Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

for

Orthogonal, Dual Protein Labeling by Tandem Cycloaddition of Strained Alkenes and Alkynes to ortho-Quinones and Azides

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Table of Contents

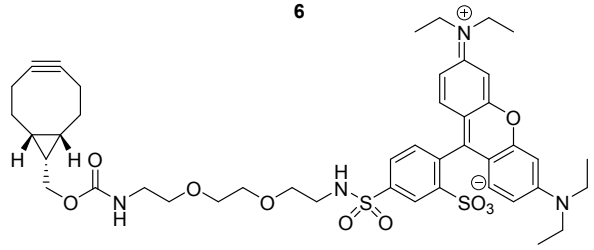
Methods and materials

Chemicals

Unless stated otherwise, all chemicals and solvents were obtained from Sigma-Aldrich or Fisher Scientific and used as received. Mushroom tyrosinase enzyme was obtained from Sigma-Aldrich. 2-Mercaptoethanol (BME), tetramethylenediamine (TEMED) were obtained from Acros. Sodium dodecyl sulfate (SDS), bromophenol blue, coomassie brilliant blue G250 were obtained from VWR. Acrylamide was obtained from Serva. Precision Plus Protein Dual Color Standards was obtained from Bio-Rad. Glycine was obtained from Applichem. BCN-POE3-NH-lissamine rhodamine B conjugate (**6**) and was obtained from SynAffix. BCN-HydraSpace™-PEG2-vc-PABC-MMAE conjugate (**7**) and 1-UDP-6-azido-GalNAc (**8**) were kindly donated by SynAffix.

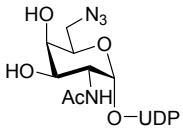
BCN-POE3-NH-lissamine rhodamine B

6



1-UDP-6-azido-GalNAc

8



BCN-HydraSpace™-PEG_2-vc-PABC-MMAE

7

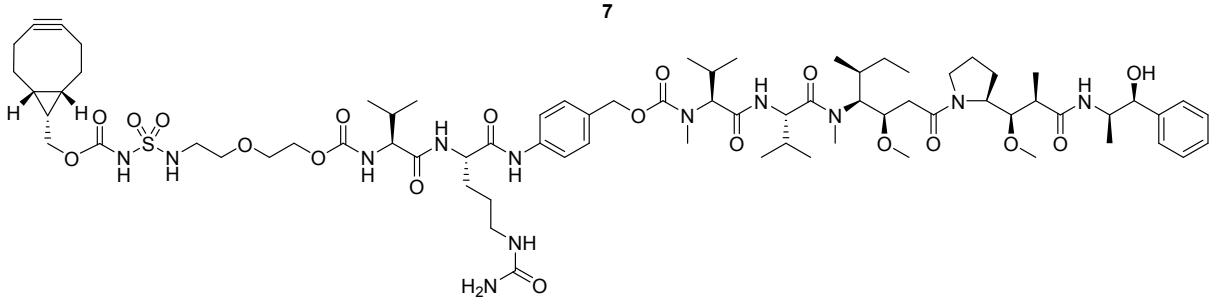


*Figure S1. Structure of supplied BCN compounds, **6** and **7**, and azide-bearing GalNac **8**.*

General SPOCQ method

In a typical experiment, to a solution of the antibody (10 mg/mL) in PBS buffer (pH 5.5), the cpTCO-label / BCN-label (3-5 eq.) in DMSO was added and cooled to 4 °C. Finally, mTyr (1.0 mg/mL) in phosphate buffer pH 5.5 (50 mM phosphate, 150 mM NaCl) was added. For example, cpTCO-Lissamine SPOCQ on Tras[HC]G_4Y:

- 8 µL 12.5 mg/mL Tras[HC]G_4Y in PBS pH 5.5
- 1 µL 5.0 mg/mL cpTCO-Lissamine in DMSO
- 1 µL 10.0 mg/mL mTyr in phosphate buffer* pH 5.5

Resulting in 10.0 mg/mL Tras[HC]G_4Y (67 µM 1 eq.), 0.5 mg/mL cpTCO-Lissamine (0.58 mM, 4.3 eq. per G_4Y), 1.0 mg/mL mTyr (7.7 µM, 0.11 eq) in 9:1 PBS/DMSO solution.

* Mushroom Tyrosinase is stored in phosphate buffer, as sodium chloride slowly inhibits the enzyme, which will cause problems with long-term storage.[1]

Dual labelling methods

SPOCQ labelling was performed according to the general SPOCQ method, with spin-filtration after 90 minutes to remove excess cpTCO-lissamine.

SPAAC was perfomed by adding 10 eq. BCN-label in DMF (final ratio 85:15 H_2O/DMF) and incubating overnight at room temperature. After which, spin-filtration was performed to remove excess BCN-toxin.

SDS-PAGE analysis

After the indicated reaction time expired, the sample was incubated with SDS-PAGE sample buffer (2×) including 5% 2-mercaptoethanol for 5 minutes at 95 °C. The denatured sample was then applied for SDS-PAGE analysis (12% acrylamide gel). Bio-Rad dual colour standard was used as a reference protein ladder.

HPLC measurements

Prior to HPLC analysis, samples were reduced by incubating a solution of 10 µg antibody in 50 µL 10 mM DTT and 0.1 M Tris pH 8.0 for 15 minutes at 37 °C. Reverse phase HPLC was performed on a Agilent 1100 HPLC using a ZORBAX Poroshell 300SB-C8 (1 x 75 mm, 5 µm) column run at 0.8 ml/min at 70 °C using a 16.8 minute linear gradient from 20% to 55% buffer B (with buffer A = 90% MilliQ, 10% Acetonitrile, 0.1% TFA and buffer B = 90% Acetonitrile, 10% MilliQ, 0.1% TFA). Protein absorption spectra were measured at 215 nm.

MS analysis

Prior to MS analysis, samples were reduced by incubating a solution of 25 µg antibody in 25 µL PBS pH 7.4 with 1 µL 50 U/µL FabRICATOR® for 1 hour at 37 °C. Next, the samples were analysed by electrospray ionization time-of-flight (ESI-TOF) on a JEOL AccuTOF LC-plus JMS-T100LP. Deconvoluted spectra were obtained using Magtran software.

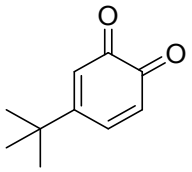
Glycan Remodelling

100 µL 21.1 mg/mL Tras[HC]G_4Y, 3 µL 6.4 mg/mL EndoX (1%), 47 µL 3.33 mg/mL glycosyltransferase (7.5%), 35 µL 0.1 M UDP-sugar **8** (25 eq.), 1.6 µL 1.0 M $MnCl_2$ were mixed and stirred overnight at 30 °C.

Antibody was purified afterwards by using Hitrap protein A HP (protA) column. The protA column was pre-equilibrated with tris buffered saline pH 7.2, remodelled Tras[HC]G_4Y was applied to the column and washed with 10 column volumes of tris buffered saline pH 7.2. Product was eluted with 5 column volumes of 0.1 M glycine buffer pH 2.7, obtained product fractions were diluted with 0.1 volume equivalents of 1.0 Tris buffer pH 9.0. The product fractions were concentrated and buffer exchanged to 50 mM potassium phosphate pH 5.5 containing 150 mM NaCl.

Synthesis

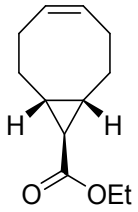
4-*tert*-Butyl-1,2-benzoquinone (**1**)



4-tertbutylcatechol (5.79 g, 34.8 mmol, 1 eq.) was dissolved in 160 mL 1:1 THF/H_2O. 12 mL methanol was added and the sample turned clear. $NaIO_4$ (7.51 g, 35.1 mmol, 1.01 eq.) was added, the reaction turned dark-red and a precipitate appeared. The reaction was stirred for 30 minutes, after which the mixture was transferred to a separatory funnel, where it was extracted with 500 mL DCM twice. The combined organic layers were washed with 250 ml H_2O twice and dried over Na_2SO_4, filtered and concentrated *in vacuo*. **1** was obtained was as a red-brown solid (5.42 gram, 33.0 mmoL, 95%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.19 (dd, J = 10.4, 2.4 Hz, 1H), 6.38 (d, J = 10.4 Hz, 1H), 6.27 (d, J = 2.4 Hz, 1H), 1.23 (s, 9H).

Ethyl (1*R*,4*Z*,8*S*,9*r*)-bicyclo[6.1.0]non-4-ene-9-carboxylate (**9**)



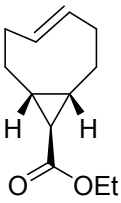
Compound **9** was made according to literature.[2]

Cyclooctadiene (44.1 g, 50 mL, 408 mmol, 8 eq.) and $Rh_2(OAc)_4$ (584 mg, 1.32 mmol, 0.025 eq.) was mixed with DCM (20 mL), to which was added ethyl diazoacetate (5.81 g 87% by wt → 51.0 mmol, 1 eq.) in a solution of DCM (50 mL) dropwise over 3 hours. After which the solution was stirred over the weekend at rt. DCM was evaporated *in vacuo* and cyclooctadiene was removed by filtration over a glass filter filled with silica, elution of product was done with 1:200 EtOAc/n-heptane. The filtrate was concentrated *in vacuo* and purified by flash column chromatography (EtOAc/n-heptane 1:20), to afford **9** (4.88 g, 6.95 mmol) in 14% yield.

^{1}H NMR (400 MHz, $CDCl_3$): δ 5.67–5.60 (m, 2H), 4.10 (q, *J* = 7.2 Hz, 2H), 2.35–2.25 (m, 2H), 2.24–2.15 (m, 2H), 2.13–2.02 (m, 2H), 1.60–1.52 (m, 2H), 1.53–1.43 (m, 2H), 1.25 (t, *J* = 7.2 Hz, 3H), 1.18 (t, *J* = 4.8 Hz, 1H).

NMR is in accordance with literature values.

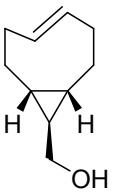
Ethyl (1*R*,4*E*,8*S*,9*r*,*R*p)-bicyclo[6.1.0]non-4-ene-9-carboxylate (cpTCO-CO_2Et) (10)



Isomerization and purification of **9** was performed according to literature.[3]

10 was obtained as a colorless oil (3.50 g, 18.0 mmol 74% yield).

cpTCO-OH (**2**)

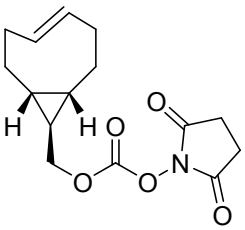


10 (701 mg, 3.61 mmol, 1 eq.) was dissolved in 15 mL dry Et_2O, to which a suspension of $LiAlH_4$ (123 mg, 3.24 mmol, 0.9 eq.) in 12 mL Et_2O in a dropwise fashion. The reaction was stirred at RT and followed by TLC (9:1 H/E). After 30 minutes the reaction was cooled to 0 °C and quenched with 10 mL water. The mixture was extracted with 2 × 25 mL DCM, the combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. After flash column chromatography (9:1 → 8:2 heptane/EtOAc), **2** was obtained as a colourless oil (432.2 mg product, 2.84 mmol, 79% yield).

^{1}H NMR (400 MHz, $CDCl_3$): δ 5.88 (ddd, *J* = 16, 9, 6 Hz, 1H), 5.13 (ddd, *J* = 16, 10, 4 Hz, 1 H), 3.51 (d, *J* = 7 Hz, 2H), 2,42–2,34 (m, 1H), 2.33–2.17 (m, 3H), 1.98–1.87 (m, 2H), 0.93–0.80 (m, 1H), 0.64–0.55 (m, 1H), 0.55–0.45 (m, 1H), 0.45–0.33 (m, 1H).

NMR is in accordance with literature values.[4]

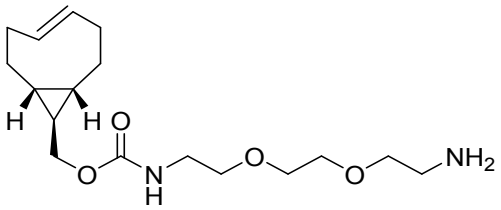
cpTCO-NHS (**11**)



2 (432 mg, 2.84 mmol, 1 eq.) was dissolved in 20 mL dry DCM. To which was added 1.19 mL dry triethylamine (1.2 mL, 3 eq) and disuccinimidyl carbonate (1.46 g, 5.60 mmol, 2 eq). The reaction was stirred overnight at RT, and completion was shown by TLC (3:1 heptane/EtOAc). The reaction was concentrated *in vacuo* and purified by flash column chromatography (3:1 heptane/EtOAc). **11** was obtained as a white solid, 636 mg (2.17 mmol, 76%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 5.85 (ddd, *J* = 16, 9, 6 Hz, 1H), 5.14 (ddd, *J* = 16, 10, 4 Hz, 1H), 4.26–4.14 (m, 2H), 2.84 (s, 5H), 2.46–2.37 (m, 1H), 2.38–2.13 (m, 3H), 1.99–1.87 (m, 2H), 0.92–0.87 (m, 1H), 0.59–0.49 (m, 4H).

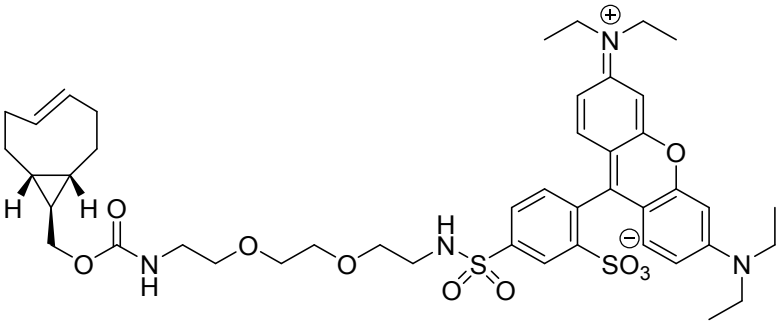
cpTCO-PEG$_2$-NH$_2$ (**12**)



Dissolve **11** (66.2 mg, 0.226 mmol, 1 eq.) in 3 mL dry DCM. Et_3N (94 µL, 2 eq) and 2,2′-(Ethylenedioxy)bis(ethylamine) (303 µL, 334 mg, 2.26 mmol, 10 eq) were added and the mixture was stirred overnight at RT. The reaction was diluted to 10 mL with DCM and extracted with H_2O (5×) to remove free 2,2′-(Ethylenedioxy)bis(ethylamine). The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. Product **12** was obtained as a colourless oil (63.3 mg, 0.194 mmol, 86%).

