-9-ylmethanol 8 (5.3 $\mu \mathrm{l}$, 700 equiv.) in DMF was added to $34.7 \mu \mathrm{l}$ of previously prepared Diazo-Ubiquitin ( 20 $\mu \mathrm{M}$, 1 equiv., $\mathrm{NaP}_{\mathrm{i}} 50 \mathrm{mM}, \mathrm{pH} 8$ ). The reaction mixture was vortexed and shaken at room temperature for 18 h . An aliquot of the reaction mixture was then analysed by LC-MS. Full conversion of the Ubiquitin starting material was observed to the formation of one cycloaddition product (calculated mass: 8917 Da ; observed mass: 8922 Da), Figure S19.


Figure S19 ESI-MS of the reaction of diazo-Ubiquitin with 8 after 18 h at RT.

## 21. Diazo-C2Am 1,3-dipolar cycloaddition




A 106 mmol solution of ((1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methanol 8 ( $3.75 \mu \mathrm{l}$, 700 equiv.) in DMF was added to $36.25 \mu$ of previously prepared solution of DiazoC2Am ( $20 \mu \mathrm{M}$, 1 equiv.). The reaction mixture was vortexed and shaken at room temperature for 6 h . An aliquot of the reaction mixture was then analysed by LC-MS and $>95 \%$ conversion of the Diazo-C2Am adduct to the corresponding product was observed (Calculated mass: 16575; Observed mass: 16576), Figure S20.


Figure S20 ESI-MS of the reaction of diazo-C2Am with 8 after 6 h at RT.

## 22. Diazo-Annexin V 1,3-dipolar cycloaddition




A 53 mM solution of (( $1 R, 8 \mathrm{~S}, 9 \mathrm{~s})$-bicyclo[6.1.0]non-4-yn-9-yl)methanol $8(1.5 \mu \mathrm{l}, 100$ equiv.) in DMF was added to $38.5 \mu$ l of previously prepared solution of Diazo-Annexin $\mathrm{V}(20 \mu \mathrm{M}, 1$ equiv.). The reaction mixture was vortexed and shaken at room temperature for 18 h . An aliquot of the reaction mixture was then analysed by LC-MS and $>95$ \% conversion of the Diazo-Annexin V adduct to the corresponding product was observed (Calculated mass: 36156; Observed mass: 36167), Figure S21.


Figure S21 ESI-MS of the reaction of diazo-Annexin V with 8 after 18 h at RT.


Annexin V-diazo ( $20 \mu \mathrm{M}$ )


A 106 mmol solution of (( $1 R, 8 \mathrm{~S}, 9 \mathrm{~s})-$ bicyclo[6.1.0]non-4-yn-9-yl)methanol 8 ( $3.75 \mu \mathrm{l}$, 700 equiv.) in DMF was added to $36.25 \mu$ of previously prepared solution of DiazoAnnexin $\mathrm{V}(20 \mu \mathrm{M}$, 1 equiv.). The reaction mixture was vortexed and shaken at room temperature for 3 h . An aliquot of the reaction mixture was then analysed by LC-MS and $>95$ \% conversion of the Diazo-Annexin V adduct to the corresponding product was observed (Calculated mass: 36156; Observed mass: 36167), Figure S22.


Figure S22 ESI-MS of the reaction of diazo-Annexin V with 8 after 3 h at RT.

## 23. Diazo-Albumin 1,3-dipolar cycloaddition



A 53.3 mmol solution of ((1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methanol 8 (1.5 $\mu \mathrm{l}$, 100 equiv.) in DMF was added to $38.5 \mu$ of previously prepared solution of Albumindiazo ( $20 \mu \mathrm{M}, 1$ equiv.). The reaction mixture was vortexed and shaken at room temperature for 18 h . An aliquot of the reaction mixture was then analysed by LC-MS and $>95 \%$ conversion of the Diazo-Albumin adduct to the corresponding product was observed (Calculated mass: 66695; Observed mass: 66709), Figure 23.


Figure S23 ESI-MS of the reaction of diazo-Albumin with 8 after 18 h at RT.

## 24. Procedure for Nanobody 2Rb17c bioconjugation



A $21.2 \mu \mathrm{~L}$ aliquot of a stock solution of Nanobody $2 \mathrm{Rb} 17 \mathrm{c}(118 \mu \mathrm{M})$ was added to an eppendorf containing $25.8 \mu \mathrm{~L}$ of $\mathrm{NaP}_{\mathrm{i}}$ buffer ( pH 8.0 , 50 mM ). $3.1 \mu \mathrm{~L}$ of TCEP ( 50 equiv., 40 mM solution in DMF) was added and the mixture reacted for 30 min at room temperature. Afterwards, $0.5 \mu \mathrm{~L}$ of (E)-5-diazo-1-phenylpent-2-ene-1,4dione 3 (10 equiv., 50 mM DMF stock solution) was added and the reaction was stirred for 3 h at room temperature. At the end, a $10 \mu \mathrm{~L}$ aliquot was analysed by LC-MS and complete conversion to the expected product was observed (calculated mass: 15060; observed mass: 15061), Figure S24.


Figure S24 ESI-MS of the reaction of Nanobody with $\mathbf{3}$ after 3 h at RT.

## 25. Diazo-Nanobody 2Rb17c 1,3-dipolar cycloaddition



Nanobody-diazo (41 $\mu \mathrm{M}$ )






12 (100 equiv)
(pH 8.0, 50 mM )
$18 \mathrm{~h}, \mathrm{RT}$

>95\% conversion

A 41.3 mmol solution of commercially available DBCO-Cy5 12 ( $2.0 \mu \mathrm{l}, 100$ equiv.) in DMF was added to $19 \mu$ of previously prepared diazo-nanobody adduct ( $41 \mu \mathrm{M}, 1$ equiv.). The reaction mixture was vortexed and shaken at room temperature for 18 h . An aliquot of the reaction mixture was then analysed by LC-MS. Full conversion of the nanobody-DABCO-Cy product was observed (Calculated mass: 16067 Da; Observed mass: 16069 Da), Figure S25.


Figure S25 ESI-MS of diazo-nanobody reaction with DABCO-Cy5 12 after 18 h at RT.

## 26. Diazo-Nanobody 2Rb17c stability

A $10 \mu \mathrm{~L}$ aliquot of the conjugate $2 \mathrm{Rb} 17 \mathrm{c}-3(10 \mu \mathrm{M})$ in NaP i buffer ( $\mathrm{pH} 8.0,50 \mathrm{mM}$ ) was thawed. $1 \mu \mathrm{~L}$ of reconstituted human plasma (Sigma Aldrich) was added at room temperature and the resulting mixture vortexed for 30 seconds. The resulting reaction mixture was then mixed at RT overnight. After $24 \mathrm{~h}, 10 \mu \mathrm{~L}$ of the reaction mixture was analysed by LC-MS. No significant degradation of the adduct was observed (Figure S26). The nanobody can be stored for 4 months at $-20^{\circ} \mathrm{C}$ (Figure S27).


Figure S26 ESI-MS of diazo-nanobody stability in plasma.


Figure S27 ESI-MS of diazo-nanobody after 4 months stored at $-20^{\circ} \mathrm{C}$.

## 27. SDS-PAGE

The incubation solution ( 5.0 mL ) was transferred to tube, and NuPAGE LDS Sample Buffer ( $4 \mathrm{x}, 2.5 \mathrm{~mL}$ ), NuPAGE Reducing Agent (10x, 1 mL ), and $\mathrm{H} 2 \mathrm{O}(1.5 \mathrm{~mL}$ ) were added to the tube. The solution was heated at $70^{\circ} \mathrm{C}$ for 10 min . The heated solution was loaded to NuPAGE Bis-Tris mini gel (10x 10 cm ) with 4-12\% gradient polyacrylamide concentration, and then the conjugation reaction was analysed by electrophoresis (200 V). The buffering system employed was 1x SDS Running Buffer (NuPAGE MES SDS Running Buffer, 20x, pH 7.3, 50 to 950 mL deionised water). For reduced samples, 500 mL of NuPAGE antioxidant was added to each 200 mL 1x SDS running buffer. After 35 min , the intensity of fluorescence was analysed. Then, the gel was stained with $0.5 \%$ of Ruby. The gel was mixed overnight at room temperature and read the day after. After wash the gel, coomassie ( $0.5 \%$ ) was added and the gel was read 2 h after mixing at room temperature (Figure S 28 ).


Figure S28 SDS-PAGE of the fluorescently labelled-nanobody.

## 28. In-cell click microscopy



SKBR-3 cells were maintained in McCoy's 5A medium (ThermoFisher) supplemented with $10 \% ~(\mathrm{v} / \mathrm{v}$ ) Fetal Bovine Serum and 1\% (v/v) Penicillin/Streptomycin in a humidified $5 \% \mathrm{CO}_{2}$ atmosphere at $37^{\circ} \mathrm{C}$. MCF-7 cells were maintained in Dulbecco's Modified Eagle Medium (ThermoFisher) with GlutaMAX supplemented with 10\% (v/v) Fetal Bovine Serum and $1 \%$ (v/v) Penicillin/Streptomycin in a humidified $5 \% \mathrm{CO}_{2}$ atmosphere at $37^{\circ} \mathrm{C}$. Cells were passaged every 2-3 days by use of $0.25 \%$ Trypsin (ThermoFisher). For microscopy, cells were seeded onto ibidi® 8 well $\mu$-slides at a concentration of $1 \times 10^{5}$ cells $/ \mathrm{ml}$ in $200 \mu \mathrm{~L}$ media. After 1 day, media was replaced by $200 \mu \mathrm{~L}$ fresh media with or without 200 nM 2Rb17c-3. The plate was then incubated for 4 hours at $37{ }^{\circ} \mathrm{C}$. Following incubation, DBCO-Cy5 dye 12 (Sigma-Aldrich) was
added to obtain an in-plate dye concentration of $5 \mu \mathrm{M}$. The cell plate was then further incubated for 1 hour at $37^{\circ} \mathrm{C}$. Following this, the dye was removed, and cells were washed 3 times with $300 \mu$ FluoroBrite ${ }^{\text {TM }}$ DMEM media/well for 5 minutes at $37^{\circ} \mathrm{C}$. The cells were stained with $5 \mu \mathrm{~g} / \mathrm{ml}$ Hoechst 33342 nuclear stain for 15 minutes at room temperature. Staining solution was then removed and $200 \mu$ FluoroBrite ${ }^{\text {TM }}$ DMEM media was added per well. Cells were immediately imaged using a Leica SP5 inverted confocal microscope through a 63 x water-immersion objective lens (NA=1.20). Hoechst nuclear stain was imaged with 405 nm excitation and 418-515 nm emission. Cy5 dye was imaged with 633 nm excitation and 639-714 nm emission. Identical microscopy settings were used for treated and control images. Images were processed using ImageJ software (version 1.52a, NIH). Treated and control images were processed in an identical manner.
(a)
(i) SKBR-3 + 2Rb17c-3 + DBCO-Cy5

(ii) SKBR-3 - 2Rb17c-3 + DBCO-Cy5

(iii) MCF-7 + 2Rb17c-3 + DBCO-Cy5

(iv) MCF-7 - 2Rb17c-3 + DBCO-Cy5

Cy5


Hoechst
Merge
Brightfield




Figure S29 Click-imaging of diazo-nanobody 2Rb17c with high expressing HER2 cells (SKBR-3) and low expressing HER2 cells (MCF-7). (a) Confocal fluorescence of SKBR-3 cells incubated with or without 2Rb17c-3 conjugate then with DBCO-Cy5 click dye 12. Images show Cy5, Hoechst-33342 nuclear stain, merge of Cy5 and Hoechst33342 and Brightfield image. (i) SKBR-3 + 2Rb17c-3 + DBCO-Cy5, (ii) SKBR-3 + DBCO-Cy5 only (iii) MCF-7 + 2Rb17c-3 + DBCO-Cy5, (iv) MCF-7 + DBCO-Cy5 only (b) Washout experiment performed in SKBR-3 cells where 2Rb17c-3 is removed and cells washed ( $1 \times \mathrm{PBS}$ ) prior to dye addition, (i), or 2Rb17c-3 is not removed before dye addition (as for other images), (ii). Similar in cell-fluorescence is observed, suggesting the diazo-dye click reaction occurs inside the cell.


Figure S30 Relative pixel intensity of fluorescence images (SKBR-3, a) i, a) ii normalised to mean fluoresence of image treated with 2Rb17c-3 conjugate.

## 29. ${ }^{1} \mathrm{H}$ NMR kinetics experiments for diazo and diazoester with DIBONE.



The reactants were mixed in equal ratios at a concentration of 0.00319 M in deuterated solvent (MeCN). The components were combined, the NMR tube was inverted once and inserted into the spectrometer, and the scan was initialized after initial mixing. A 64-scan NMR spectrum was acquired every 30 min , and the integrations were used to calculate concentrations from the known initial concentrations. The second-order rate constants were then determined from the slope of the plot of [DIBONE] ${ }^{-1} v s$ time (Figure 31 and 32).


Figure S31 Second-Order rate constants for the reaction of diazo compound (pink) and diazoester (blue) with DIBONE in $\mathrm{CD}_{3} \mathrm{CN}$. $k_{\text {diazo }}=0.0018 \mathrm{M}^{-1} \mathrm{~s}^{-1}$ and $k_{\text {diazoester }}=$ $0.0036 \mathrm{M}^{-1} \mathrm{~s}^{-1}$.


Figure S32 ${ }^{1}$ NMR kinetics experiments for $\mathbf{3}$ with DIBONE.

## 30. ${ }^{1}$ H NMR Competition Experiments



Competitive kinetics were analysed by dissolving 0.025 mmol each diazoacetophenone (diazoester) and ethyl 2-diazo-2-phenylacetate (diazo) in $\mathrm{CD}_{3} \mathrm{CN}$ $(0.50 \mathrm{~mL})$ at room temperature. To this solution was added a solution of DIBONE (5.5 $\mathrm{mg}, 0.025 \mathrm{mmol}) \mathrm{CD}_{3} \mathrm{CN}(0.50 \mathrm{~mL})$, and the reaction mixture was analysed by ${ }^{1} \mathrm{H}$ NMR spectroscopy. Figure 33-36.


Figure S33 ${ }^{1} \mathrm{H}$ NMR of the reaction between diazoacetophenone 10 and DIBONE. Time zero ( $\mathrm{t}_{0}$ ) and after 3, 12 and $24 \mathrm{~h}\left(\mathrm{CDCl}_{3}\right)$.


Figure S34 ${ }^{1} \mathrm{H}$ NMR of the reaction between ethyl 2-diazo-2-phenylacetate and DIBONE. Time zero ( $\mathrm{t}_{0}$ ) and after 3,12 and $24 \mathrm{~h}\left(\mathrm{CDCl}_{3}\right)$.


Figure S35 Competition Kinetics (diazo vs diazoester). Equimolar mixture of diazo and diazoester. Resulting NMR spectra after adding 1 equiv. of DIBONE ( $\mathrm{CDCl}_{3}$ ).


Figure S36 Resulting graph extracted from ${ }^{1} \mathrm{H}$ NMR integrals for the consumption of DIBONE and formation of pyrazoles from diazo and diazoester showing that the diazoester is more reactive (in accordance with the kinetics experiments).

## 31. Reaction of 13 with Veltis-V0354


$6.6 \mu \mathrm{l}$ of a stock solution of Albumin V0354 ( $1507 \mu \mathrm{M}$ ) was added to an Eppendorf containing $491.4 \mu \mathrm{l}$ of $\mathrm{NaP} \mathrm{i}_{\mathrm{i}}$ buffer ( $\mathrm{pH} 8.0,50 \mathrm{mM}$ ). The resulting mixture was vortexed, and afterwards a 10 mM solution of ethyl $13(2 \mu \mathrm{~L}$, 2 equiv.) in DMF was added. The reaction mixture was then shaken for 3 h at $37^{\circ} \mathrm{C}$. After this time, an aliquot of the reaction mixture in $\mathrm{NaP}_{\mathrm{i}}$ buffer (pH 8.0, 50 mM ) was analysed by LC-MS. $90 \%$ conversion of the Albumin V0354 was observed (Calculated mass: 66722 Da; Observed mass: 66758 Da), Figure 37.


Figure S37 ESI-MS of the reaction of Albumin-V0354 with 13 after 2 h at $37^{\circ} \mathrm{C}$.

## 32. Reaction in the presence of GSH


$5.3 \mu \mathrm{l}$ of a stock solution of Albumin V0354 $(150.7 \mu \mathrm{M})$ was added to an Eppendorf containing $33.5 \mu \mathrm{l}$ of NaPi buffer ( $\mathrm{pH} 8.0,50 \mathrm{mM}$ ). The resulting mixture was vortexed, and afterwards a 10 mM solution of 3 ( $0.4 \mu \mathrm{~L}, 5$ equiv.) and a 1 mM solution of reduced GSH ( $0.8 \mu \mathrm{~L}, 1$ equiv.) in DMF was added to the Eppendorf at the same time. The reaction mixture was then shaken for 2 h at RT . After this time, a $10 \mu \mathrm{~L}$ aliquot of the reaction mixture in NaPi buffer ( $\mathrm{pH} 8.0,50 \mathrm{mM}$ ) was analysed by LC-MS. >95\% conversion of the Albumin V0354 was observed (Calculated mass: 66545 Da; Observed mass: 66545 Da), Figure 38. The same protocol was used when 10 equiv. and 50 equiv. of GSH (10 mM) was added, Figure 39 (10 equiv.) and Figure 40 (50 equiv.).


Figure S38 ESI-MS of the reaction of Albumin-V0354 with 3 in the presence of 1 equiv. of GSH after 2 h at RT.


Figure S39 ESI-MS of the reaction of Albumin-V0354 with $\mathbf{3}$ in the presence of 10 equiv. of GSH after 2 h at RT.


Figure S40 ESI-MS of the reaction of Albumin-V0354 with 3 in the presence of 50 equiv. of GSH after 2 h at RT.

$5.3 \mu \mathrm{l}$ of a stock solution of Albumin V0354 $(150.7 \mu \mathrm{M})$ was added to an Eppendorf containing $33.5 \mu \mathrm{l}$ of NaPi buffer ( $\mathrm{pH} 8.0,50 \mathrm{mM}$ ). The resulting mixture was vortexed, and afterwards a 10 mM solution of ethyl $13(0.2 \mu \mathrm{~L}, 2.5$ equiv.) and a 1 mM solution of reduced GSH ( $0.8 \mu \mathrm{~L}, 1$ equiv.) in DMF was added to the Eppendorf at the same time. The reaction mixture was then shaken for 2 h at RT. After this time, a $10 \mu \mathrm{~L}$ aliquot of the reaction mixture in NaPi buffer ( $\mathrm{pH} 8.0,50 \mathrm{mM}$ ) was analysed by LCMS. >95\% conversion of the Albumin V0354 was observed (Calculated mass: 66722 Da; Observed mass: 66766 Da ), Figure 41. The same protocol was used when 10 equiv. and 50 equiv. of GSH ( 10 mM ) was added, Figure 42 (10 equiv.) and Figure 43 (50 equiv.).