^{1}H NMR (400 MHz, Methanol-d4): δ 5.86 (ddd, *J* = 16, 9, 6 Hz, 1H), 5.13 (ddd, *J* = 16, 10, 4 Hz, 1H), 3.91 (d, *J* = 6 Hz, 2 H); 3.70 (t, *J* = 6 Hz, 1H), 3.64–3.58 (m, 4H), 3.55–3.49 (m, 2H), 3.47–3.42 (m, 2H); 3.30–3.24 (m, 2H); 2.40–2.30 (m, 1H), 2.29–2.15 (m, 3H), 2.04–1.98 (m, 1H), 1.97–1.85 (m, 3H), 0.96–0.82 (m, 1H), 0.68–0.52 (m, 2H), 0.50–0.38 (m, 2H).

cpTCO-lis (**13**)



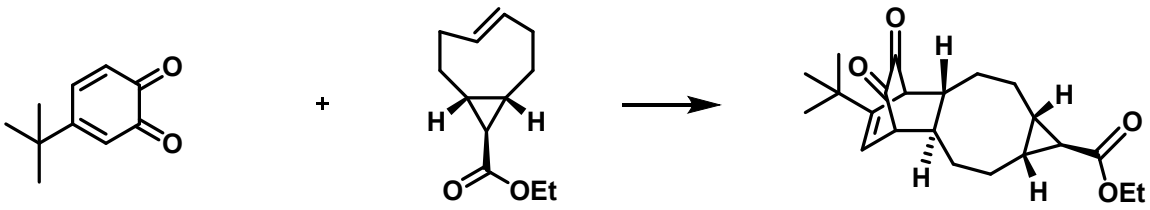
12 (14.9 mg, 0.0458 mmol, 2 eq.) was dissolved in 2 mL dry DCM. Lissamine rhodamine B sulfonylchloride (13.2 mg, 0.0229 mmol, 1 eq) and Et_3N (13 µL, 9.3 mg, 0.092 mmol, 4 eq) were added. The reaction was stirred overnight, concentrated *in vacuo* and purified by flash column chromatography (DCM → 10% MeOH → 25% MeOH). **13** was obtained as a red/pink product (7.8 mg, 9.0 µmol, 39%).

^{1}H NMR (400 MHz, Methanol-d_4) δ 8.67 (d, *J* = 1.8 Hz, 1H), 8.13 (dd, *J* = 8.0, 1.9 Hz, 1H), 7.54–7.46 (m, 1H), 7.13 (d, *J* = 9.5 Hz, 2H), 7.05–6.91 (m, 3H), 6.81 (s, 1H), 5.84 (ddd, *J* = 16, 9, 6 Hz, 1H), 5.10 (ddd, *J* = 16, 10, 4 Hz, 1H), 3.88 (d, *J* = 6.5 Hz, 2H), 3.73–3.49 (m, 15H), 3.32–3.13 (m, 5H), 3.00 (s, 1H), 2.87 (s, 1H), 2.37–2.11 (m, 2H), 1.97–1.82 (m, 3H), 1.38–1.26 (m, 13H), 1.15–1.01 (m, 2H), 0.95–0.79 (m, 2H), 0.61–0.50 (m, 1H), 0.46–0.35 (m, 2H).

MS (ESI) for $C_{44}H_{58}N_4O_{10}S_2$ $[M + Na]^+$: calcd 889.35, found 889.35

NMR analysis of the cpTCO-SPOCQ reaction

Experimental



cpTCO-CO_2Et (**10**) was dissolved in $CDCl_3$ (10.0 mg/mL, 51.5 mM), *tert*-butyl quinone (**1**) was dissolved in $CDCl_3$ (8.45 mg/mL, 51.5 mM). The samples were mixed 1:1 and measured with NMR (Figure S3).

Results and discussion

Though only one product is shown in the reaction scheme, there are a multitude of different products possible. This is due to the regional isomers of *tert*-butyl, the two isomers of the *trans*-oriented protons located at the newly formed fused carbon-carbons bonds, and the cpTCO's inherent stereocentres (which can point up or down), illustrated in Figure S2. This would lead to 8 different products.

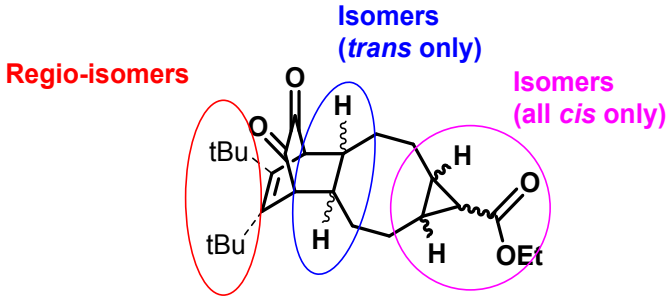


Figure S2. Identification of isomeric groups of the product.

Analysis of the alkene proton next to the *tert*-butyl group indicates that only 4 different products are formed in a ratio of **1:1:2.7:3.6** (Figure S4); δ 6.13 (dd, *J* = 6.9, 2.2 Hz), 6.05 (dd, *J* = 6.9, 2.2 Hz), 5.98 (dd, *J* = 6.5, 1.8 Hz), 5.95 (dd, *J* = 6.5, 1.1 Hz).
It is feasibly however, that the cpTCO's inherent stereocentres (as indicated in purple) do not effect this proton. The most obvious protons to exhibit different chemical shifts for all 8 isomers, are the *trans*-oriented protons (as indicated in blue), these are located at 2-2.7 ppm. However, if 8 different products are present, this would result in up to 16 different proton signals. No 16 unique protons were found in that area (Figure S5).

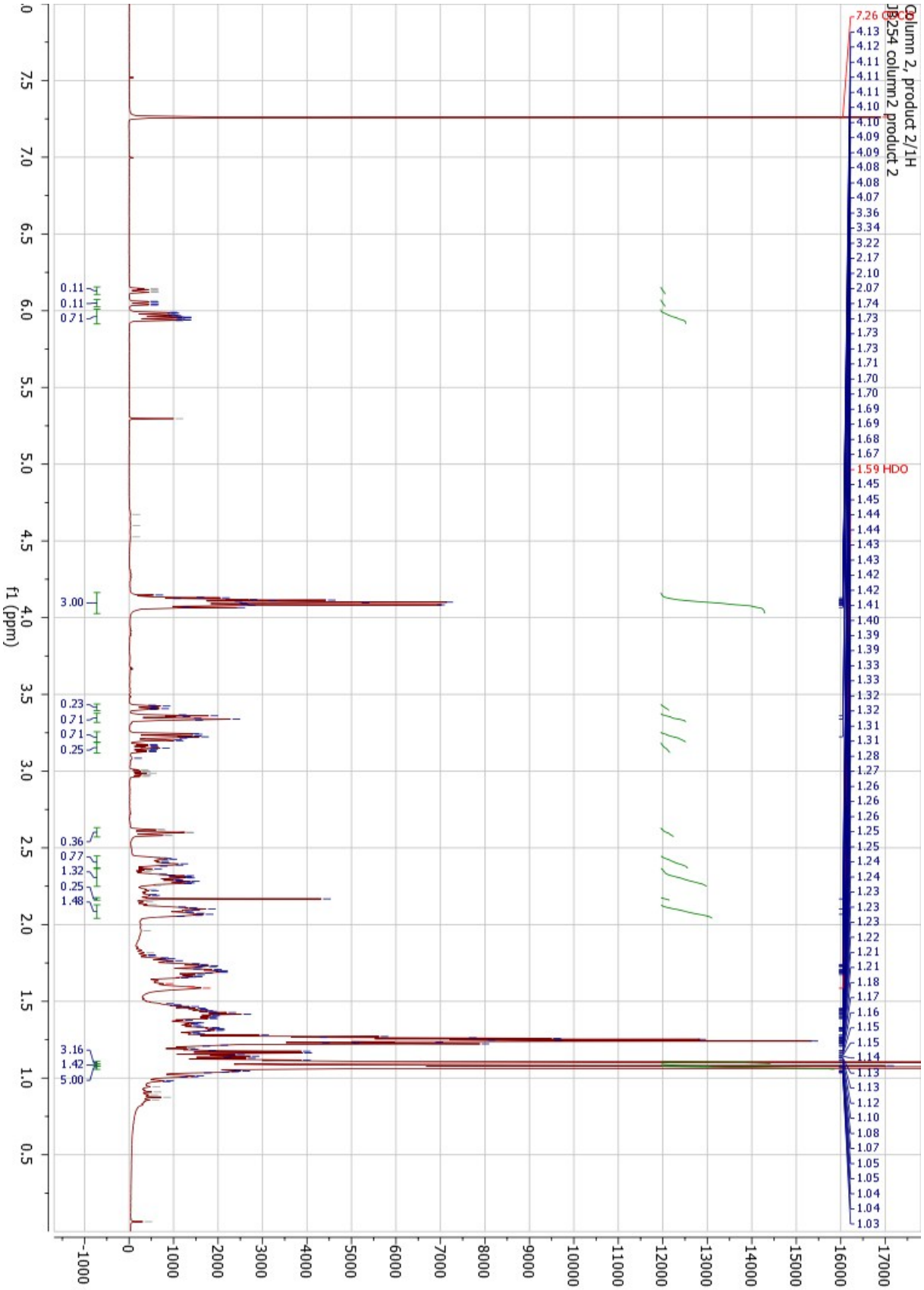


Figure S3. Full [1]H-NMR spectrum of the product.

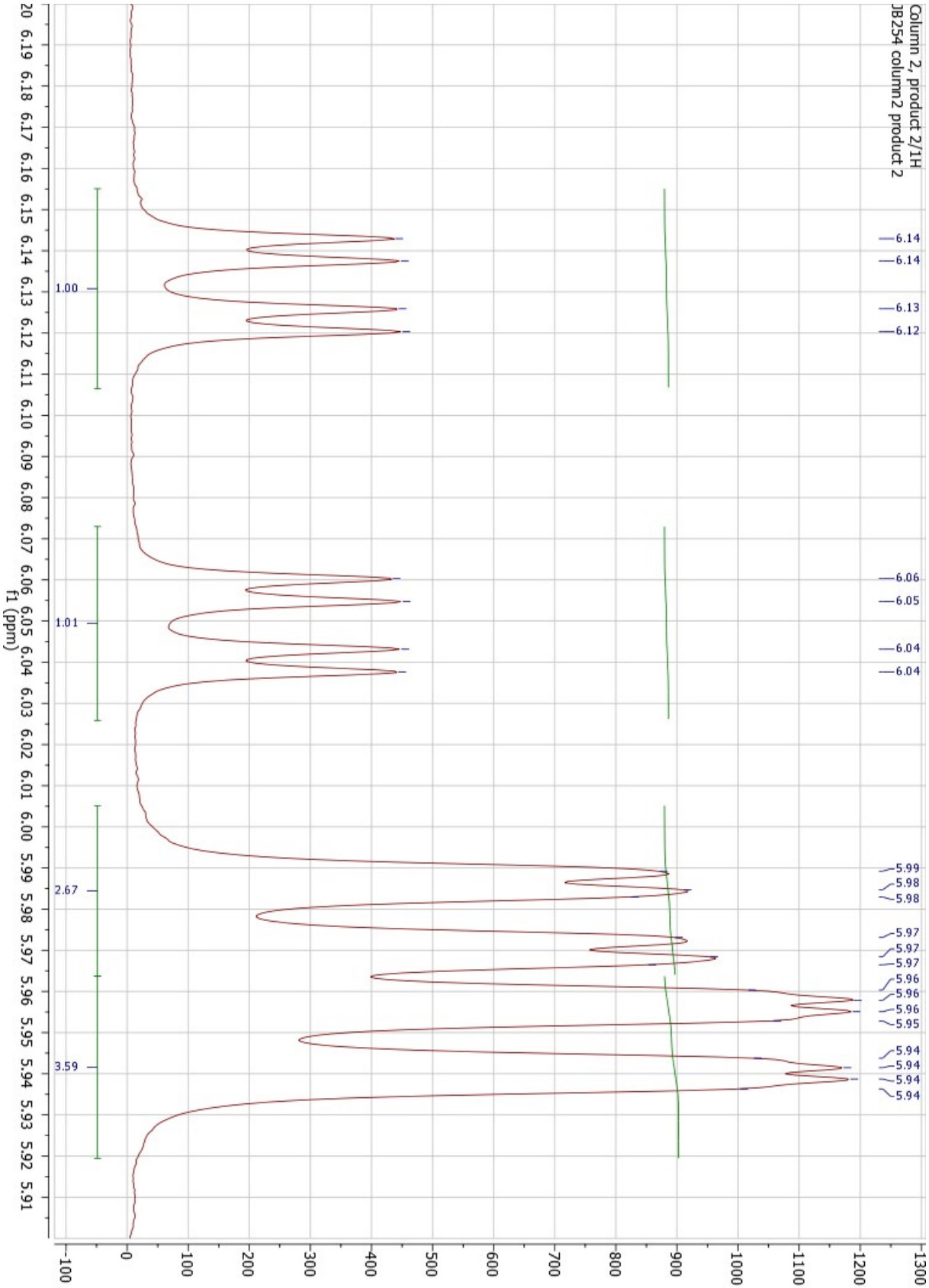


Figure S4. [1]H-NMR spectrum, zoomed in at 6.20–5.90 ppm.

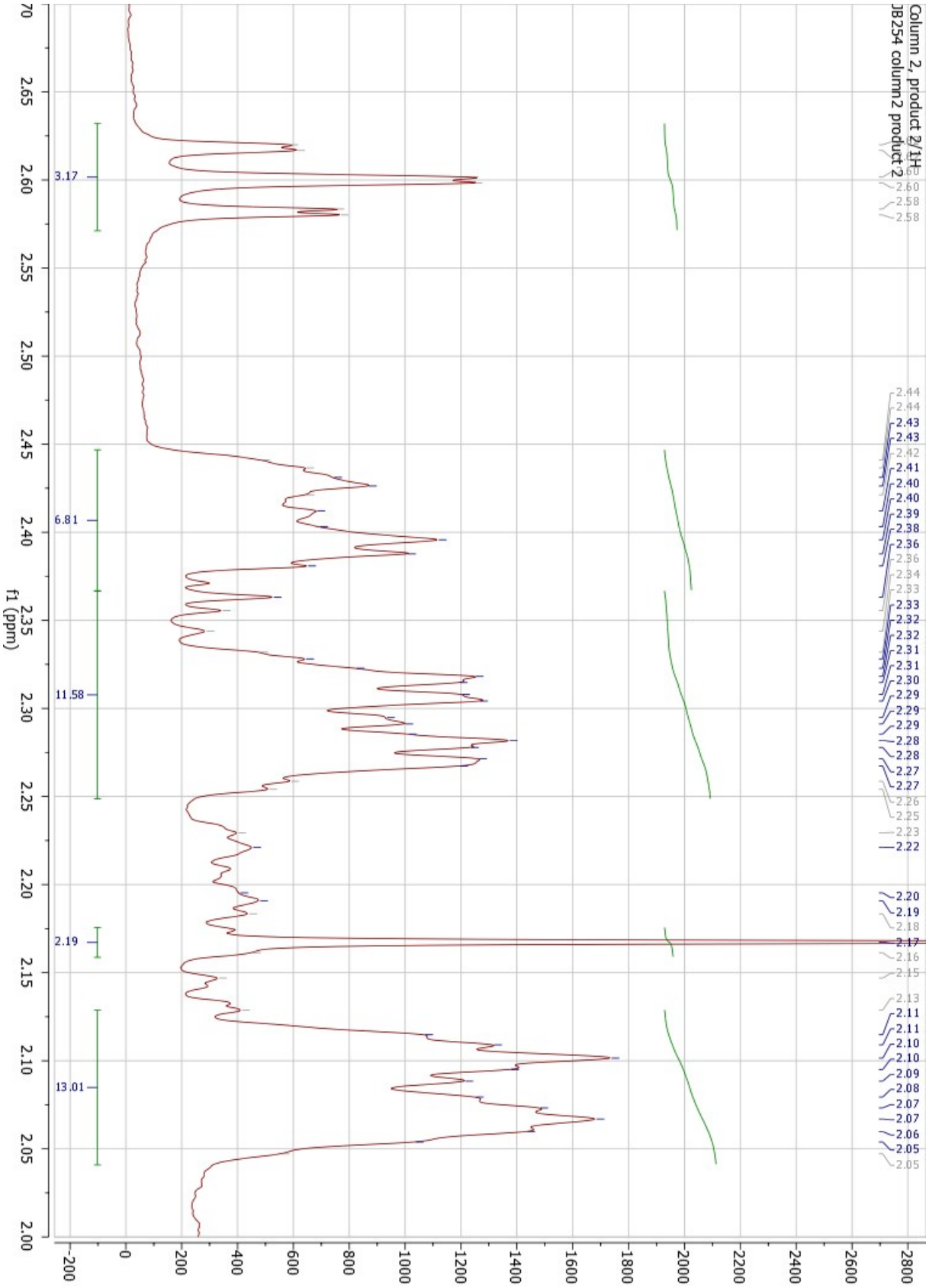


Figure S5. [1]H-NMR spectrum, zoomed in at 2.70–2.00 ppm

SDS-PAGE gels

cpTCO-SPOCQ time-resolved gel.

Reaction was performed according to ‘General SPOCQ method’.

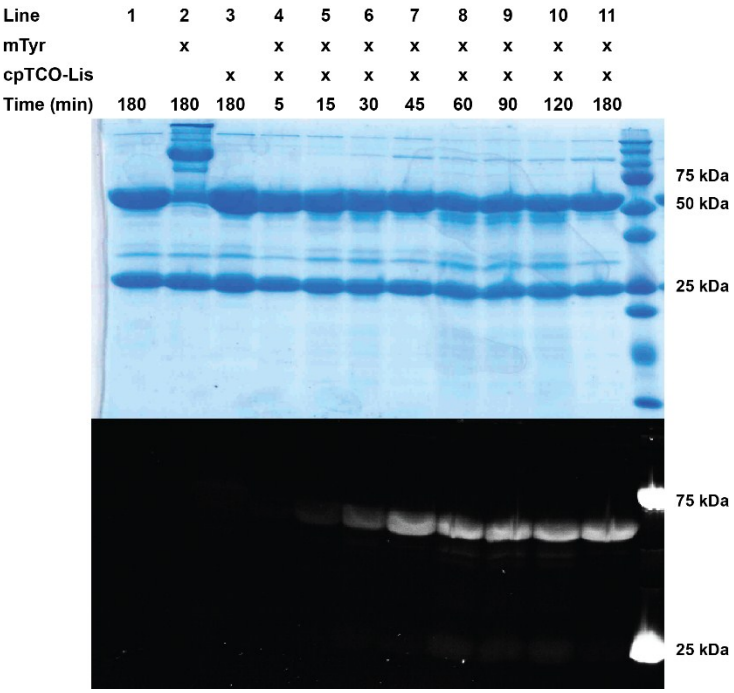


Figure S6. Full SDS-PAGE picture of time-resolved SPOCQ reaction on Tras[HC]G_4Y with cpTCO-Lis (Fig. 2 in manuscript).

Optimization SPOCQ conditions by using N-terminal SPOCQ.

To give a rough estimation on the amount of side reactions, N-terminal SPOCQ on MYG_4-bearing trastuzumab was performed (antibody abbreviated as MYG_4[LC]Tras) (Figure S7). Without the use of any strained alkynes/alkenes, the N-terminal quinone will readily react with other nucleophiles, such as the N-terminus of the heavy chain. A slower click reaction would be less capable of ‘trapping’ the intermediate quinone, and would result in more side-reactions. This would be visible by increased presence of bands at 75 kDa on SDS-PAGE gel. As an alternative, side-reactions can be suppressed by lowering the reaction rate of the Michael addition of *e.g.* amines to said quinones by lowering pH, temperature etc.

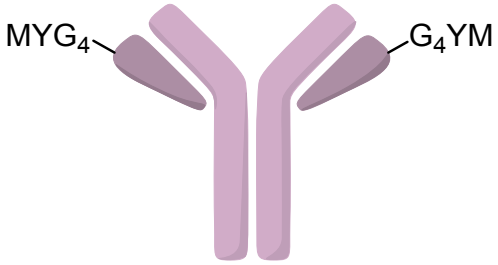


Figure S7. Schematic representation of MYG_4[LC]Tras.

1.6 µL of a 29.8 mg/mL MYG_4[LC]Tras solution was taken for each reaction, 0.2 µL 10 mg/mL BCN-lissamine was added, finally 0.2 µL 3.0 mg/mL mTyr was added, and the reaction was set to react overnight at various temperatures and pH values (Figure S8).

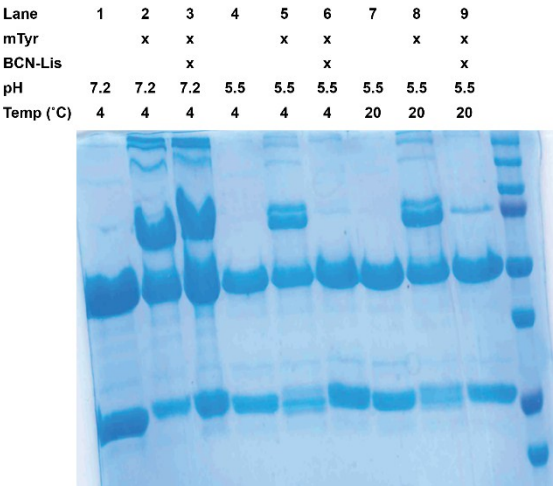


Figure S8. Effect of different pH values and temperatures on SPOCQ and its side-reactions.

Side-reactions of quinones can occur due to Michael addition of nucleophilic amino acids on the proteins,[6] which in the case of trastuzumab are only lysine, histidine and the N-termini. In lanes 2, 5 and 8 (where we only add mTyr) we can see a large band at 75 kDa. Which can be interpreted as the coupling of the heavy chain to the quinone-bearing light chain, via its N-terminus or any lysine or histidine residue. At pH 7, this effect greatly hampers product yield (lane 3). At lower pH, this effect is greatly reduced (lane 9), and can be further reduced by cooling down the sample (lane 6). Formation of higher mass units is due to oligomerization / polymerization of the reactive quinone-bearing light chains. It is noteworthy that lower pH and temperature would lower the oxidation rate of tyrosine into quinones by mushroom tyrosinase,[1] but a higher yield / purity can be expected.

Ideal conditions are at pH 5.5 and at 4 °C.

Comparing BCN-Lissamine and cpTCO-lissamine conjugation on N-terminal SPOCQ

1 µL of a 29.8 mg/mL MYG_4[LC]Tras solution in PBS was taken for each reaction, 0.2 µL of 10 mg/mL cpTCO-lissamine or 10 mg/mL BCN-lissamine was added, the sample was diluted to 1.8 µL with PBS for reactions to which mTyr would be added, or 2.0 µL for non-mTyr reactions. Finally, 0.2 µL 10 mg/mL mTyr was added, and the reaction was set to react overnight at 4 °C. Dilute to 5 µL with PBS, add 5 µL 19:1 SB/BME. Denature at 95 °C for 5 minutes, run SDS-PAGE gel.

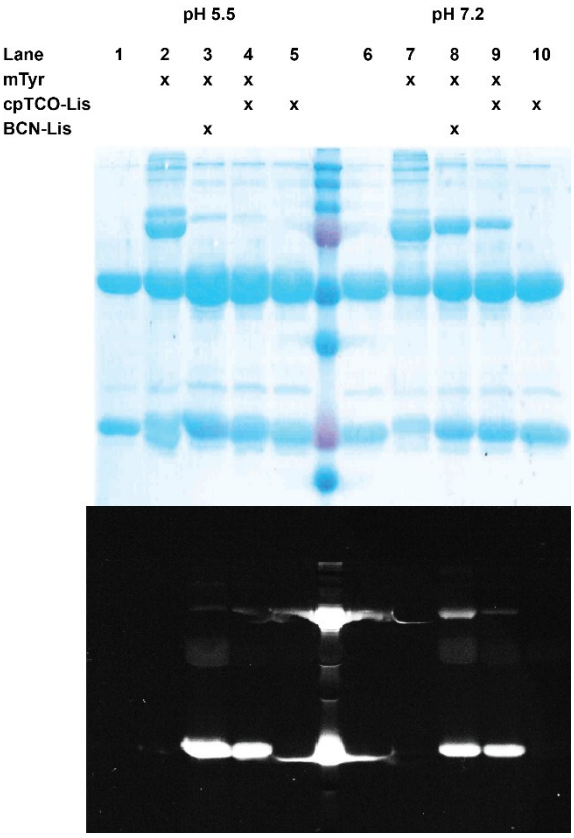


Figure S9. Comparison of BCN-lissamine and cpTCO-lissamine SPOCQ on MYG$_4$[LC]Tras.

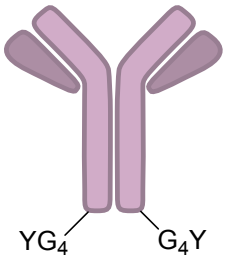
Considering the nature of the side-reactions (see '*Optimization SPOCQ conditions by using N-terminal SPOCQ*'), it can be rationalized that faster reacting probes will reduce the lifetime of the formed quinones, and in turn reduce the time these side-reactions can occur. This means; higher reaction rate results into less side products. If we compare BCN-lissamine with cpTCO-lissamine at pH 5.5 (lane 3 and 4) and pH 7.2 (lane 8 and 9), we clearly see less side products for the cpTCO-lissamine SPOCQ.

HPLC Measurements (Single Labelling)

Sample preparation (BCN and cpTCO yield determination)

Prior to HPLC analysis, samples were reduced by incubating a solution of 10 µg antibody in 30 µL of a 67 mM DTT solution in 0.1 M Tris pH 8.0 for 30 minutes at 37 °C. Reverse phase HPLC was performed on a Agilent 1220 HPLC Infinity using a Thermo Scientific™ MAbPac™ RP column (3.0 x 50 mm, 4 µm) column run at 0.6 mL/min at 80 °C using a 10 minute linear gradient from 20% to 45% buffer B (with buffer A = 95% MilliQ, 5% Acetonitrile, 0.1% TFA and buffer B = 95% Acetonitrile, 5% MilliQ, 0.1% TFA). Protein absorption spectra were measured at 215 nm (Figure S10).

Tras[HC]G_4Y HPLC



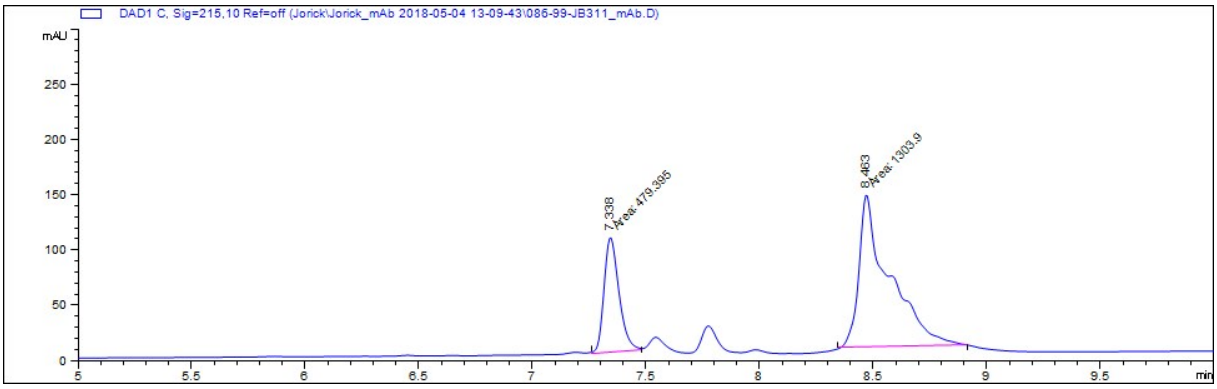


Figure S10. HPLC measurement of Tras[HC]G_4Y without glycan remodelling.

The two main peaks correspond to light chain (7.34 min) and heavy chain (8.4–8.9 min), based on comparison of surface areas, 479 versus 1304 (or 27 versus 73 percent), to the expected absorption values by calculation of the amino acid sequence:

[LC]

DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPS
VFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

[HC]

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYW
GQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI
EKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS
PGKGGGGY

A molar absorption at 280 nm is calculated to be 25900 M^{-1} cm^{-1} for light chain and 82280 M^{-1} cm^{-1} for heavy chain. Peak broadening of heavy chain can be rationalized by glycan heterogeneity. Two unknown minor peaks at 7.55 and 7.8 can also be observed.