Figure S41 ESI-MS of the reaction of Albumin-V0354 with 13 in the presence of 1 equiv. of GSH after 2 h at RT.


Figure S42 ESI-MS of the reaction of Albumin-V0354 with 13 in the presence of 10 equiv. of GSH after 2 h at RT.


Figure S43 ESI-MS of the reaction of Albumin-V0354 with 13 in the presence of 50 equiv. of GSH after 2 h at RT.
33. diazo/diazoester-Albumin V0354 1,3-dipolar cycloaddition


A 40 mmol solution of DBCO-PEG-OH ( $1 \mu \mathrm{l}, 100$ equiv.) in DMF was added to $19 \mu \mathrm{l}$ of previously prepared solution of Albumin-diazo $(20 \mu \mathrm{M}, \mathrm{NaPi}, \mathrm{pH} 8.0,50 \mathrm{mM})$. The reaction mixture was vortexed and shaken at room temperature for 24 h . An aliquot of the reaction mixture was then analysed by LC-MS and >90 \% conversion of the Diazo-Albumin V0354 adduct to the corresponding product was observed (Calculated mass: 67063; Observed mass: 67072, Figure 44.


Figure S44 ESI-MS of the reaction of Albumin-diazo with 100 equiv. of DBCO-PEGOH .


A 40 mmol solution of BCN-PEG- $\mathrm{NH}_{2}(1 \mu \mathrm{l}, 100$ equiv.) in DMF was added to $19 \mu \mathrm{l}$ of previously prepared solution of Albumin-diazo ( $20 \mu \mathrm{M}, \mathrm{NaPi}, \mathrm{pH} 8.0,50 \mathrm{mM}$ ). The reaction mixture was vortexed and shaken at room temperature for 24 h . An aliquot of the reaction mixture was then analysed by LC-MS and >90 \% conversion of the Diazo-Albumin V0354 adduct to the corresponding product was observed (Calculated mass: 66879; Observed mass: 66891, Figure 45.


Figure S45 ESI-MS of the reaction of Albumin-diazo with 100 equiv. of BCN-PEG$\mathrm{NH}_{2}$.


A 20 mmol solution of L-Homopropargylglycine, ( $2 \mu \mathrm{l}$, 100 equiv.) in DMF was added to $18 \mu \mathrm{l}$ of previously prepared solution of Albumin-diazo $(20 \mu \mathrm{M}, \mathrm{NaPi}, \mathrm{pH} 8.0,50$ $\mathrm{mM})$. The reaction mixture was vortexed and shaken at room temperature for 24 h . An aliquot of the reaction mixture was then analysed by LC-MS and no product was observed (Calculated mass: 66672; Observed mass: 66562, Figure 46.


Figure S46 ESI-MS of the reaction of Albumin-diazo with 100 equiv. of L-HPG.



(20 $\mu \mathrm{M}$ )

A 40 mmol solution of DBCO-PEG-OH ( $1 \mu \mathrm{l}, 100$ equiv.) in DMF was added to $19 \mu \mathrm{l}$ of previously prepared solution of Albumin-diazoester ( $20 \mu \mathrm{M}, \mathrm{NaPi}, \mathrm{pH} 8.0,50 \mathrm{mM}$ ). The reaction mixture was vortexed and shaken at room temperature for 24 h . An aliquot of the reaction mixture was then analysed by LC-MS and $>70 \%$ conversion of the Diazo-Albumin V0354 adduct to the corresponding product was observed (Calculated mass: 67230; Observed mass: 67254, Figure 47.


Figure S47 ESI-MS of the reaction of Albumin-diazoester with 100 equiv. of DBCO-PEG-OH.


A 40 mmol solution of BCN-PEG- $\mathrm{NH}_{2}(1 \mu \mathrm{l}, 100$ equiv.) in DMF was added to $19 \mu \mathrm{l}$ of previously prepared solution of Albumin-diazoester ( $20 \mu \mathrm{M}, \mathrm{NaPi}, \mathrm{pH} 8.0,50 \mathrm{mM}$ ). The reaction mixture was vortexed and shaken at room temperature for 24 h . An aliquot of the reaction mixture was then analysed by LC-MS and $>60 \%$ conversion of the Diazo-Albumin V0354 adduct to the corresponding product was observed (Calculated mass: 67046; Observed mass: 67066, Figure 48.


Figure S48 ESI-MS of the reaction of Albumin-diazoester with 100 equiv. of BCN-PEG-NH2.


 $(20 \mu \mathrm{M})$

A 20 mmol solution of L-Homopropargylglycine, ( $2 \mu \mathrm{l}, 100$ equiv.) in DMF was added to $18 \mu \mathrm{l}$ of previously prepared solution of Albumin-diazoester $(20 \mu \mathrm{M}, \mathrm{NaPi}, \mathrm{pH} 8.0$, 50 mM ). The reaction mixture was vortexed and shaken at room temperature for 24 h . An aliquot of the reaction mixture was then analysed by LC-MS and no product was observed (Calculated mass: 66849; Observed mass: 66732, Figure 49.


Figure S49 ESI-MS of the reaction of Albumin-diazoester with 100 equiv. of L-HPG.

## 34. General methods for Hella cell lysates

## Preparation of HeLa cell lysates

Cells were cultured as described above. Approximately $1.2 \times 10^{7}$ cells (T-175 flask) were pelleted ( $150 \times \mathrm{g}, 5 \mathrm{~min}$ ) and washed with PBS $(3 \times 10 \mathrm{~mL})$. RiPa buffer with protease inhibitor ( $700 \mu \mathrm{~L}$, Invitrogen) was then added and incubated on ice for 45 min . The resulting solution was centrifuged $(10,000 \times \mathrm{g}, 10 \mathrm{~min})$ to clarify the lysate and then purified using a Zeba size exclusion spin column (2 mL, 7 kDa MWCO, ThermoFisher) pre-equilibrated with PBS. Protein concentration was determined using BCA protein assay and typically gave around $2-3 \mathrm{mg} / \mathrm{mL}$ protein. The eluent was aliquoted and frozen until use.

## Gel electrophoresis and western blot

Samples were separated by SDS-PAGE using NuPage 4-12\% Bis-Tris protein gels (Invitrogen) with MES running buffer ( 200 V ) and transferred to a polyvinylidene difluoride (PVDF) membrane ( $0.2 \mu \mathrm{M}$, iBlot, ThermoFisher) at 20 V . After transfer, the PVDF membrane was first washed with water $(\times 3)$ then blocked with BSA ( $3 \%$ in trisbuffered saline Tween-20 (TBST) for 1 h at room temperature with gentle rocking. The membrane was washed with TBST ( $3 \times 5 \mathrm{~min}$ ) then incubated with 1:1,000 Alexa Fluor® 555 streptavidin ( $1 \mathrm{mg} / \mathrm{mL}$ stock in PBS, Invitrogen) in TBST for 30 min at room temperature with gentle rocking. The PVDF membrane was washed with TBST ( $2 \times 5$ min ), water ( $2 \times 5 \mathrm{~min}$ ), and imaged by fluorescence using ChemiDoc MP Imaging System. To assess equal protein loading, PVDF membranes were stained with Sypro Ruby blot stain (Invitrogen) according to manufacturer's instructions and imaged by fluorescence using ChemiDoc MP Imaging System.

## Labelling of Cys-proteins in HeLa cell lysates

Incubation of the probes $\mathbf{3}$ and $\mathbf{1 3}$ with the cell lysate followed by cycloaddition with DBCO


Incubation of the probes $\mathbf{3}$ and $\mathbf{1 3}$ with the cell lysate followed by reaction with the alkyne in the presence or absence of cooper


HeLa cell lysate was prepared as described above. Protein concentration was assessed using BCA assay (Pierce) to be approximately $2100 \mu \mathrm{~g} / \mathrm{mL}$ and diluted to approximately $1000 \mu \mathrm{~g} / \mathrm{mL}$. To the cell lysate ( $50 \mu \mathrm{~L}$ ) in PBS, probes 3 or 13 (desired concentration in DMSO) were added and the reaction vortexed for 10 seconds then left to stir for 2 h at room temperature. Samples were then treated with Biotin-PEGDBCO or Biotin-PEG-alkyne (in the presence or not of CuAAC) reagents before western blot as described below. For performing western blot, about 20 ug of total protein was added per lane, each sample should be normalized to contain same amount of total protein. If poor signal, can increase protein loading by increasing initial protein concentration of cell lysates. Samples were then prepared for SDS-PAGE by mixing sample ( $17 \mu \mathrm{~L}$, normalised) with lithium dodecyl sulfate (LDS $4 \times$ ) ( $6.5 \mu \mathrm{~L}$, Invitrogen), and DTT NuPAGE samples reducing agent (2.5 $\mu \mathrm{L}$, Invitrogen) and
heating to $70{ }^{\circ} \mathrm{C}$ for 5 min . Samples were then resolved by electrophoresis and analysed by Alexa Fluor® 555 streptavidin western blot as described above.