Tras[HC]G_4Y after SPOCQ with BCN-lissamine

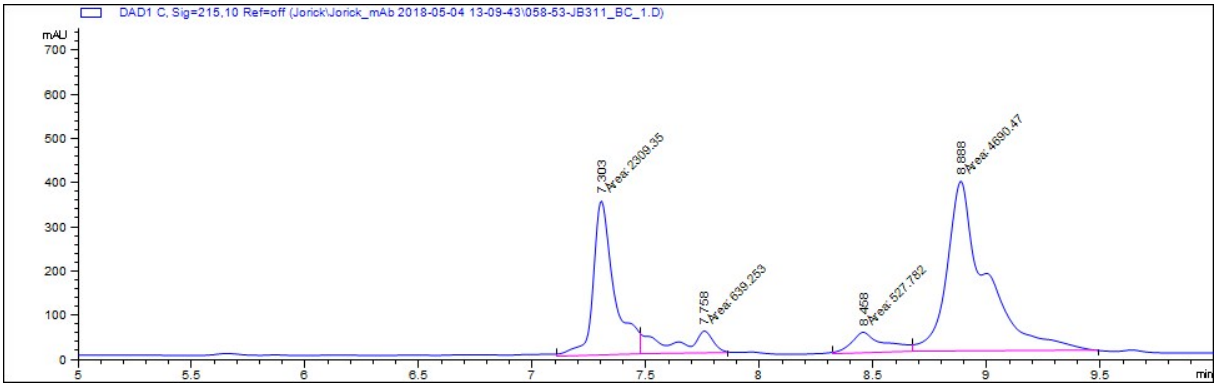


Figure S11. HPLC measurement of Tras[HC]G_4Y without glycan remodelling after SPOCQ with BCN-lissamine.

After SPOCQ with BCN-lissamine, most of the heavy chain has shifted to a later retention time (8.7–9.2 minutes, Fig. S11). Light chain versus heavy chain is 28 versus 72 percent, which corresponds to the values observed in the starting material. A yield of about 90% may be calculated based on relative peak areas of the peaks corresponding to the HC's.

Tras[HC]G_4Y after SPOCQ with cpTCO-lissamine

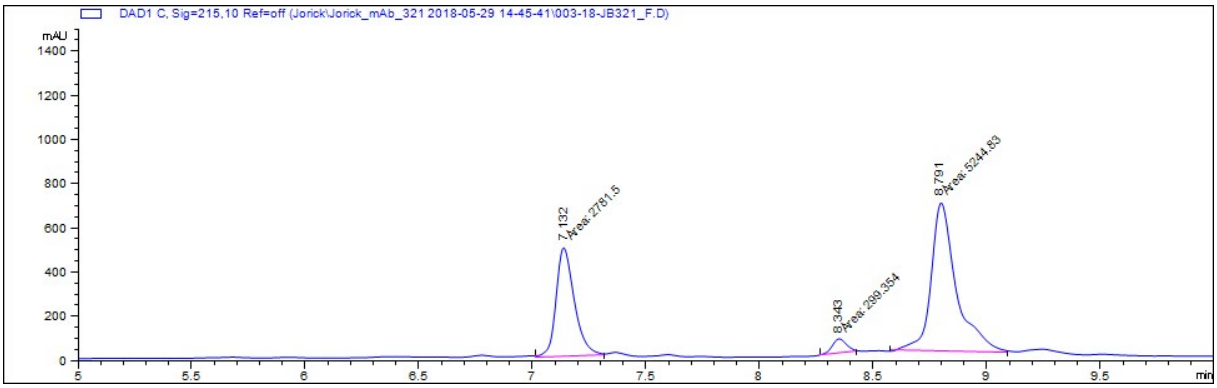


Figure S12. HPLC measurement of Tras[HC]G_4Y without glycan remodelling after SPOCQ with cpTCO-lissamine.

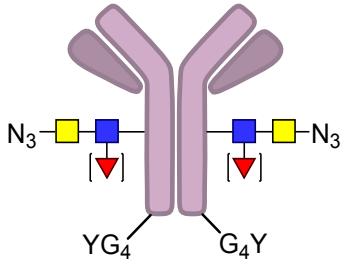
As with the BCN-SPOCQ, a shift of heavy chain retention time is observed (Fig. S12). Based on relative peak areas of the peaks corresponding to the HC's, a yield of 95% is calculated.

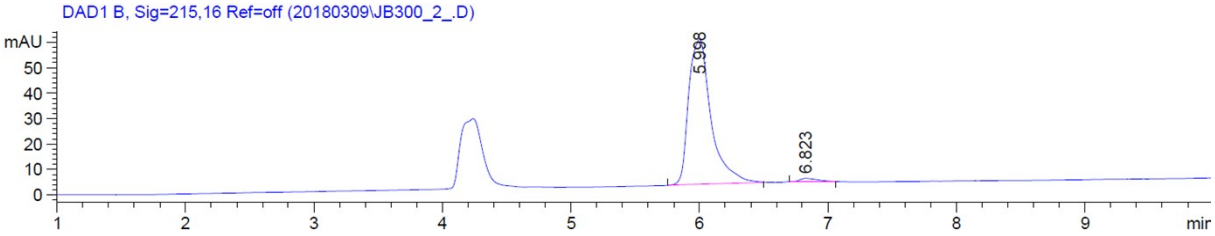
HPLC Measurements (Dual labelling)

Sample preparation

Prior to HPLC analysis, samples were reduced by incubating a solution of 10 µg antibody in 50 µL of a 10 mM DTT solution in 0.1 M Tris pH 8.0 for 15 minutes at 37 °C. Reverse phase HPLC was performed on a Agilent 1100 HPLC using a ZORBAX Poroshell 300SB-C8 (1 x 75 mm, 5 µm) column run at 0.8 ml/min at 70 °C using a 16.8 minute linear gradient from 20% to 55% buffer B (with buffer A = 90% MilliQ, 10% Acetonitrile, 0.1% TFA and buffer B = 90% Acetonitrile, 10% MilliQ, 0.1% TFA). Protein absorption spectra were measured at 215 nm.

HPLC analysis Glycan remodelled Tras[HC]G_4Y





Signal 2: DAD1 B, Sig=215,16 Ref=off

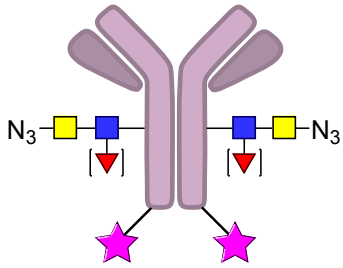
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.998	BB	0.1880	695.78778	57.18351	97.8292
2	6.823	BB	0.1562	15.43900	1.42700	2.1708
Totals :				711.22679	58.61050	

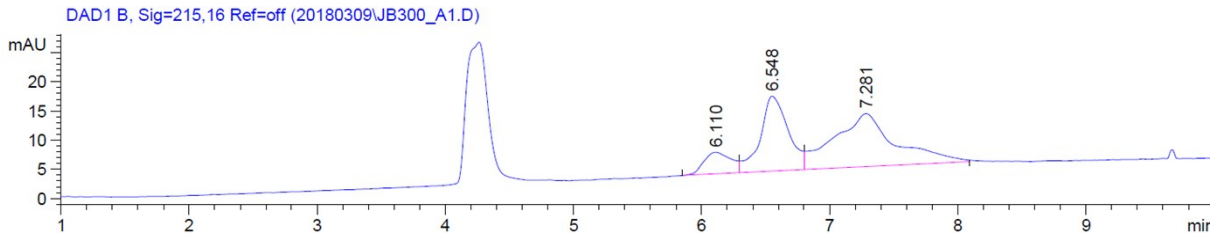
Figure S13. Schematic representation of glycan remodelled Tras[HC]G_4Y, and it's HPLC spectrum with peak integration (heavy chain only).

HPLC analysis conjugation method A

In Method A, first SPOCQ with compound **1** was performed. Subsequently, SPAAC with compound **7** was done.

HPLC of SPOCQ reaction product:





Signal 2: DAD1 B, Sig=215,16 Ref=off

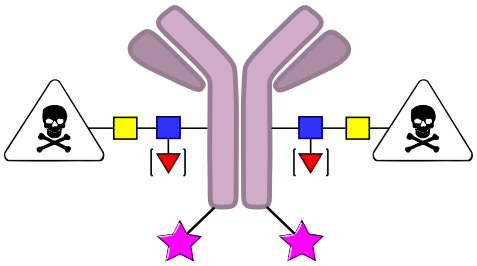
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	6.110	BV	0.2060	55.99022	3.75408	9.9915
2	6.548	VV	0.2300	197.14027	12.91893	35.1799
3	7.281	VB	0.4366	307.24786	9.12960	54.8286
Totals :				560.37836	25.80261	

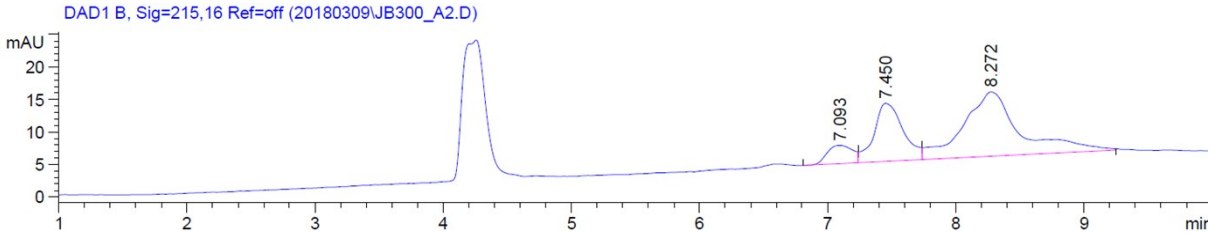
Figure S14. Schematic representation of glycan remodelled Tras[HC]G_4Y after C-terminal SPOCQ with cpTCO-lissamine, and it's HPLC spectrum with peak integration (heavy chain only).

The peak at 6.110 corresponds to heavy chain antibody fragments which do not have cpTCO-Lissamine conjugated to it, as they have almost the same retention time as the starting material (Figure S13). The other peaks, which corresponds to 90% surface area, corresponds to the heavy chain antibody fragments after SPOCQ reaction. Multiple peaks are most likely due to the different regio- and stereoisomers that are formed during the reaction (see: 'NMR analysis cpTCO-SPOCQ').

90% conversion of cpTCO-SPOCQ is observed.

HPLC of subsequent SPAAC reaction product:





Signal 2: DAD1 B, Sig=215,16 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.093	PV	0.2152	39.49880	2.82298	8.0579
2	7.450	VV	0.2387	141.70636	8.94885	28.9085
3	8.272	VB	0.4097	308.98380	9.91050	63.0336
Totals :				490.18895	21.68233	

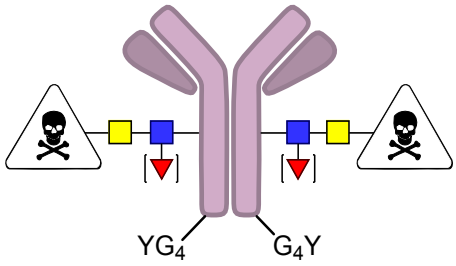
Figure S15. Schematic representation of glycan remodelled Tras[HC]G_4Y after C-terminal SPOCQ with cpTCO-lissamine and subsequent SPAAC with BCN-MMAE, and it's HPLC spectrum with peak integration (heavy chain only).

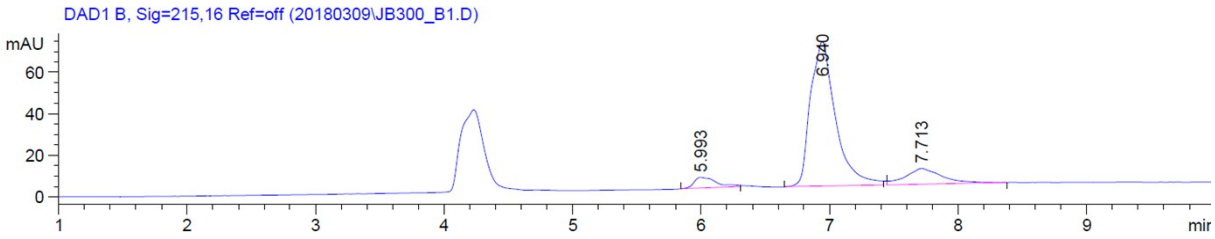
When performing subsequent SPAAC with the highly hydrophobic BCN-MMAE compound, a noticeable shift in retention time was observed, indicating most of the starting material is converted. The pattern of peaks is nearly identical as to the starting material (Figure S14).

HPLC analysis conjugation method B

In Method B, first SPAAC with compound **7** was performed. Subsequently, SPOCQ with compound **1** was done.

HPLC of SPAAC reaction product:





Signal 2: DAD1 B, Sig=215,16 Ref=off

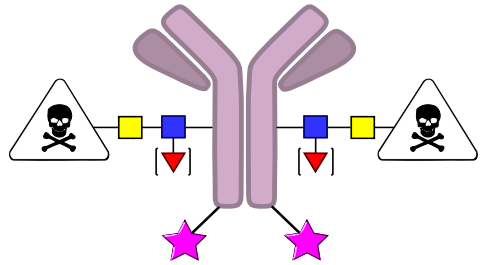
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	5.993	BB	0.1649	63.61626	5.19527	5.4760
2	6.940	PB	0.1821	944.76532	69.72007	81.3247
3	7.713	BB	0.2555	153.33818	7.66240	13.1992

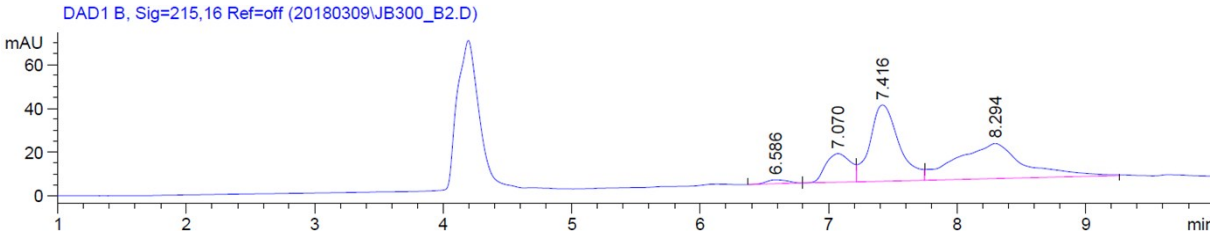
Figure S16. Schematic representation of glycan remodelled Tras[HC]G_4Y after SPAAC with BCN-MMAE, and it's HPLC spectrum with peak integration (heavy chain only).

The peak at 5.993 min corresponds to heavy chain antibody fragments that do not have BCN-MMAE conjugated to it (Figure S13). The other two peaks, which corresponds to 95% surface area, correspond to the heavy chain antibody fragments after SPAAC reaction.

95% conversion of BCN-SPAAC is observed.

HPLC of subsequent SPOCQ reaction product:





Signal 2: DAD1 B, Sig=215,16 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	6.586	BV	0.1739	23.27377	1.94138	1.7297
2	7.070	VV	0.2194	180.52756	13.21194	13.4170
3	7.416	VV	0.2362	551.59589	34.92854	40.9953

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
4	8.294	VB	0.4817	590.11194	16.09604	43.8579

Figure S17. Schematic representation of glycan remodelled Tras[HC]G_4Y after SPAAC with BCN-MMAE and subsequent C-terminal SPOCQ with cpTCO-lissamine, and it's HPLC spectrum with peak integration (heavy chain only).

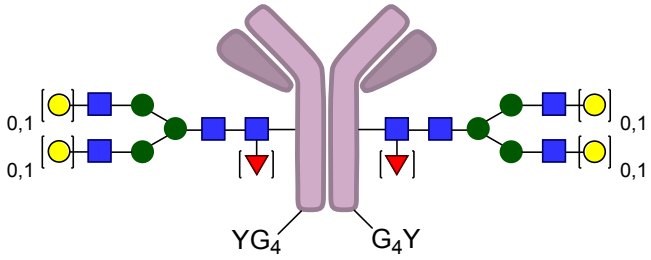
The HPLC-spectrum is near identical to the sample where SPOCQ was done prior to SPAAC (Figure S17). Indicating the order in which the reaction was performed does not affect the product.

MS measurements

Sample preparation

Prior to MS analysis, samples were reduced by incubating a solution of 25 µg antibody in 25 µL PBS pH 7.4 with 1 µL 50 U/µL FabRICATOR® for 1 hour at 37 °C. Next, the samples were analysed by electrospray ionization time-of-flight (ESI-TOF) on a JEOL AccuTOF LC-plus JMS-T100LP. Deconvoluted spectra were obtained using Magtran software.

Tras[HC]G_4Y



Full MS spectra:

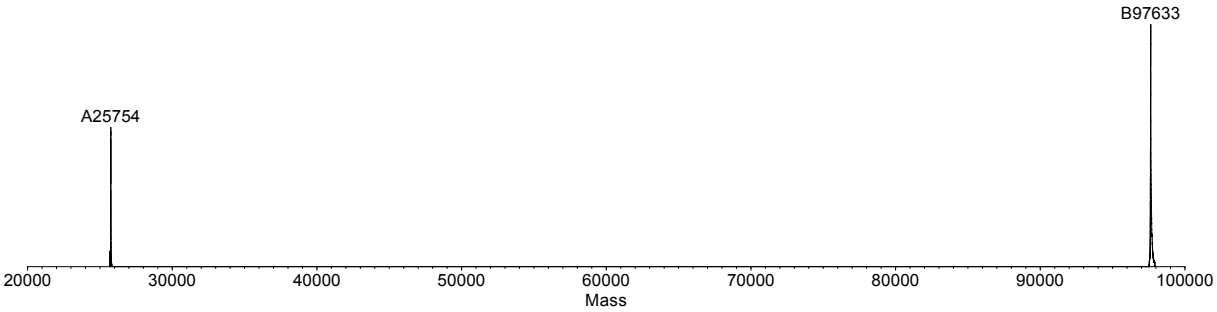


Figure S18. Full MS-spectrum of Tras[HC]G_4Y after fabricator treatment.

FAB'2 fragment calculated mass: **97629**
FC fragment calculated mass (Non glycosylated): **24306**
With glycan profile:

Table S1. Glycan profile and it's corresponding masses of fabricator-treated Tras[HC]G_4Y

Glycan profile	Glycan mass	HC mass
G0	1316.5	25605
G0F	1462.5	25751
G1	1478.5	25767
G1F	1624.6	25913
G2	1640.6	25929
G2F	1786.7	26075

The main peak seems to belong to the G0F glycan.

MS FC_only:

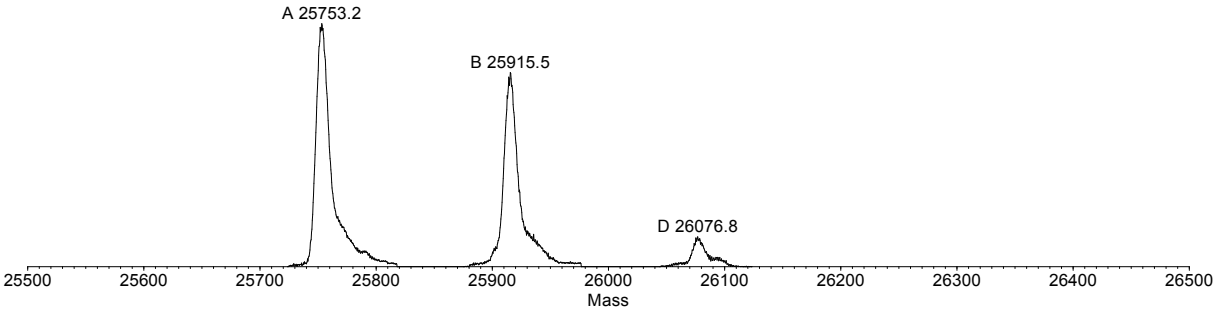


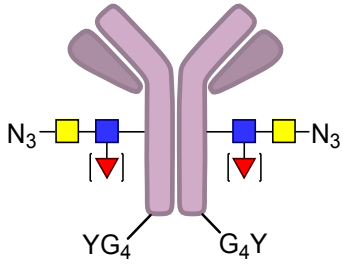
Figure S19. MS-spectrum of FC fragment of Tras[HC]G_4Y after fabricator treatment.

Table S2. Glycan profile and it's corresponding masses of fabricator-treated Tras[HC]G_4Y, compared to measured masses.

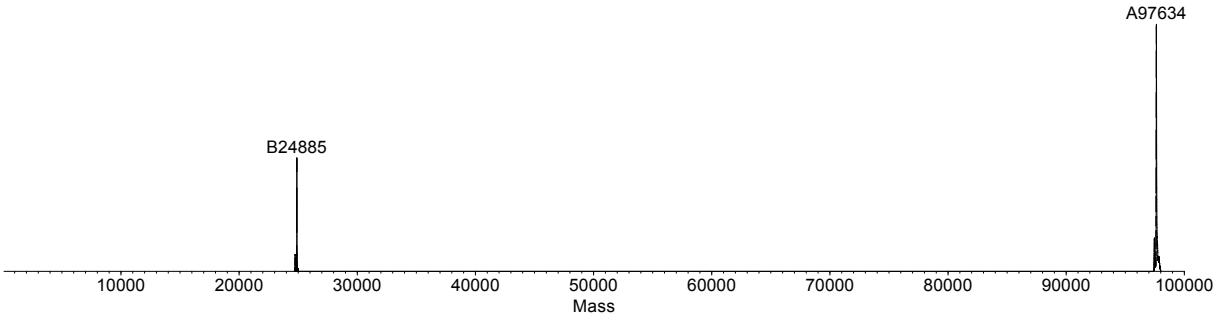
Glycan profile	**Calculated**	**Found**
G0F	25751	25753
G1F	25913	25916
G2F	26075	27077

Data seems in accordance with calculated value, with a consistent +2 Da deviation.

Tras[HC]G_4Y after glycan remodelling



Full MS spectra:



FC Fragment only:

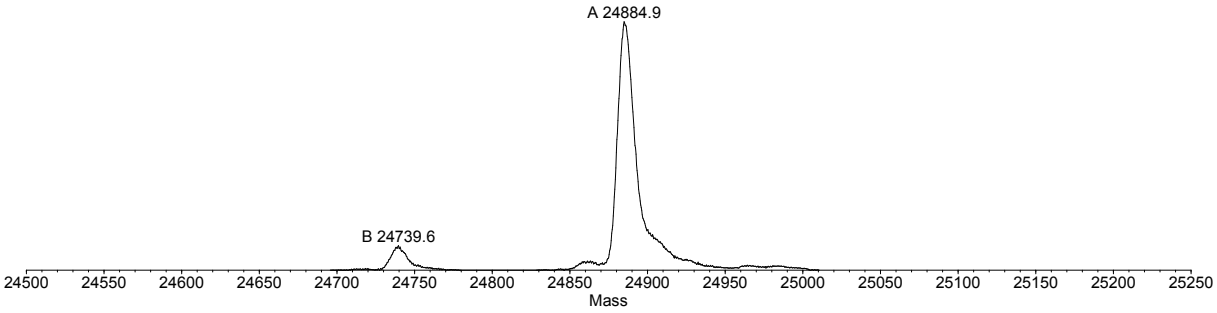


Figure S20. MS-spectrum of Tras[HC]G_4Y after glycan remodelling and subsequent fabricator treatment.

Table S3. Glycan profile and it's corresponding masses of fabricator-treated Tras[HC]G_4Y after glycan remodelling, compared to measured masses. Note: -18 Da has been calculated in product mass due to loss of water in the formation of the glycosidic bond.

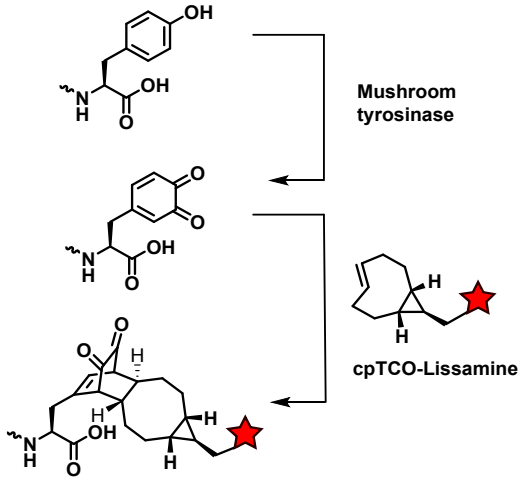
Glycan profile	Protein mass	+ glycan	Calculated Mass	Found
A	24510	246.2	24738	24740
A_F	24656	246.2	24884	24885

Data seems in accordance with calculated value, with a +1–2 Da deviation.

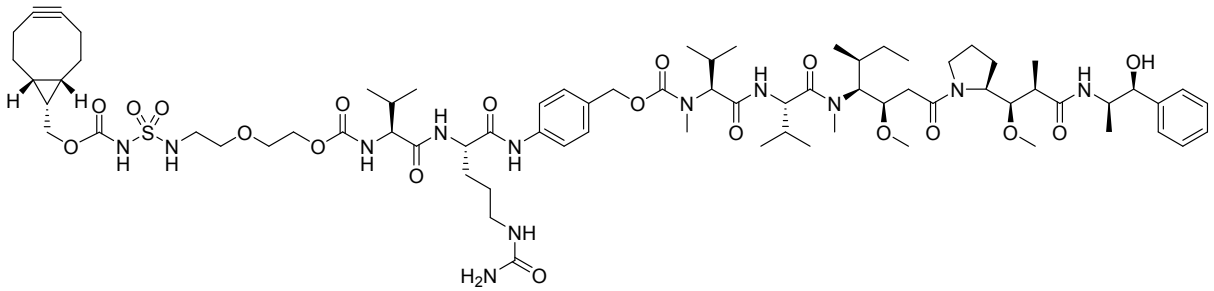
Conjugation masses

SPOCQ with cpTCO: Oxidation to quinone yields a +14 Da increase in mass, cycloaddition with cpTCO-lissamine yields another 867 Da. SPOCQ with cpTCO yields a total of +881 Da. SPAAC with BCN-MMAE yields an increase in mass of 1509 Da. However, in-line fragmentation near the para-amino benzyl alcohol is an occurrence in ESI-TOF; this splits off a 718 Da fragment and CO_2, only yielding an increase 747 Da.

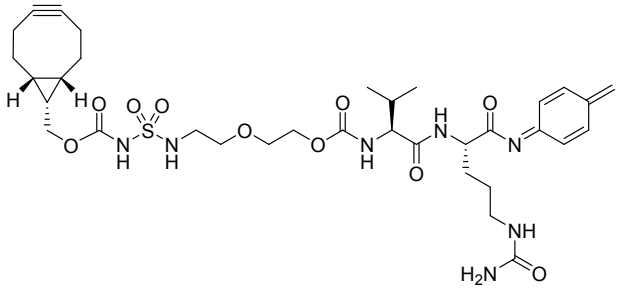
SPOCQ and SPAAC yield a total of 2390 Da, with



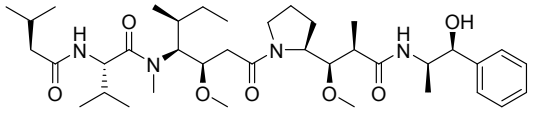
Scheme S1. Reaction scheme of the SPOCQ reaction with cpTCO (1-pot reaction).