## Preparation of stock solutions

Probe in the desired concentration in DMSO. Dithiothreitol (DTT) ( $30.2 \mathrm{mg}, 0.19 \mathrm{mmol}$ ) was dissolved in $\mathrm{H}_{2} \mathrm{O}(982 \mu \mathrm{~L})$ to give a 200 mM stock solution.

## CuAAC reagents

Sodium ascorbate ( 5.2 mg ) was dissolved in $\mathrm{H}_{2} \mathrm{O}(484 \mu \mathrm{~L})$ to give a final concentration of $50 \mathrm{mM} . \mathrm{CuSO}_{4} \cdot 5 \mathrm{H}_{2} \mathrm{O}(4.2 \mathrm{mg})$ was dissolved in $\mathrm{H}_{2} \mathrm{O}(336 \mu \mathrm{~L})$ to give a final concentration of 50 mM . THTPA ( 6.2 mg ) was dissolved in $\mathrm{H}_{2} \mathrm{O}(1.43 \mathrm{~mL})$ to give a final concentration of 10 mM . Biotin-PEG-DBCO and Biotin-PEG-alkyne were prepared in DMSO to give a concentration of 10 mM .

## Reaction with biotin-PEG-alkyne catalysed by Copper (CuAAC reaction)

To each sample was added the biotin-PEG-alkyne ( 0.1 mM final concentration from 10 mM solution in DMSO), sodium ascorbate ( 1 mM final concentration from a 50 mM solution in $\mathrm{H}_{2} \mathrm{O}$ ), THTPA ( 0.1 mM final concentration from a 10 mM solution in $\mathrm{H}_{2} \mathrm{O}$ ) and the samples vortexed for 10 seconds. A solution of $\mathrm{CuSO}_{4}(1 \mathrm{mM}$ final concentration from a 50 mM solution in $\mathrm{H}_{2} \mathrm{O}$ ) was added and the samples vortexed for 10 seconds to initiate the reaction. Samples were left for 2 h at room temperature in the dark with constant shaking. Samples were then purified with a zeba spin column ( 7 kDa MWCO, 2 mL ) pre-equilibrated with PBS to remove excess Biotin-PEG-alkyne and stored in the dark and in the freezer until further use for affinity purification.

## Reaction with biotin-PEG-alkyne without Copper (Cu Free reaction)

To each sample was added the biotin-PEG-alkyne ( 0.1 mM final concentration from 10 mM solution in DMSO) and the samples vortexed for 10 seconds. Samples were then purified with a zeba spin column ( 7 kDa MWCO, 2 mL ) pre-equilibrated with PBS to remove excess Biotin-PEG-alkyne and stored in the dark and in the freezer until further use for affinity purification.

## Reaction with Biotin-PEG-DBCO without Copper (SPAAC reaction)

To each sample was added the biotin-PEG-DBCO ( 0.1 mM final concentration from 10 mM solution in DMSO) and the samples vortexed for 10 seconds. Samples were then purified with a zeba spin column ( 7 kDa MWCO, 2 mL ) pre-equilibrated with PBS to remove excess Biotin-PEG-DBCO and stored in the dark and in the freezer until further use for affinity purification.


Figure S50. Assessment of $\mathbf{3}$ and 13 as probes for cysteine in HeLa cell lysates. Probes were added to the lysates as solutions in DMSO. The reactions were carried out for 2 h at RT. Probes 3 and 13 was modified with a biotin-containing alkyne or DBCO before SDS-PAGE. Western blots were developed using an Alexa Fluor 555 streptavidin conjugate, SPYRO Ruby and colorimetric using ChemiDoc MP Imaging System.

## 35. NMR spectra





Figure S51. ${ }^{1} \mathrm{H}$ and ${ }^{13} \mathrm{C}$ NMR spectra of diazocarbonylacrylic 3


Figure S52. ${ }^{1} \mathrm{H}$ and ${ }^{13} \mathrm{C}$ NMR spectra of diazoacetophenone 10




Figure S53. ${ }^{1} \mathrm{H}$ and ${ }^{13} \mathrm{C}$ NMR spectra of N -Boc-diazo-michael adduct 7a.




Figure S54. ${ }^{1} \mathrm{H}$ and ${ }^{13} \mathrm{C}$ NMR spectra of N -Cbz-diazo-michael adduct 7 b .


Figure $\mathbf{S 5 5} .{ }^{1} \mathrm{H}$ and ${ }^{13} \mathrm{C}$ NMR spectra of cycloaddition product 9 .

$\begin{array}{lllllllllllllllllllllllllll}40 & 230 & 220 & 210 & 200 & 190 & 180 & 170 & 160 & 150 & 140 & 130 & 120 & 110 & 100 & 90 & 80 & 70 & 60 & 50 & 40 & 30 & 20 & 10 & 0 & -1 \\ \text { Chemical Shift }\end{array}$
Figure S56. ${ }^{1} \mathrm{H}$ and ${ }^{13} \mathrm{C}$ NMR spectra of cycloaddition product 11.




Figure S57. ${ }^{1} \mathrm{H}$ and ${ }^{13} \mathrm{C}$ NMR spectra of Fmoc-4-lodo-aniline.


Figure S58. ${ }^{1} \mathrm{H}$ and ${ }^{13} \mathrm{C}$ NMR spectra of Fmoc-4-diazoester-aniline.



Figure S59. ${ }^{1} \mathrm{H}$ and ${ }^{13} \mathrm{C}$ NMR spectra of 13.


Figure $\mathbf{S 6 0} .{ }^{1} \mathrm{H}$ NMR spectrum of DIBONE.

## 36. Quantum mechanical calculations

Computational Details. Full geometry optimizations were carried out with Gaussian $16^{10}$ using the M06-2X hybrid functional ${ }^{11}$ and $6-31+G(d, p)$ basis set in combination with ultrafine integration grids. Bulk solvent effects in water were considered implicitly through the IEF-PCM polarizable continuum model. ${ }^{12}$ The possibility of different conformations was taken into account. Frequency analyses were carried out at the same level used in the geometry optimizations, and the nature of the stationary points was determined in each case according to the appropriate number of negative eigenvalues of the Hessian matrix. Scaled frequencies were not considered. Massweighted intrinsic reaction coordinate (IRC) calculations were carried out by using the Gonzalez and Schlegel scheme ${ }^{13,14}$ in order to ensure that the TSs indeed connected the appropriate reactants and products. Gibbs free energies $(\Delta G)$ were used for the discussion on the relative stabilities of the considered structures. Free energies calculated using the gas phase standard state concentration ( $1 \mathrm{~atm}=1 / 24.5 \mathrm{M}$ ) were converted to reproduce the standard state concentration in solution (1 M) by adding or subtracting $1.89 \mathrm{kcal} \mathrm{mol}^{-1}$ for bimolecular additions and decompositions, respectively. The lowest energy conformer for each calculated stationary point was considered in the discussion; all the computed structures can be obtained from authors upon request. Cartesian coordinates, electronic energies, entropies, enthalpies, Gibbs free energies, and lowest frequencies of the calculated structures are available below.

Table of energies, entropies and lowest frequencies of the calculated lowestenergy structures

| Structur <br> e | $\begin{gathered} \mathrm{E}_{\text {elec }} \\ (\text { Hartree })^{\mathrm{a}} \end{gathered}$ | $E_{\text {elec }}+$ ZPE <br> (Hartree) ${ }^{\text {a }}$ | H <br> (Hartree) ${ }^{\text {a }}$ | $\begin{gathered} \mathrm{S} \\ \left(\mathrm{cal} \mathrm{~mol}^{1}\right. \\ \left.\mathrm{K}^{-1}\right) \\ \hline \end{gathered}$ | $\underset{(\text { Hartree })^{\mathbf{a}}}{\mathbf{G}}$ | Lowest freq. (cm ${ }^{-1}$ ) | $\begin{gathered} \text { \# of } \\ \text { ima } \\ \text { g } \\ \text { freq. } \end{gathered}$ |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| 8' | $349.942515$ | -349.755639 | -349.746738 | 85.8 | -349.787503 | 173.0 | 0 |
| A | $155.909761$ | -155.824919 | -155.818280 | 73.8 | -155.853330 | 31.5 | 0 |
| 12' | $\begin{gathered} - \\ 785.285661 \end{gathered}$ | -785.029737 | -785.013885 | 121.3 | -785.071519 | 49.3 | 0 |
| B | $539.258573$ | -539.065623 | -539.053642 | 109.0 | -539.105417 | 10.6 | 0 |
| 7' | $301 . \overline{291008}$ | -301.219435 | -301.212071 | 80.2 | -301.250187 | 45.9 | 0 |
| 7'-8'-ts | $651.215215$ | -650.955608 | -650.939846 | 121.7 | -650.997680 | -451.3 | 1 |
| 7'-A-ts | $457.161376$ | -457.003028 | -456.990280 | 106.3 | -457.040765 | -527.9 | 1 |
| 7'-12'-ts | $\begin{gathered} 1086.56126 \\ 1 \end{gathered}$ | $1086 . \overline{-}$ | $1086 . \overline{-}$ | 155.8 | $1086.283580$ | -438.4 | 1 |
| 7'-B-ts | $\begin{gathered} - \\ 840.512414 \\ \hline \end{gathered}$ | -840.246896 | -840.228113 | 141.6 | -840.295384 | -493.8 | 1 |
| 10 | $492.963308$ | -492.837425 | -492.827387 | 95.4 | -492.872704 | 46.7 | 0 |
| 10-8'-ts | $842.887573$ | -842.573882 | -842.555198 | 138.5 | -842.620985 | -461.5 | 1 |
| 10-A-ts | $648.833056$ | -648.620484 | -648.604937 | 121.9 | -648.662855 | -534.1 | 1 |
| 10-12'-ts | $1278.23466$ | $\stackrel{-}{1277.851648}$ | $1277 . \overline{-} 26159$ | 167.9 | $1277 . \overline{-} 05921$ | -465.3 | 1 |
| 10-B-ts | $1032.18689$ $6$ | $1031 . \overline{867135}$ | $1031.845616$ | 154.4 | $1031 . \overline{-} 18985$ | -501.6 | 1 |
| 13' | $607.467949$ | -607.308348 | -607.295749 | 109.0 | -607.347541 | 24.5 | 0 |
| 13'-8'-ts | $957.396300$ | -957.048998 | -957.027733 | 150.7 | -957.099341 | -442.2 | 1 |
| 13'-12'-ts | $\begin{gathered} 1392.74043 \\ 5 \end{gathered}$ | $1392.323632$ | $1392.295479$ | 182.2 | $1392.382069$ | -441.0 | 1 |