Exact Mass: 1508.82
Molecular Weight: 1509.87



Exact Mass: 747.33
Molecular Weight: 747.87

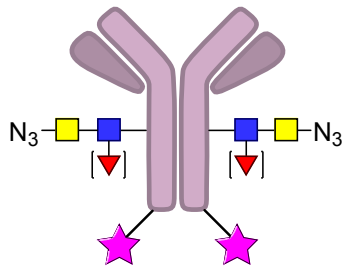


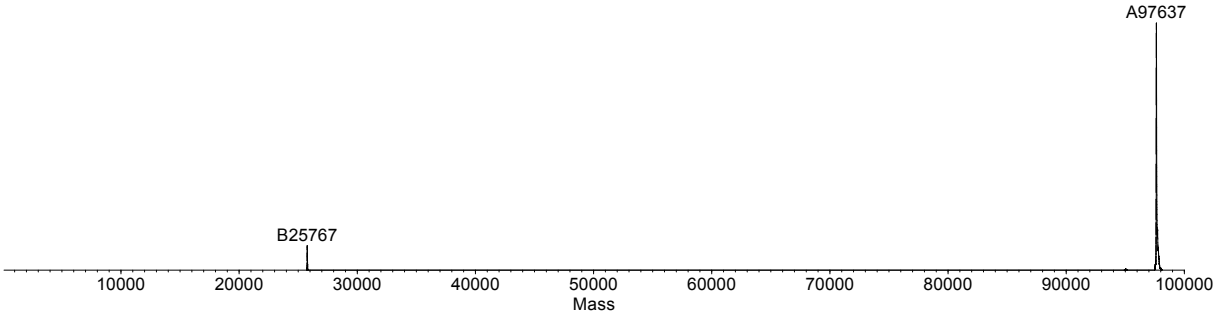
Exact Mass: 688.48
Molecular Weight: 688.95

*Figure S21. BCN-MMAE compound **7** and its fragmentation pattern in ESI-TOF.*

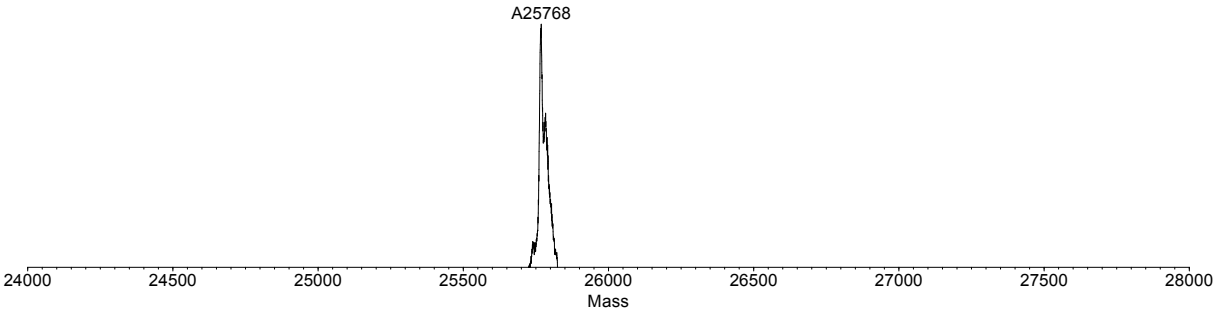
Method A: SPOCQ with cpTCO on Tras[HC]G_4Y, followed by SPAAC

MS of SPOCQ reaction product, Full MS spectrum:





FC_only:



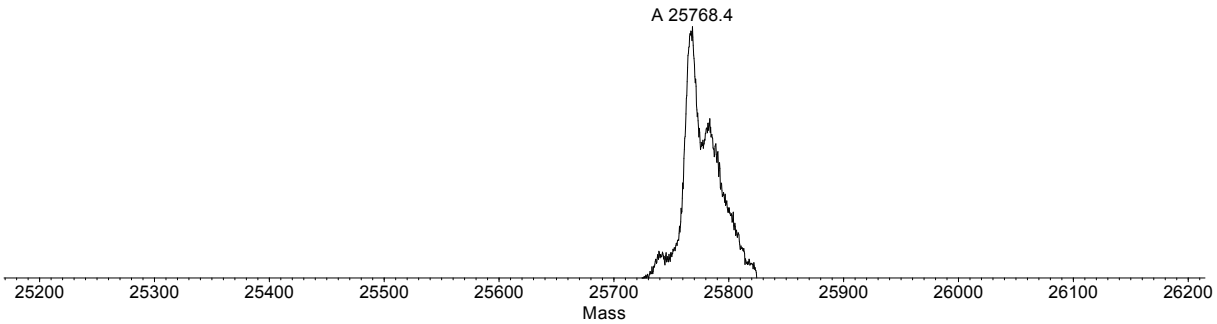
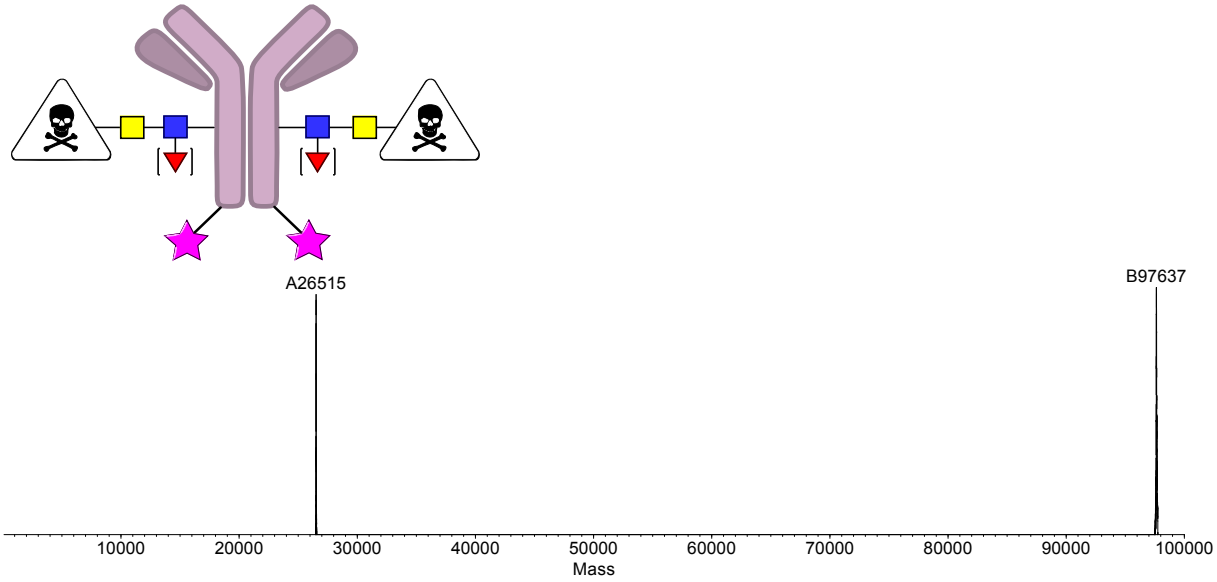


Figure S22. MS-spectrum of FC fragment of Tras[HC]G_4Y after glycan remodelling, SPOCQ reaction and subsequent fabricator treatment.

MS of subsequent SPAAC reaction product, Full MS:



FC_only:

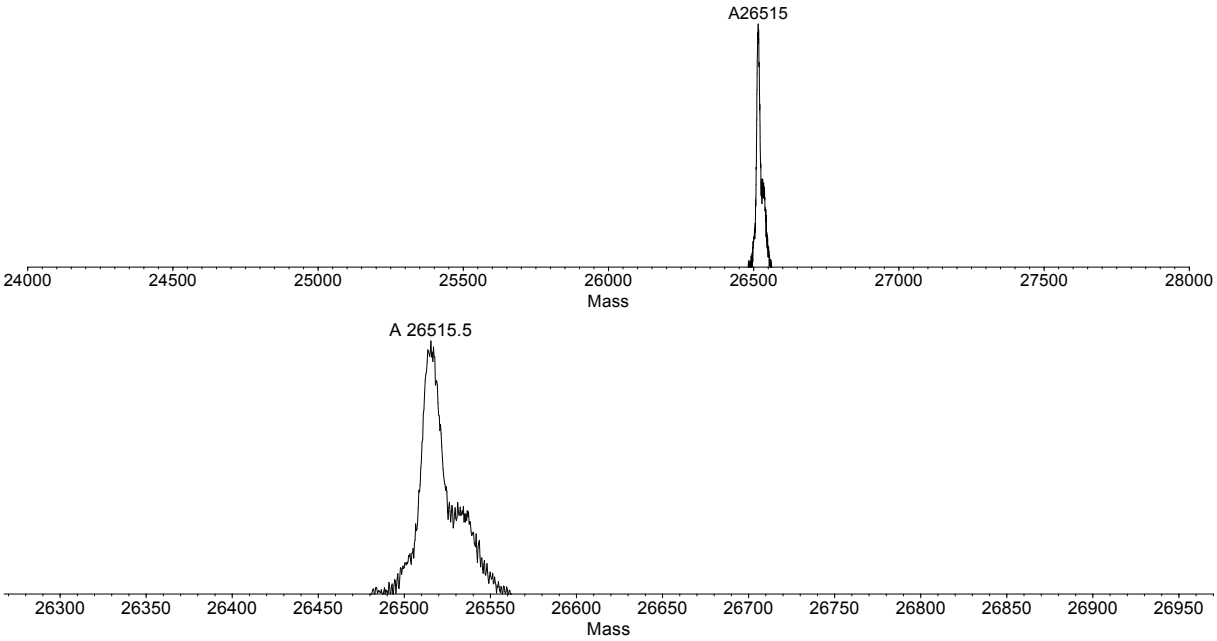


Figure S23. MS-spectrum of FC fragment of Tras[HC]G_4Y after glycan remodelling, SPOCQ reaction, then SPAAC reaction and subsequent fabricator treatment.

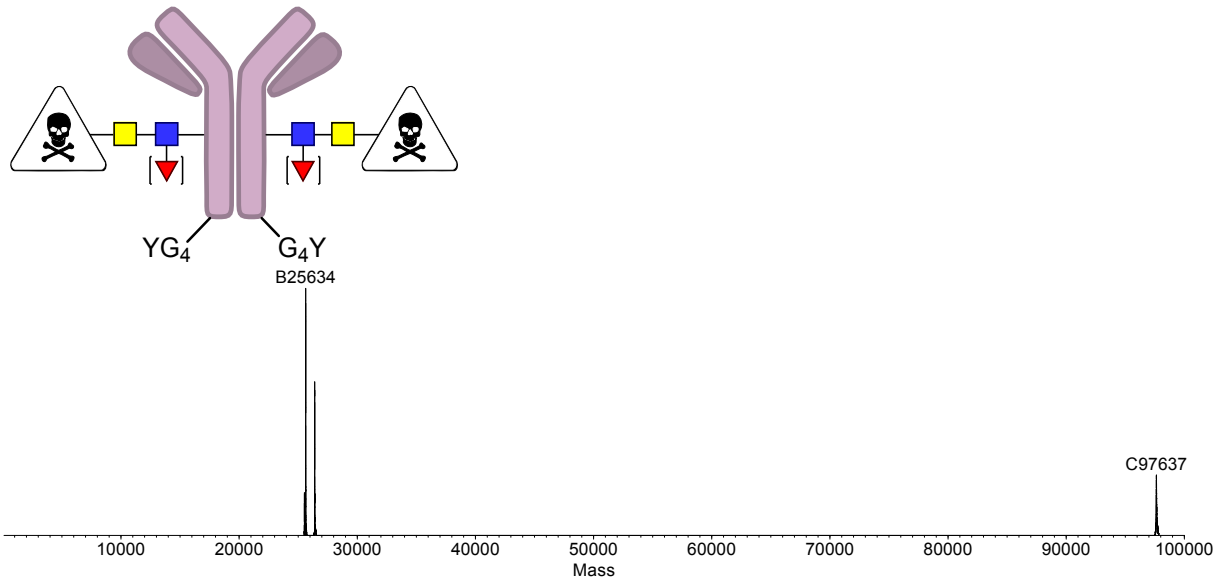
Table S4. Calculated and measured masses of fabricator-treated Tras[HC]G_4Y after glycan remodelling, SPOCQ reaction, then SPAAC reaction and subsequent fabricator treatment.

Mass	**Tras[HC]G_4Y**	**SPOCQ**	**SPAAC**	**SPAAC (fragmented label)**
Calculated (Da)	24884	25766	27275	26514
Measured (Da)	24885	25768	NA	26515

Correct mass was found after SPOCQ, with a deviation of +2 Da. SPAAC yielded the correct mass as well, with a deviation of +1 Da, but only the fragmented MMAE was found.

Method B: SPAAC on Tras[HC]G_4Y, followed by SPOCQ with cpTCO

MS of SPAAC reaction product, Full MS:



FC_only

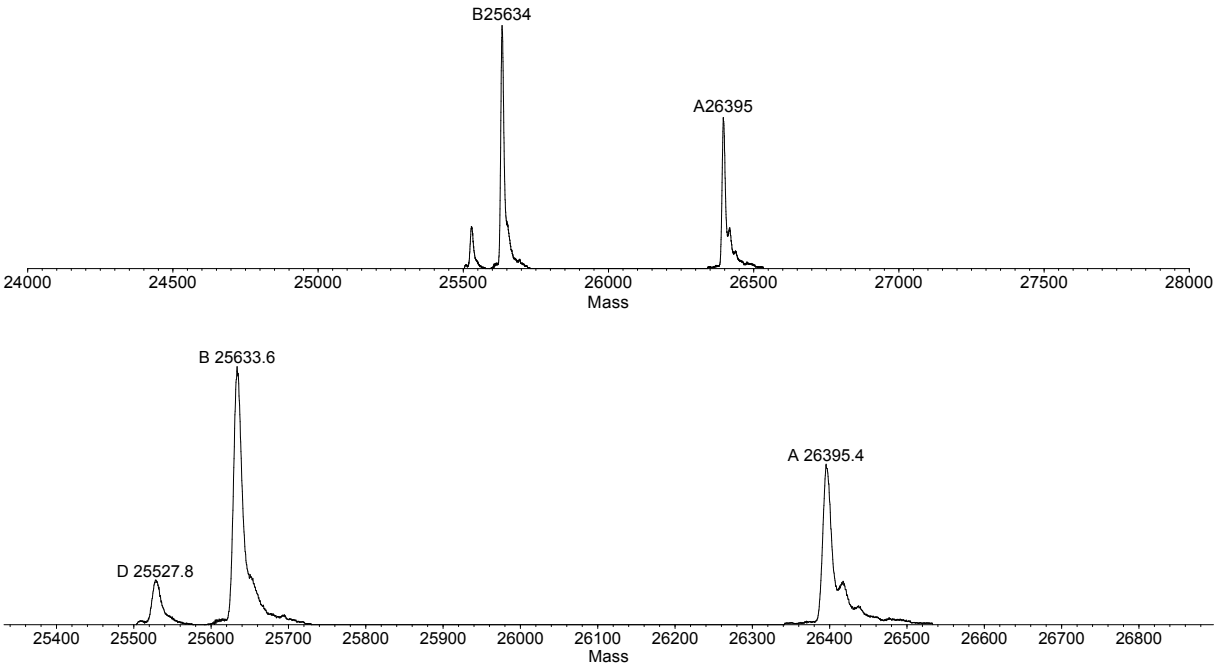
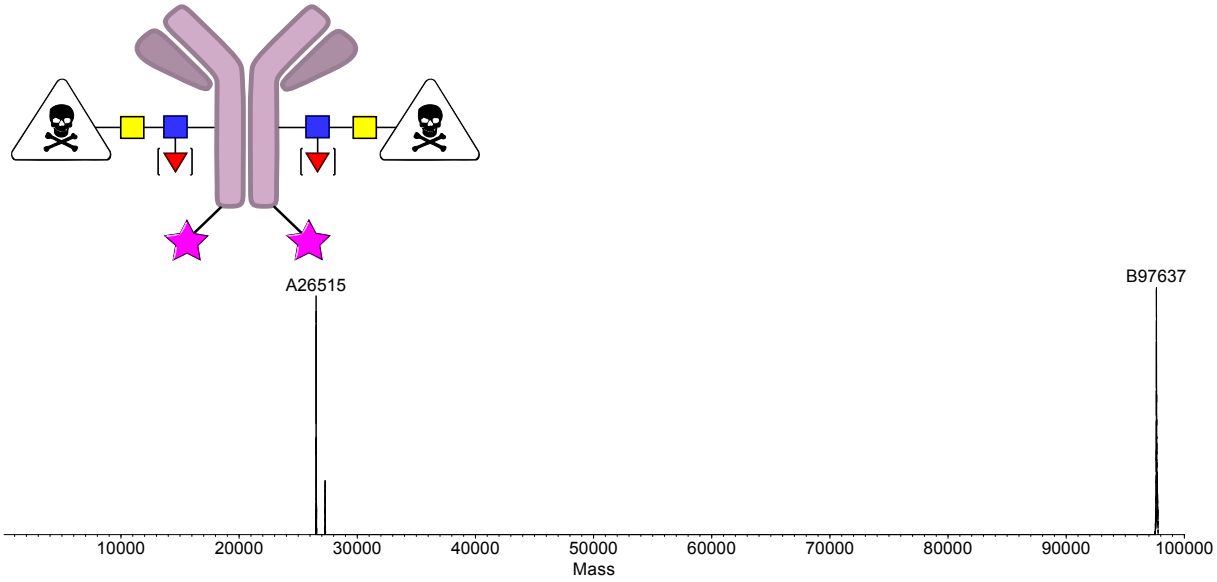


Figure S24. MS-spectrum of FC fragment of Tras[HC]G_4Y after glycan remodelling, SPAAC reaction and subsequent fabricator treatment.