${ }^{\text {a }}$ Energy values calculated with $\mathrm{PCM}\left(\mathrm{H}_{2} \mathrm{O}\right) / \mathrm{M} 06-2 \mathrm{X} / 6-31+\mathrm{G}(\mathrm{d}, \mathrm{p}) .1$ Hartree $=627.51 \mathrm{kcal} \mathrm{mol}^{-1}$.




$12^{\prime}$
A


$\Delta G^{t}=41 \cdot 3: k_{\text {ele }}=3.0 \cdot 10^{-11}$
7-12'-ts
7'-B-ts
$7-8{ }^{\prime}$-ts
$=27.0 ; k_{\text {wel }}=1.0$
$\Delta \mathrm{G}^{\ddagger}=25.8 \cdot \mathrm{k}_{\text {kel }}=7$
$\Delta \mathrm{G}^{\mathrm{t}}=39.7: \mathrm{K}_{\text {ele }}=5.0 \cdot 10^{-10}$

10-A-ts
$\Delta \mathrm{G}^{\ddagger}=41.5 ; k_{\text {rel }}=2.010^{-11}$

10-12'-ts
$\Delta \mathrm{G}^{\mathrm{t}}=25.9 ; \mathrm{K}_{\text {tel }}=6.4$


13'-12'-ts
$\Delta \mathrm{G}^{\mathrm{t}}=24.1: k_{\sim}=132$

Figure S61. Lowest-energy structures calculated with $\mathrm{PCM}\left(\mathrm{H}_{2} \mathrm{O}\right) / \mathrm{M} 06-2 \mathrm{X} / 6-31+\mathrm{G}(\mathrm{d}, \mathrm{p})$ for the 1,3-dipolar cycloaddition reactions between diazo-compounds $\mathbf{7 '}^{\prime}$ and 10 with strained alkynes $\mathbf{8}^{\prime}$ and $\mathbf{1 2 '}^{\prime}$ and unstrained alkynes $\mathbf{A}$ and B. Distances are given in angstrom $(\AA)$ and free energies $(\Delta \mathrm{G})$ in $\mathrm{kcal} \mathrm{mol}^{-1}$. Relative kinetic rate constants ( $k_{\mathrm{rel}}$ ) were derived from the corresponding activation free energies $\left(\Delta \mathrm{G}^{\ddagger}\right)$ using the Eyring equation at 298 K .

Table of activation free energies ( $\Delta \mathbf{G}^{\ddagger}$ ), Frontier Molecular Orbital energies $\left(\Delta \mathrm{E}_{\text {NED }}, \Delta \mathrm{E}_{\text {IED }}\right.$ ) and distortion ( $\Delta \mathrm{E}_{\text {dist }} /$ /interaction ( $\Delta \mathrm{E}_{\text {int }}$ ) energies calculated for the lowest-energy transition structures with PCM $\left(\mathrm{H}_{2} \mathrm{O}\right) / \mathrm{M} 06-2 \mathrm{X} / 6-31+\mathrm{G}(\mathrm{d}, \mathrm{p})$.

| diazo- <br> compound | alkyne | $\boldsymbol{\Delta} \mathbf{G}^{\ddagger}$ | $\boldsymbol{\Delta} \mathrm{E}^{\ddagger}$ | $\boldsymbol{\Delta} \mathrm{E}_{\text {dist }}$ | $\boldsymbol{\Delta} \mathrm{E}_{\text {int }}$ | $\boldsymbol{\Delta} \mathrm{E}_{\text {NED }^{\mathbf{a}}}$ | $\boldsymbol{\Delta} \mathrm{E}_{\text {IED }^{\mathbf{b}}}$ |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | $\mathbf{8}$ | 27.0 | 11.5 | 21.6 | -10.1 | 200.6 | 176.1 |
| $\mathbf{7 '}$ | $\mathbf{A}$ | 41.3 | 24.7 | 37.0 | -12.3 | 206.4 | 182.8 |
|  | $\mathbf{1 2}$ | 25.8 | 9.7 | 23.3 | -13.6 | 165.8 | 158.9 |
|  | $\mathbf{B}$ | 39.7 | 23.3 | 36.9 | -13.5 | 167.4 | 156.8 |
|  | $\mathbf{8}$ | 26.5 | 11.5 | 22.1 | -10.6 | 200.2 | 164.2 |
| $\mathbf{1 0}$ | $\mathbf{A}$ | 41.5 | 25.1 | 37.6 | -12.5 | 206.0 | 170.9 |
|  | $\mathbf{1 2}$ | 25.9 | 9.0 | 25.3 | -16.3 | 165.4 | 146.9 |
|  | $\mathbf{B}$ | 39.0 | $\mathbf{2 2 . 0}$ | 37.5 | -15.5 | 167.0 | 144.8 |

${ }^{a}$ NED: Normal Electron Demand (LUMOakyne - HOMOdiazo-compound). ${ }^{\text {al }}$ IED: Inverse Electron Demand (LUMOdiazo-compound - $\mathrm{HOMO}_{\text {akkne) }}$. Energies are given in $\mathrm{kcal} \mathrm{mol}^{-1}$. The lowest HOMO-LUMO gap for each reaction is highlighted in blue.


Figure S62. Frontier Molecular Orbital (FMO) energies for the cycloaddition between bicyclononyne 8' and diazoacetone 7' calculated at PCM( $\left.\mathrm{H}_{2} \mathrm{O}\right) / \mathrm{M} 06-2 \mathrm{X} / 6-31+\mathrm{G}(\mathrm{d}, \mathrm{p})$ level. Molecular orbital isosurfaces were generated at $\mathrm{PCM}\left(\mathrm{H}_{2} \mathrm{O}\right) / \mathrm{M} 06-2 \mathrm{X} / 6-31 \mathrm{G}(\mathrm{d}, \mathrm{p})$ level due to plotting issues encountered with diffuse functions. Despite the $\mathrm{LUMO}_{\text {diazo- }}$ compound $-\mathrm{HOMO}_{\text {alkyne }}$ energy gap is the smallest calculated one, the more appropriate symmetry of the LUMO+1 and LUMO+2 orbitals of diazoacetone (i.e. having bigger coefficients at the diazo carbon atom), which lead to effectively larger HOMO-LUMO energy gaps, suggests that diazoketones (as well as diazoesters and diazoamides) are ambiphilic dipoles with no clear preference for NED (Normal Electronic Demand) or IED (Inverse Electron Demand) pathways.

## Cartesian coordinates of the lowest-energy calculated structures

## Structure 8'

| C | 0.754661 | 1.098731 | -0.437612 |
| :--- | ---: | ---: | ---: |
| C | -0.762645 | 1.093480 | -0.437704 |
| C | 0.612626 | -1.763766 | -0.154879 |
| C | -1.661002 | 0.284587 | 0.481367 |
| C | -0.599597 | -1.767068 | -0.154527 |
| C | -1.928308 | -1.154057 | -0.031721 |
| H | -1.186498 | 1.215995 | -1.433789 |
| H | -1.217406 | 0.218316 | 1.481254 |
| H | 1.177737 | 1.224231 | -1.433659 |
| H | -2.618932 | 0.808399 | 0.588991 |
| H | -2.575798 | -1.694282 | 0.664796 |
| H | -2.439720 | -1.127126 | -1.000148 |
| C | 1.658573 | 0.296122 | 0.481526 |
| H | 1.215190 | 0.226383 | 1.481277 |
| H | 2.612631 | 0.826891 | 0.589573 |
| C | 1.936636 | -1.140352 | -0.031727 |
| H | 2.447990 | -1.109484 | -1.000078 |
| H | 2.588211 | -1.675756 | 0.664715 |
| C | -0.008171 | 2.292466 | 0.075600 |
| H | -0.008728 | 2.433064 | 1.154376 |
| H | -0.011316 | 3.212517 | -0.499251 |

## Structure A

| C | 0.605238 | 0.000005 | 0.000042 |
| :--- | ---: | ---: | ---: |
| C | -0.605237 | -0.000014 | -0.000009 |
| C | -2.071641 | 0.000005 | -0.000009 |
| H | -2.460360 | -1.021389 | 0.007359 |
| H | -2.460320 | 0.517075 | 0.880879 |
| H | -2.460358 | 0.504334 | -0.888220 |
| C | 2.071641 | 0.000001 | -0.000008 |
| H | 2.460353 | 1.021418 | -0.003020 |
| H | 2.460391 | -0.508129 | 0.886020 |
| H | 2.460294 | -0.513290 | -0.883114 |

## Structure 12'

| C | -0.561449 | 0.436204 | -1.224384 |
| :--- | ---: | ---: | ---: |
| C | -0.698917 | -1.738427 | 0.484444 |
| C | 1.751309 | 0.111083 | -0.253134 |
| C | 0.514155 | -1.797872 | 0.447708 |
| C | 1.799462 | -1.244755 | 0.160305 |
| H | -0.156582 | -0.377536 | -1.831221 |
| H | -0.743997 | 1.293122 | -1.878778 |
| C | -1.886403 | 0.001790 | -0.615207 |
| C | -1.972203 | -1.150496 | 0.204803 |
| C | 3.024260 | -1.916255 | 0.206167 |
| C | 4.194815 | -1.253279 | -0.152581 |
| H | 5.144268 | -1.776265 | -0.110551 |
| C | 4.146018 | 0.074499 | -0.578083 |
| H | 5.056504 | 0.586007 | -0.871855 |
| C | 2.926938 | 0.749898 | -0.639039 |
| H | 2.883373 | 1.778356 | -0.984309 |
| C | -3.205112 | -1.597216 | 0.690171 |
| C | -4.366287 | -0.902448 | 0.363835 |
| H | -5.324806 | -1.247258 | 0.737521 |
| C | -4.294745 | 0.237622 | -0.437770 |
| H | -5.199163 | 0.782672 | -0.688082 |


| C | -3.062633 | 0.683862 | -0.918017 |
| :--- | ---: | ---: | ---: |
| H | -3.011221 | 1.577128 | -1.534203 |
| H | -3.244176 | -2.481344 | 1.317732 |
| H | 3.048968 | -2.953503 | 0.522280 |
| N | 0.504003 | 0.810345 | -0.265746 |
| C | 0.279514 | 1.919086 | 0.506888 |
| O | -0.739244 | 2.589744 | 0.348305 |
| C | 1.295715 | 2.270517 | 1.570643 |
| H | 1.753368 | 1.385422 | 2.015857 |
| H | 2.091119 | 2.887504 | 1.143788 |
| H | 0.781664 | 2.852466 | 2.335109 |

## Structure B

| C | -0.607018 | -0.000001 | -0.000018 |
| :--- | ---: | ---: | ---: |
| C | 2.749700 | -1.213048 | 0.006063 |
| C | 0.607018 | -0.000015 | -0.000008 |
| C | 2.041224 | -0.000009 | 0.000000 |
| C | -2.749693 | -1.213043 | -0.006067 |
| C | -2.041224 | 0.000001 | -0.000008 |
| C | 2.749685 | 1.213041 | -0.006063 |
| C | 4.141554 | 1.208382 | -0.006060 |
| H | 4.681572 | 2.149716 | -0.010831 |
| C | 4.840619 | 0.000010 | 0.000009 |
| H | 5.925884 | 0.000017 | 0.000012 |
| C | 4.141570 | -1.208369 | 0.006070 |
| H | 4.681600 | -2.149697 | 0.010844 |
| C | -2.749692 | 1.213045 | 0.006059 |
| C | -4.141562 | 1.208376 | 0.006069 |
| H | -4.681585 | 2.149707 | 0.010844 |
| C | -4.840619 | 0.000001 | 0.000009 |
| H | -5.925884 | 0.000000 | 0.000015 |
| C | -4.141562 | -1.208375 | -0.006061 |
| H | -4.681587 | -2.149705 | -0.010829 |
| H | -2.201647 | 2.149764 | 0.010789 |
| H | 2.201633 | 2.149755 | -0.010799 |
| H | -2.201647 | -2.149762 | -0.010804 |
| H | 2.201663 | -2.149771 | 0.010793 |

## Structure 7'

| C | 0.290795 | -0.807640 | -0.022660 |
| :--- | ---: | ---: | ---: |
| H | 0.201967 | -1.882949 | -0.036934 |
| N | 2.521822 | 0.173153 | 0.019910 |
| N | 1.508923 | -0.310948 | 0.000187 |
| C | -0.790822 | 0.155485 | -0.016375 |
| O | -0.589172 | 1.369372 | -0.009662 |
| C | -2.184782 | -0.421714 | 0.014312 |
| H | -2.869269 | 0.265467 | -0.484746 |
| H | -2.230845 | -1.406925 | -0.452514 |
| H | -2.494838 | -0.522789 | 1.059153 |

## Structure 7'-8'-ts

C $\quad 2.658366-1.048071-0.512073$
C $\quad 3.079765 \quad 0.384368$-0.268454
C $\quad-0.158986-0.137858$-0.245629
$\begin{array}{lllll}\text { C } & 2.492463 & 1.284337 & 0.800114\end{array}$
C $\quad 0.192805 \quad 1.032307-0.041250$
$\begin{array}{lllll}\text { C } & 1.221604 & 2.020851 & 0.334256\end{array}$

| H | 3.351579 | 0.922317 | -1.175798 |
| :--- | ---: | ---: | ---: |
| H | 2.254545 | 0.701358 | 1.697234 |
| H | 2.685989 | -1.341495 | -1.560734 |
| H | 3.246295 | 2.025309 | 1.094702 |
| H | 0.852829 | 2.681967 | 1.126021 |
| H | 1.449858 | 2.663236 | -0.525519 |
| C | 1.593729 | -1.781101 | 0.279399 |
| H | 1.609736 | -1.457783 | 1.326508 |
| H | 1.825261 | -2.853661 | 0.275847 |
| C | 0.173019 | -1.578621 | -0.281645 |
| H | 0.117564 | -1.948002 | -1.313653 |
| H | -0.550014 | -2.164893 | 0.295814 |
| C | -2.348145 | 0.020116 | -0.952103 |
| H | -2.366362 | -0.380867 | -1.959204 |
| N | -1.639469 | 2.247571 | -0.559785 |
| N | -2.287709 | 1.351568 | -0.857628 |
| C | -2.992437 | -0.717568 | 0.144376 |
| O | -3.166427 | -1.923476 | 0.024719 |
| C | -3.323821 | 0.030160 | 1.410665 |
| H | -3.710060 | -0.670783 | 2.149445 |
| H | -2.425487 | 0.521340 | 1.798737 |
| H | -4.069163 | 0.806206 | 1.212505 |
| C | 4.001977 | -0.748468 | 0.100002 |
| H | 4.106901 | -0.959861 | 1.161858 |
| H | 4.900151 | -0.913257 | -0.485583 |

## Structure 7'-A-ts

| C | -0.820512 | 1.051359 | -0.046997 |
| :--- | ---: | ---: | ---: |
| C | -1.783045 | 0.292621 | 0.161906 |
| C | 0.757504 | -0.274571 | -0.951955 |
| H | 0.944831 | 0.120705 | -1.945264 |
| N | -1.102508 | -1.654605 | -0.548318 |
| N | -0.053542 | -1.345822 | -0.905041 |
| C | 1.823513 | -0.187934 | 0.061130 |
| O | 2.671181 | 0.688504 | -0.045545 |
| C | 1.760075 | -1.117689 | 1.245820 |
| H | 2.559345 | -0.867161 | 1.942000 |
| H | 0.789809 | -1.019619 | 1.743948 |
| H | 1.863690 | -2.157536 | 0.921802 |
| C | -0.124575 | 2.358403 | -0.057501 |
| H | -0.783506 | 3.136914 | 0.336069 |
| H | 0.782806 | 2.333166 | 0.551707 |
| H | 0.172871 | 2.636360 | -1.072590 |
| C | -3.135889 | -0.119631 | 0.584197 |
| H | -3.698772 | 0.730524 | 0.979227 |
| H | -3.690406 | -0.544050 | -0.257335 |
| H | -3.080191 | -0.889694 | 1.358712 |

## Structure 7'-12'-ts

| C | 1.450061 | -1.696753 | -0.842836 |
| :--- | ---: | ---: | ---: | ---: |
| C | 0.662658 | 1.068415 | -0.604302 |
| C | -0.994746 | -1.657114 | -0.383875 |
| C | -0.525180 | 0.706248 | -0.741309 |
| C | -1.448735 | -0.401238 | -0.844779 |
| H | 0.997605 | -1.540547 | -1.825729 |
| H | 1.968748 | -2.660244 | -0.857930 |
| C | 2.458219 | -0.601007 | -0.571614 |
| C | 2.073283 | 0.759151 | -0.506701 |


| C | -1.791196 | 2.567176 | -0.423741 |
| :--- | ---: | ---: | ---: |
| H | -2.377828 | 2.774678 | -1.312432 |
| N | 0.463660 | 3.275118 | -0.261209 |
| N | -0.678852 | 3.294089 | -0.300820 |
| C | -2.494950 | 2.199674 | 0.831783 |
| O | -3.627588 | 1.753780 | 0.759309 |
| C | -1.737956 | 2.316159 | 2.128611 |
| H | -2.342884 | 1.899544 | 2.932793 |
| H | -0.786420 | 1.778412 | 2.060065 |
| H | -1.513316 | 3.365582 | 2.342934 |
| C | -2.738299 | -0.315183 | -1.384591 |
| C | -3.558634 | -1.436879 | -1.453525 |
| H | -4.554624 | -1.347943 | -1.874503 |
| C | -3.104089 | -2.669457 | -0.984112 |
| H | -3.741850 | -3.545463 | -1.037030 |
| C | -1.817180 | -2.778721 | -0.461084 |
| H | -1.436949 | -3.736091 | -0.116975 |
| C | 3.061053 | 1.741985 | -0.338909 |
| C | 4.404299 | 1.392443 | -0.237844 |
| H | 5.153543 | 2.167444 | -0.112156 |
| C | 4.781511 | 0.051651 | -0.293553 |
| H | 5.826678 | -0.228411 | -0.209458 |
| C | 3.807436 | -0.931858 | -0.455892 |
| H | 4.095776 | -1.978891 | -0.492221 |
| H | 2.771814 | 2.784025 | -0.291461 |
| H | -3.100911 | 0.639690 | -1.747120 |
| N | 0.334681 | -1.777280 | 0.115920 |
| C | 0.618032 | -2.081446 | 1.416340 |
| O | 1.771123 | -2.335171 | 1.763919 |
| C | -0.527314 | -2.070769 | 2.404323 |
| H | -1.238128 | -1.267730 | 2.196940 |
| H | -1.069230 | -3.019734 | 2.364821 |
| H | -0.106374 | -1.951554 | 3.402070 |
|  |  | 5 |  |