MS of subsequent SPOCQ reaction product, Full MS:



FC_only

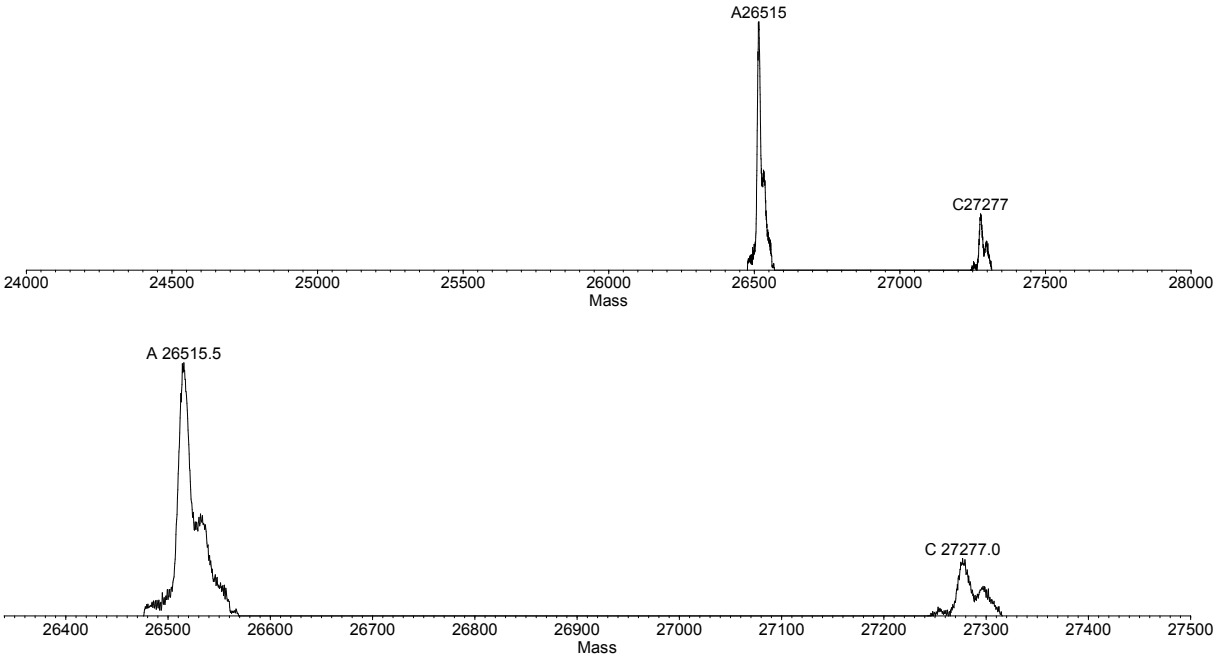


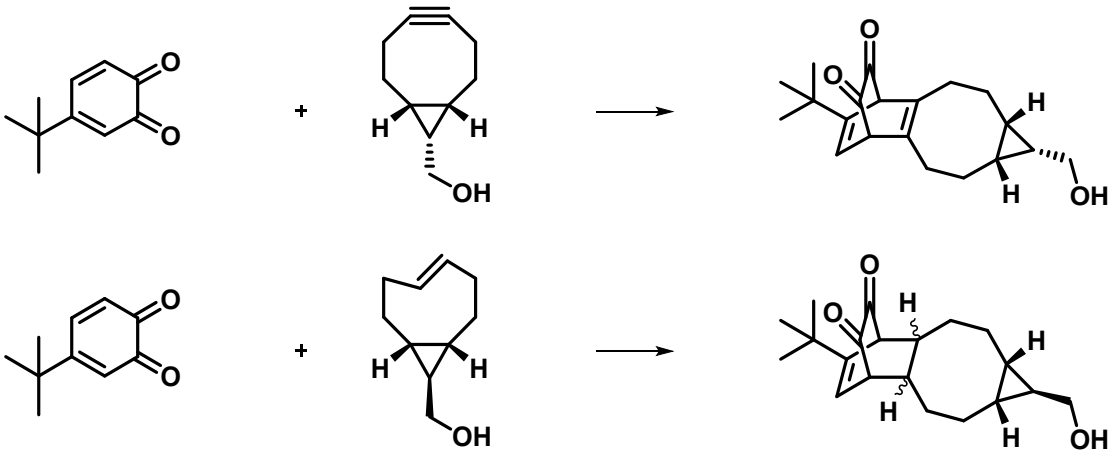
Figure S25. MS-spectrum of FC fragment of Tras[HC]G_4Y after glycan remodelling, SPAAC reaction, then SPOCQ reaction and subsequent fabricator treatment.

Table S5. Calculated and measured masses of fabricator-treated Tras[HC]G_4Y after glycan remodelling, SPAAC reaction, then SPOCQ reaction and subsequent fabricator treatment.

Mass	Tras[HC]G_4Y	SPAAC	SPAAC (fragmented)	SPOCQ	SPOCQ (fragmented label)
Calculated (Da)	24884	26393	25631	27275	26514
Measured (Da)	24885	26395	25634	27277	26515

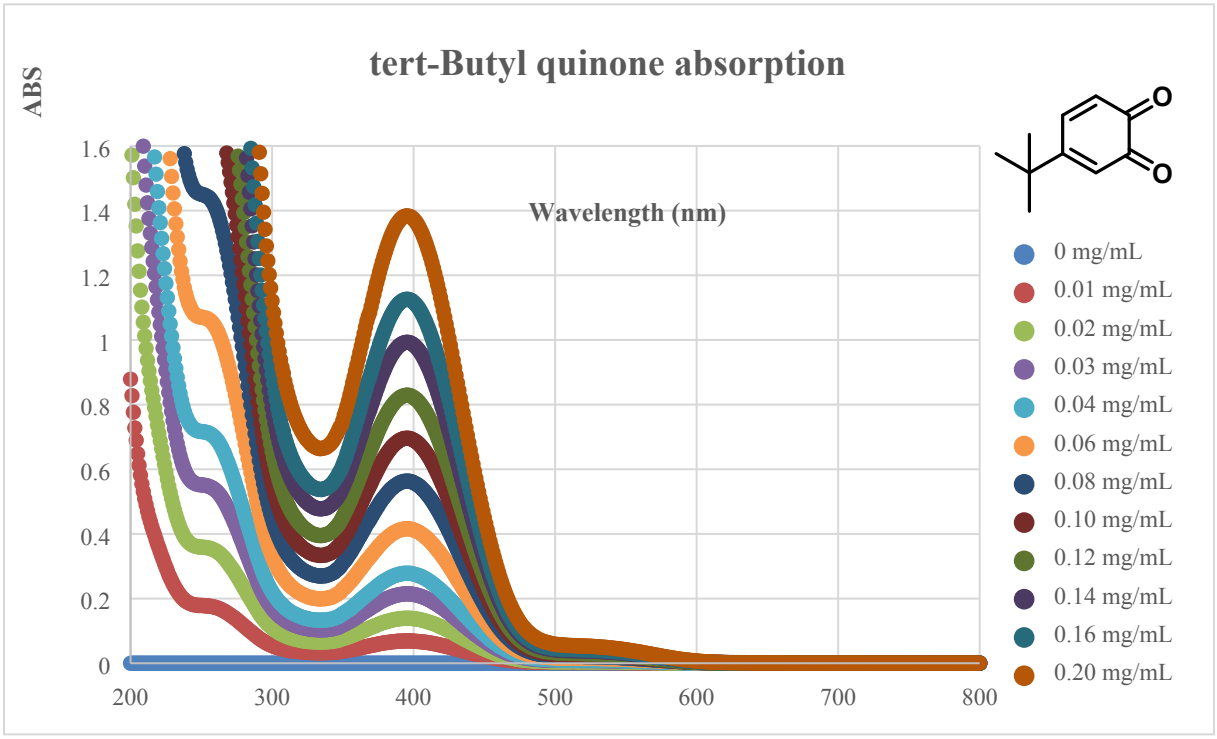
Correct mass was found after SPAAC, with a deviation of -1–2 Da. The defucosylated glycan was observed, as well as the intact MMAE group. SPAAC yielded the correct mass as well, with a deviation of +1.6–2 Da, and again both the fragmented and intact MMAE groups were found.

Kinetic studies



Scheme S2. BCN-SPOCQ and cpTCO-SPOCQ reactions as performed for kinetic studies.

Calibration curves



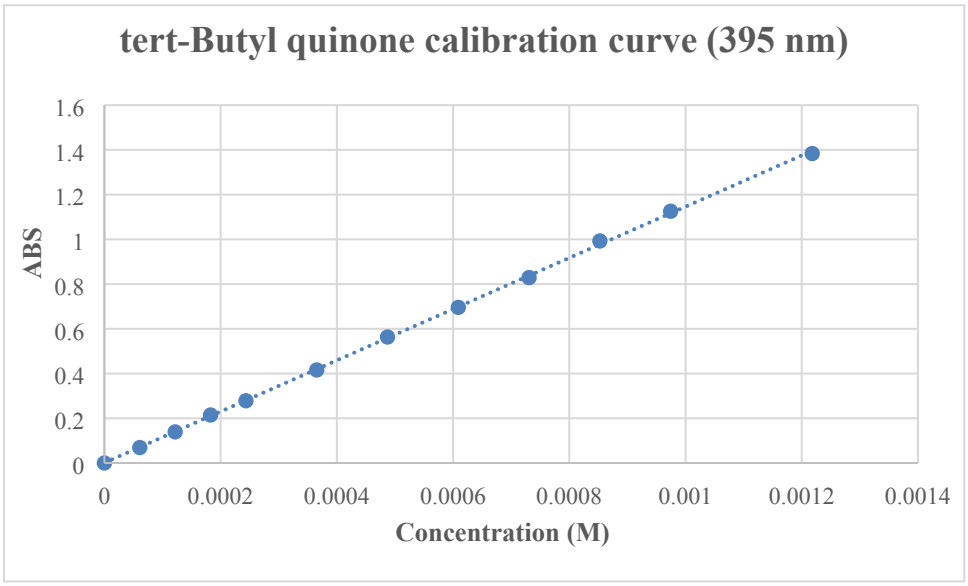
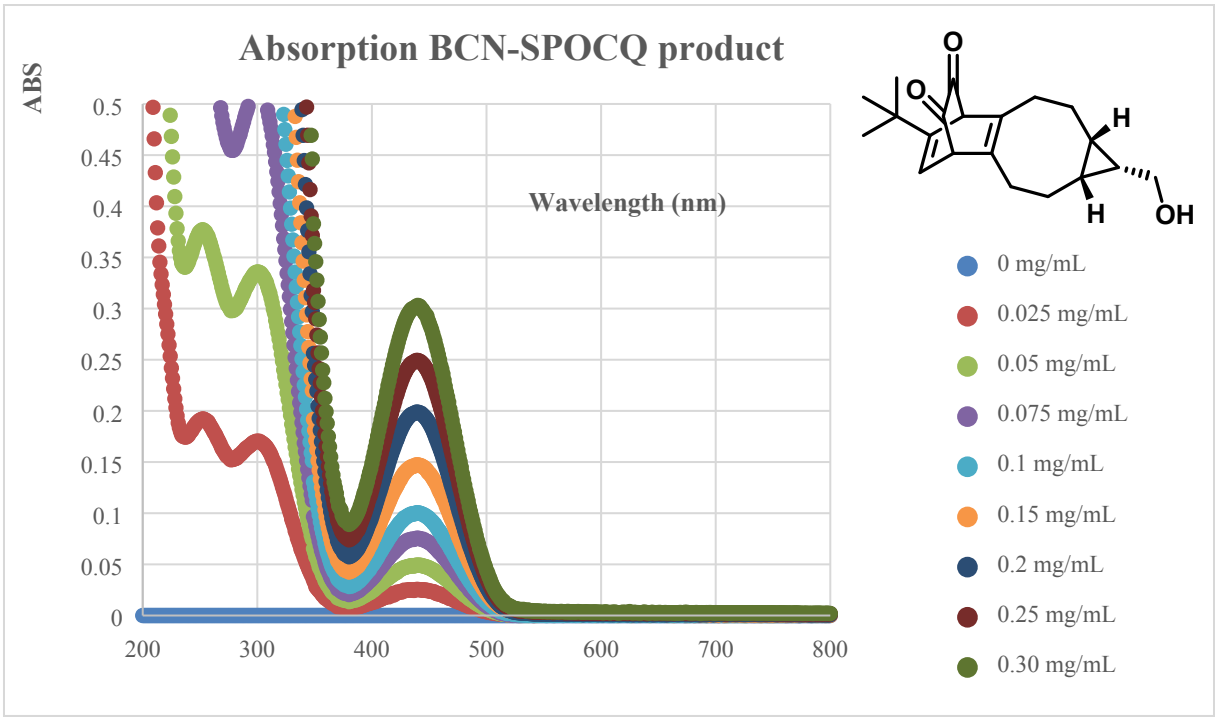


Figure S26. Absorption and calibration curve at 395 nm for tert-butyl quinone.