## Structure 7'-B-ts

| C | -0.280627 | -0.029636 | -0.382738 |
| :--- | ---: | ---: | ---: |
| C | 2.636440 | -1.120120 | 0.948332 |
| C | 0.952965 | 0.151907 | -0.318804 |
| C | 2.323999 | -0.261722 | -0.119311 |
| C | -1.799657 | -1.483161 | 0.903097 |
| C | -1.468689 | -0.859337 | -0.309206 |
| C | -1.158314 | 1.993849 | -0.704939 |
| H | -1.693046 | 1.974358 | -1.649396 |
| N | 1.175828 | 2.310880 | -0.638373 |
| N | 0.058985 | 2.552710 | -0.744997 |
| C | -1.935476 | 2.128989 | 0.548698 |
| O | -3.110320 | 1.798922 | 0.544175 |
| C | -1.208514 | 2.562550 | 1.794294 |
| H | -1.896169 | 2.532349 | 2.638333 |
| H | -0.361582 | 1.893412 | 1.981642 |
| H | -0.812736 | 3.575482 | 1.674831 |
| C | 3.350620 | 0.162506 | -0.976761 |
| C | 4.656779 | -0.278049 | -0.780330 |
| H | 5.440703 | 0.049875 | -1.455702 |
| C | 4.960682 | -1.129561 | 0.283140 |
| H | 5.981110 | -1.464845 | 0.438417 |
| C | 3.947151 | -1.545583 | 1.147858 |
| H | 4.176290 | -2.206004 | 1.978292 |


| C | -2.294845 | -1.051402 | -1.426346 |
| :--- | ---: | ---: | ---: |
| C | -3.419775 | -1.866057 | -1.334592 |
| H | -4.047107 | -2.014615 | -2.207859 |
| C | -3.744873 | -2.484963 | -0.125880 |
| H | -4.626451 | -3.113941 | -0.055779 |
| C | -2.932793 | -2.289349 | 0.991243 |
| H | -3.180628 | -2.764631 | 1.935148 |
| H | -2.046845 | -0.564644 | -2.365012 |
| H | 3.116569 | 0.834430 | -1.796469 |
| H | -1.166180 | -1.326806 | 1.771340 |
| H | 1.844514 | -1.446087 | 1.615867 |

## Structure 10

| C | 1.835982 | -0.732014 | -0.188140 |
| :--- | ---: | ---: | ---: |
| H | 1.579597 | -1.735821 | -0.486257 |
| N | 4.203518 | -0.170822 | -0.039471 |
| N | 3.121455 | -0.458439 | -0.115822 |
| C | 0.949346 | 0.381414 | 0.075683 |
| O | 1.381266 | 1.511773 | 0.310663 |
| C | -0.521931 | 0.109259 | 0.031762 |
| C | -1.053673 | -1.179099 | 0.155110 |
| C | -1.382699 | 1.201708 | -0.123046 |
| C | -2.433522 | -1.370518 | 0.119690 |
| H | -0.404576 | -2.035903 | 0.304020 |
| C | -2.759800 | 1.007578 | -0.167597 |
| H | -0.956344 | 2.195507 | -0.211744 |
| C | -3.286883 | -0.279687 | -0.046009 |
| H | -2.841191 | -2.370423 | 0.225352 |
| H | -3.422260 | 1.857282 | -0.296790 |
| H | -4.361080 | -0.431837 | -0.077545 |

## Structure 10-8'-ts

| C | 2.878755 | -1.883745 | -0.540835 |
| :--- | ---: | ---: | ---: |
| C | 3.973211 | -0.880853 | -0.250235 |
| C | 0.917269 | 0.319074 | -0.171629 |
| C | 3.926513 | 0.134521 | 0.873715 |
| C | 1.821243 | 1.132567 | 0.069014 |
| C | 3.207292 | 1.436771 | 0.474781 |
| H | 4.476659 | -0.507985 | -1.141269 |
| H | 3.426643 | -0.293143 | 1.750259 |
| H | 2.744397 | -2.095595 | -1.600802 |
| H | 4.952391 | 0.374157 | 1.180394 |
| H | 3.217499 | 2.148664 | 1.307328 |
| H | 3.736523 | 1.922188 | -0.354877 |
| C | 1.597189 | -2.009685 | 0.257645 |
| H | 1.786675 | -1.788362 | 1.314169 |
| H | 1.249512 | -3.049314 | 0.210144 |
| C | 0.468544 | -1.089435 | -0.246116 |
| H | 0.201962 | -1.344737 | -1.280142 |
| H | -0.434975 | -1.248069 | 0.353437 |
| C | -0.821844 | 1.637830 | -0.946045 |
| H | -0.975035 | 1.294783 | -1.960602 |
| N | 0.966076 | 3.109520 | -0.475685 |
| N | -0.051834 | 2.726626 | -0.831748 |
| C | -1.788313 | 1.456498 | 0.145006 |
| O | -1.778024 | 2.184934 | 1.132510 |
| C | 4.190936 | -2.342763 | 0.040134 |
| H | 4.179844 | -2.634582 | 1.087956 |


| C | 3.912705 | 1.804074 | -0.320808 |
| :--- | ---: | ---: | ---: |
| C | 5.258149 | 1.489983 | -0.155328 |
| H | 5.987288 | 2.285637 | -0.041343 |
| C | 5.661531 | 0.156002 | -0.129390 |
| H | 6.707415 | -0.098596 | 0.009058 |
| C | 4.713418 | -0.854405 | -0.281114 |
| H | 5.025400 | -1.895158 | -0.256593 |
| C | -1.680287 | -0.403435 | -1.848095 |
| C | -2.477536 | -1.536860 | -1.960953 |
| H | -3.408618 | -1.485780 | -2.516192 |
| C | -2.086525 | -2.732069 | -1.354260 |
| H | -2.710438 | -3.616043 | -1.434631 |
| C | -0.878946 | -2.797348 | -0.661685 |
| H | -0.543762 | -3.728609 | -0.214202 |
| H | -1.987789 | 0.527147 | -2.314621 |
| H | 3.597502 | 2.840311 | -0.334552 |
| N | 1.193818 | -1.738219 | 0.100506 |
| C | 1.349896 | -1.854211 | 1.451905 |
| O | 2.464181 | -2.040186 | 1.940211 |
| C | 0.116460 | -1.678316 | 2.307088 |
| H | 0.407266 | -1.778579 | 3.351673 |
| H | -0.299007 | -0.678480 | 2.136215 |
| H | -0.656660 | -2.411755 | 2.069024 |
| C | -2.951752 | 1.139313 | 0.602703 |
| C | -3.269732 | 0.007711 | 1.362700 |
| C | -3.853982 | 1.610421 | -0.357295 |
| C | -4.465337 | -0.666989 | 1.140956 |
| H | -2.565786 | -0.342967 | 2.110504 |
| C | -5.059749 | 0.944467 | -0.563048 |
| H | -3.634892 | 2.501976 | -0.936615 |
| C | -5.359700 | -0.200527 | 0.175145 |
| H | -4.699865 | -1.556407 | 1.716637 |
| H | -5.761590 | 1.316692 | -1.301888 |
| H | -6.292716 | -0.727185 | 0.001324 |
|  |  |  |  |

## Structure 10-B-ts

| C | -0.718844 | 0.255556 | -0.472870 |
| :--- | ---: | ---: | ---: |
| C | -3.556667 | 0.688773 | 1.309434 |
| C | -1.912120 | -0.091284 | -0.347828 |
| C | -3.285610 | 0.118465 | 0.054368 |
| C | 0.925051 | 1.553756 | 0.841019 |
| C | 0.361981 | 1.220783 | -0.399866 |
| C | 0.320906 | -1.572345 | -1.222187 |
| H | 0.790102 | -1.289790 | -2.156936 |
| N | -1.961593 | -2.119379 | -1.116911 |
| N | -0.844403 | -2.225705 | -1.353107 |
| C | 1.087120 | -1.966763 | -0.015326 |
| O | 0.581974 | -2.699935 | 0.826105 |
| C | -4.355924 | -0.222318 | -0.786448 |
| C | -5.667566 | 0.019881 | -0.386498 |
| H | -6.486991 | -0.239757 | -1.049348 |
| C | -5.930401 | 0.585456 | 0.862177 |
| H | -6.954079 | 0.765428 | 1.174401 |
| C | -4.871041 | 0.915535 | 1.709010 |
| H | -5.067829 | 1.353730 | 2.682451 |
| C | 0.863876 | 1.820633 | -1.564198 |
| C | 1.902774 | 2.744369 | -1.485208 |
| H | 2.280281 | 3.205837 | -2.392384 |


| H | -2.264556 | -0.697063 | -2.952167 |
| :--- | ---: | ---: | ---: |
| C | -0.792158 | -0.110487 | -1.484332 |
| H | -0.262791 | -1.073074 | -1.499006 |
| H | -0.261541 | 0.546280 | -2.181990 |
| C | 1.470627 | 0.562258 | 0.461874 |
| N | 0.242701 | 1.237993 | 2.376857 |
| N | 1.128350 | 0.982285 | 1.694447 |
| C | 1.827793 | 1.650692 | -0.467914 |
| O | 2.119091 | 1.471446 | -1.634636 |
| C | -4.540372 | -1.211735 | -1.009928 |
| H | -5.015153 | -0.400857 | -1.557940 |
| H | -5.089377 | -2.147273 | -1.019451 |
| O | 1.719625 | 2.860629 | 0.092168 |
| C | 1.930423 | 3.973710 | -0.787941 |
| H | 1.804460 | 4.859471 | -0.169947 |
| H | 2.937597 | 3.935958 | -1.205493 |
| H | 1.194937 | 3.958265 | -1.593857 |
| C | 1.939137 | -0.836795 | 0.333650 |
| C | 1.385468 | -1.816025 | 1.173476 |
| C | 2.889820 | -1.216459 | -0.622047 |
| C | 1.780067 | -3.144748 | 1.064230 |
| H | 0.635347 | -1.537144 | 1.908735 |
| C | 3.273313 | -2.553121 | -0.732728 |
| H | 3.321303 | -0.473076 | -1.280633 |
| C | 2.724826 | -3.520462 | 0.106661 |
| H | 1.343824 | -3.889100 | 1.722631 |
| H | 4.010409 | -2.834364 | -1.478224 |
| H | 3.029135 | -4.558320 | 0.017801 |


| H | 2.299800 | 0.091273 | 1.821328 |
| :--- | ---: | ---: | ---: |
| H | -2.354082 | -3.110069 | -1.274441 |
| N | -1.823597 | 1.695750 | 0.561668 |
| C | -2.229232 | 2.421232 | -0.522257 |
| O | -3.401069 | 2.384143 | -0.896908 |
| C | -1.191003 | 3.246706 | -1.248987 |
| H | -1.121416 | 4.239577 | -0.795282 |
| H | -0.200017 | 2.789655 | -1.217185 |
| H | -1.520309 | 3.361768 | -2.281540 |
| O | 1.792936 | -3.445021 | 0.628462 |
| C | 2.228182 | -4.303878 | 1.694529 |
| H | 1.665588 | -5.225670 | 1.569949 |
| H | 3.299457 | -4.489816 | 1.608734 |
| H | 2.006749 | -3.841836 | 2.657539 |
| C | 2.499693 | -0.229948 | -1.093733 |
| C | 1.780357 | 0.630032 | -1.937229 |
| C | 3.844193 | 0.043375 | -0.821199 |
| C | 2.395815 | 1.741222 | -2.501614 |
| H | 0.729922 | 0.432498 | -2.139421 |
| C | 4.453265 | 1.166907 | -1.381522 |
| H | 4.410377 | -0.609067 | -0.168069 |
| C | 3.736173 | 2.016985 | -2.220251 |
| H | 1.827644 | 2.397638 | -3.153275 |
| H | 5.495887 | 1.372227 | -1.161145 |
| H | 4.215321 | 2.889067 | -2.653278 |

## Structure 13-12-ts

| C | -2.814406 | 0.839062 | 1.233304 |
| :--- | ---: | ---: | ---: |
| C | -0.078656 | -0.453773 | 0.497668 |
| C | -3.341253 | -0.308570 | 0.401488 |
| C | -1.043461 | -1.068989 | -0.010573 |
| C | -2.470497 | -1.266451 | -0.167217 |
| H | -3.655117 | 1.461525 | 1.554655 |
| H | -2.323558 | 0.444986 | 2.127270 |
| C | -0.540755 | 1.843775 | 1.163189 |
| C | 0.355189 | 0.750293 | 1.172757 |
| C | 1.784910 | -1.384597 | -0.491366 |
| N | -0.135999 | -2.337573 | -1.501475 |
| N | 0.964722 | -2.083243 | -1.303639 |
| C | 2.366327 | -2.243403 | 0.582354 |
| O | 3.219088 | -1.872238 | 1.362422 |
| C | -3.015245 | -2.367369 | -0.846346 |
| C | -4.393233 | -2.518802 | -0.967588 |
| H | -4.795308 | -3.379728 | -1.491840 |
| C | -5.250022 | -1.564734 | -0.420970 |
| H | -6.325453 | -1.672098 | -0.519642 |
| C | -4.718159 | -0.468335 | 0.255255 |
| H | -5.381294 | 0.280710 | 0.679464 |
| C | 1.596384 | 0.915094 | 1.803847 |
| C | 1.939743 | 2.126064 | 2.397214 |
| H | 2.907558 | 2.230414 | 2.876896 |
| C | 1.049192 | 3.199578 | 2.377258 |
| H | 1.314821 | 4.143506 | 2.841448 |
| C | -0.194872 | 3.049901 | 1.769671 |
| H | -0.911845 | 3.865567 | 1.764053 |

## 37. References

[1] Pinho V. D., and Burtoloso A.C.B. (2011) Preparation of $\alpha, \beta$-Unsaturated Diazoketones Employing a Horner-Wadsworth-Emmons Reagent. J. Org. Chem. 76 (1), 289-292.
[2] Bernardim, B.; Hardman-Baldwin, A. M.; and Burtoloso, A. C. B. (2015) LED lighting as a simple, inexpensive, and sustainable alternative for Wolff rearrangements. RSC Adv. 5 (18) 13311-13314.
[3] Mbua, N. E.; Guo, J.; Wolfert, M. A.; Steet, R.; Boons, G.-J. (2011) Strain-Promoted Alkyne-Azide Cycloadditions (SPAAC) Reveal New Features of Glycoconjugate Biosynthesis. ChemBioChem. 12 (12), 1912-1921.
[4] Pastor, I. M.; Västilä, P.; Adolfsson, H. (2003) Employing the Structural Diversity of Nature: Development of Modular Dipeptide-Analogue Ligands for Ruthenium-Catalyzed Enantioselective Transfer Hydrogenation of Ketones. Chem. Eur. J. 9 (17), 4031-4045.
[5] Ye, F.; Qu, S.; Zhou, L.; Peng, C.; Wang, C.; Cheng, J.; Hossain, M. L.; Liu, Y.; Zhang, Y.; Wang, Z.-X.; Wang, J. (2015) Palladium-Catalyzed C-H Functionalization of Acyldiazomethane and Tandem Cross-Coupling Reactions. J. Am. Chem. Soc. 137 (13), 4435-4444.
[6] Alam, I. S.; Neves, A. A.; Witney, T. H.; Boren, J.; Brindle, K. M. Comparison of the C2A Domain of Synaptotagmin-I and Annexin-V As Probes for Detecting Cell Death. (2010) Bioconjugate Chem. 21 (5), 884-891.
[7] Lee, B.; Sun, S.; Jiménez-Moreno, E.; Neves, A. A.; Bernardes, G. J. L. (2018) SiteSelective Installation of an Electrophilic Handle on Proteins for Bioconjugation. Bioorg. Med. Chem. 26 (11), 3060-3064.
[8] Cal, P. M. S. D.; Sieglitz, F.; Santos, F. M. F.; Carvalho, C. P.; Guerreiro, A.; Bertoldo, J. B.; Pischel, U.; Gois, P. M. P.; Bernardes, G. J. L. (2016) Site-Selective Installation of BASHY Fluorescent Dyes to Annexin V for Targeted Detection of Apoptotic Cells. Chem. Commun. 53 (2), 368-371.
[9] Vaneycken, I.; Devoogdt, N.; Van Gassen, N.; Vincke, C.; Xavier, C.; Wernery, U.; Muyldermans, S.; Lahoutte, T.; Caveliers, V. (2011) Preclinical Screening of Anti-HER2 Nanobodies for Molecular Imaging of Breast Cancer. The FASEB J. 25 (7), 2433-2446.
[10] Gaussian 16, Revision B.01, Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Petersson, G. A.; Nakatsuji, H.; et al. (2016) Gaussian, Inc., Wallingford CT.
[11] Zhao, Y.; Truhlar, D. G. (2007) The M06 Suite of Density Functionals for Main Group Thermochemistry, Thermochemical Kinetics, Noncovalent Interactions, Excited States, and Transition Elements: Two New Functionals and Systematic Testing of Four M06-Class
Functionals and 12 Other Functionals. Theor. Chem. Account. 120 (1-3), 215-241.
[12] Scalmani, G.; Frisch, M. J. (2010) Continuous Surface Charge Polarizable Continuum Models of Solvation. I. General Formalism. J. Chem. Phys. 132 (11), 114110.
[13] Gonzalez, C.; Schlegel, H. B. (1989) An Improved Algorithm for Reaction Path Following. J. Chem. Phys. 90 (4), 2154-2161.
[14] Gonzalez, C.; Schlegel, H. B. (1990) Reaction Path Following in Mass-Weighted Internal Coordinates. J. Phys. Chem. 94 (14), 5523-5527.