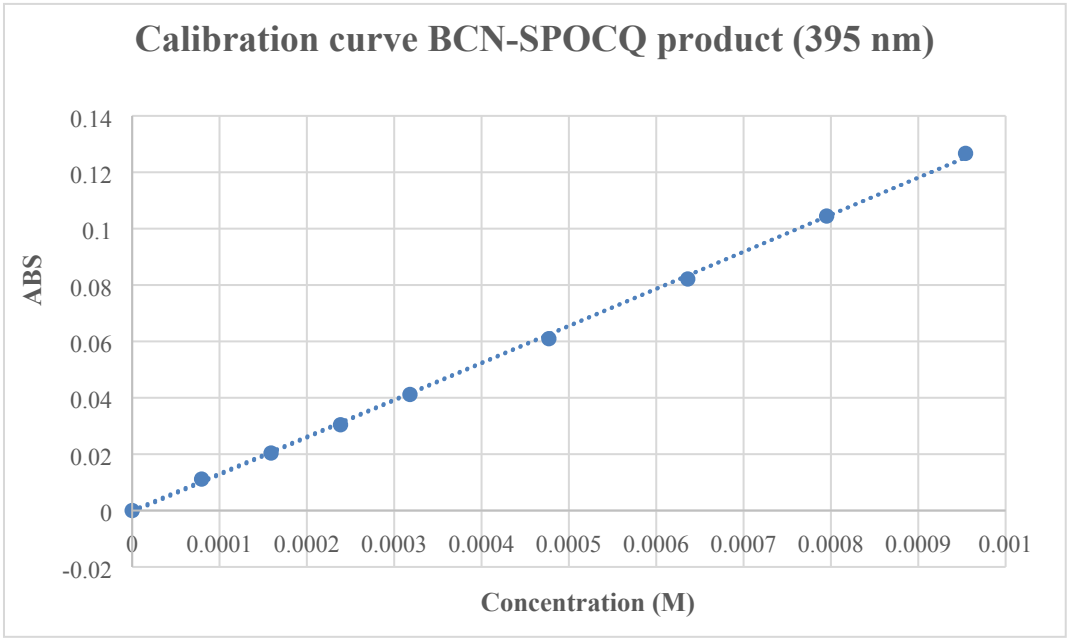
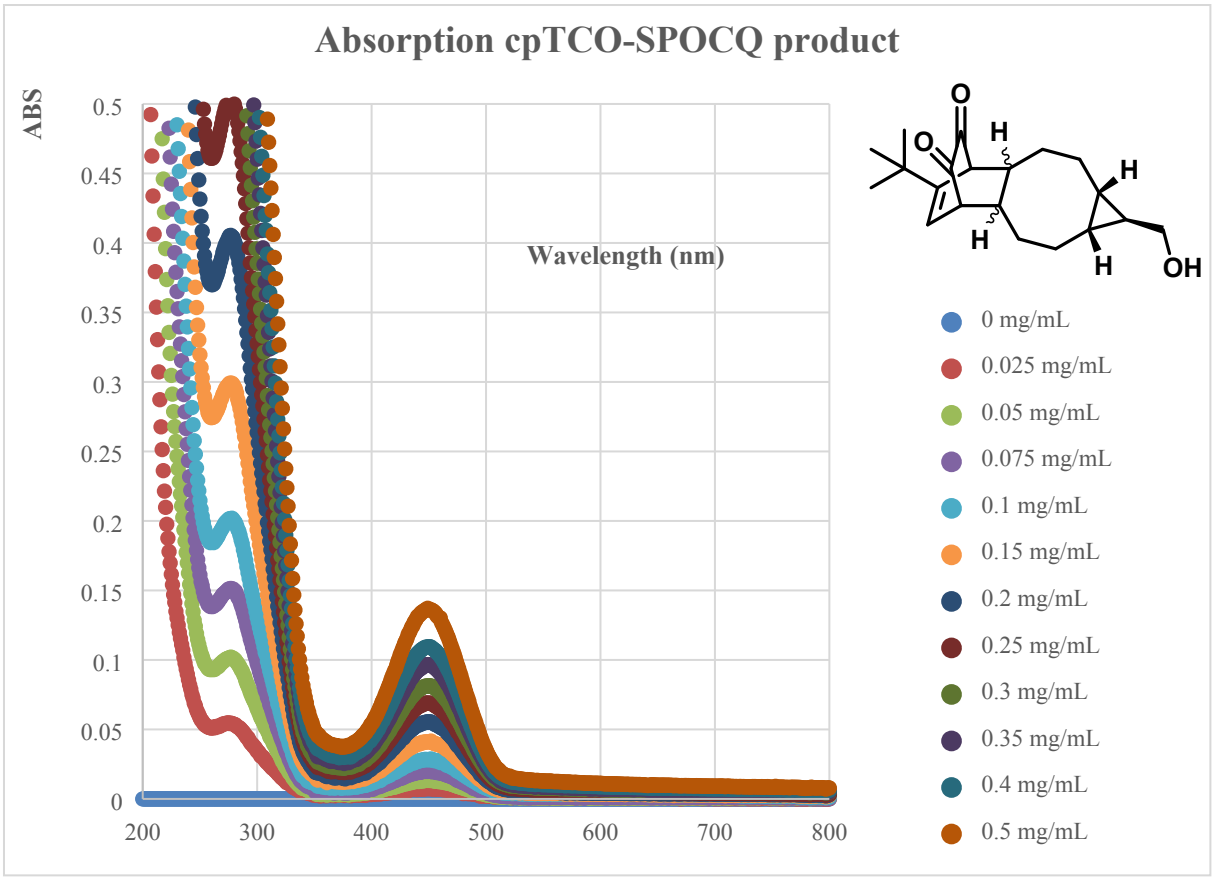


Figure S27. Absorption and calibration curve at 395 nm for the SPOCQ product of tert-butyl quinone and BCN-OH.



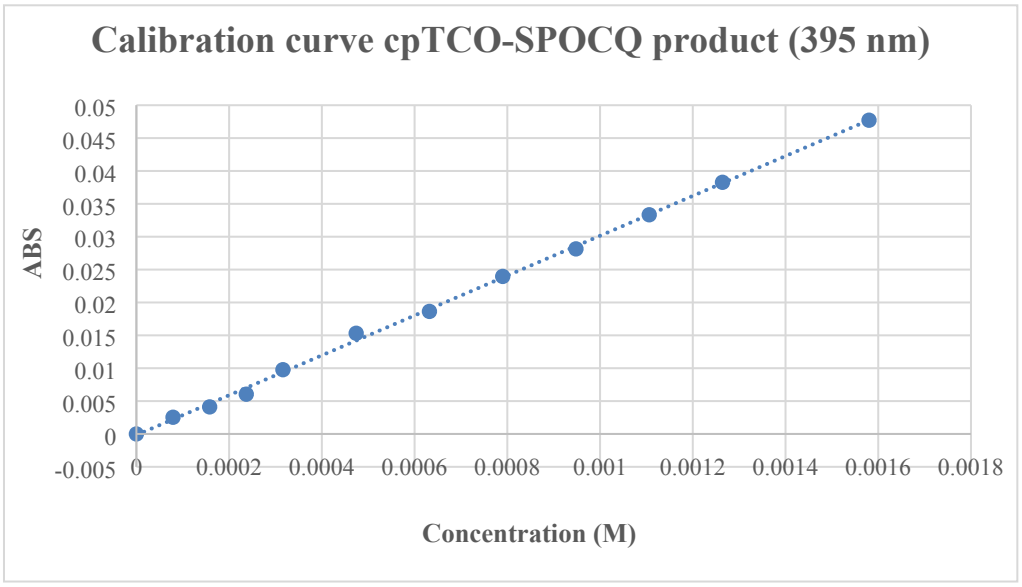


Figure S28. Absorption and calibration curve at 395 nm for the SPOCQ product of tert-butyl quinone and cpTCO-OH.

Overlay of UV-vis absorbance spectra of starting material and products.

For equimolar concentrations of quinone **1**, cpTCO-SPOCQ product **4**, and BCN-SPOCQ product **5**, the following overlay of UV-vis traces is obtained, with isosbestic points for **1** & **4** at 280 and 345 nm, and for **1** & **5** at 596 nm.

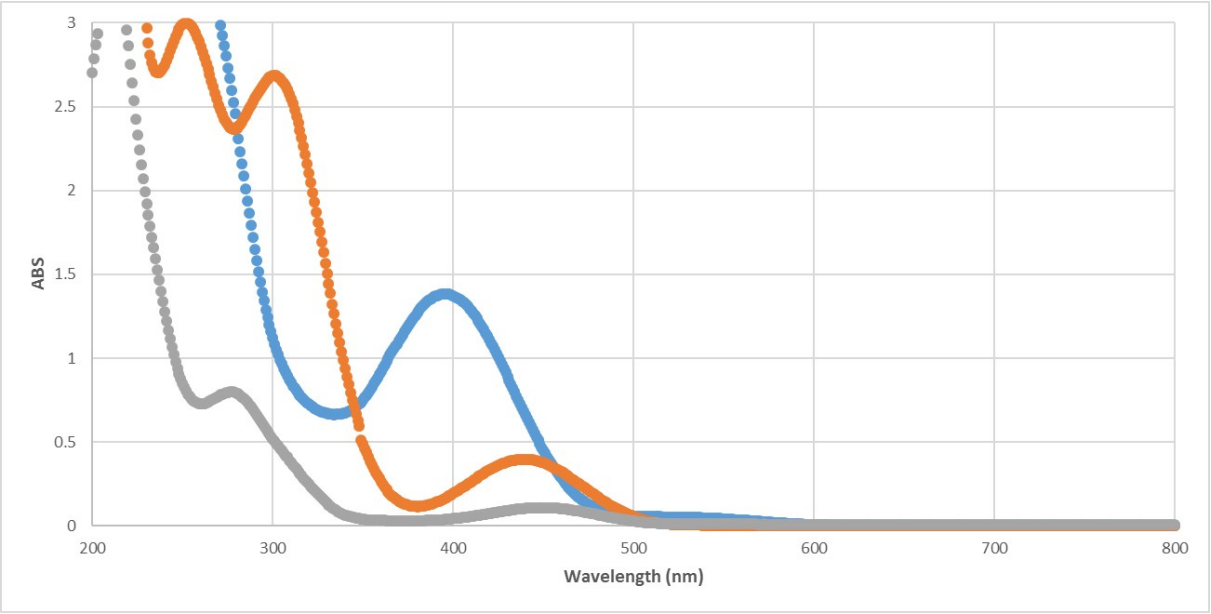


*Figure S29. Absorption spectra of quinone **1** (blue), cpTCO-SPOCQ product **4** (grey), and BCN-SPOCQ product **5** (orange).*

Procedure

Method from Borrmann et al. was applied for kinetics.[7]

Kinetics was measured with UV/VIS on a Varian Cary-100 spectrophotometer using a 1 cm quartz cuvette. A stock solution of BCN-OH or cpTCO-OH was prepared in MeOH/H_2O (1:1). 4-*tert*-butyl-1,2-benzoquinone was also dissolved in MeOH/H_2O (1:1) to a final concentration of 50 or 100 µM. For a total volume of 3 mL 60 µL of the BCN solution (final concentration 95 or 190 µM) was added to the quartz cuvette followed by the addition of 2940 µL of the 4-*tert*-butyl-1,2-benzoquinone solution. Since the product also slightly absorbs at 395 nm, we used the following equation:

$$Abs = \varepsilon_A * [A] + \varepsilon_P * [P]$$

with Abs = total absorbance at 395 nm, εA and εP = extinction coefficients of substrate A and product P, respectively, and [A] and [P] = the concentrations of substrate A and product P, respectively (mol/L). The concentration of the product was then calculated using the following equation:

$$[P] = \frac{A_0 - \frac{Abs}{\varepsilon_A}}{1 - \frac{\varepsilon_p}{\varepsilon_A}}$$

with [P] = concentration of product (mol/L), Abs = total absorbance at 395 nm, $[A]_0$ = the initial concentration of substrate A (mol/L) and ε_A and ε_P = extinction coefficients of substrate A and product P, respectively. The reaction was followed at a wavelength of 395 nm with a scanning interval of 0.33 sec and was done in triplicate. From the conversion plots thus obtained, second order rate constants were calculated according to this equation:

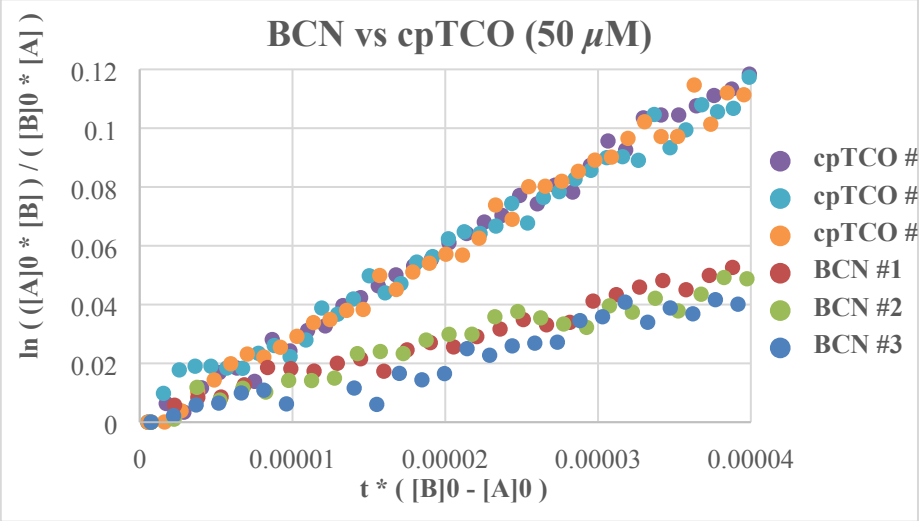
$$kt\,([B]_0 - [A]_0) = \ln\frac{[A]_0 * [B]}{[B]_0 * [A]}$$

with k = 2nd order rate constant (M^{-1} s^{-1}), t = reaction time (s), $[A]_0$ = the initial concentration of substrate A (mol/L), $[B]_0$ = the initial concentration of substrate B (mol/L).

50 µM quinone concentration.

Table S6. Kinetic data from 50 µM quinone and 95 µM strained alkene/alkyne.

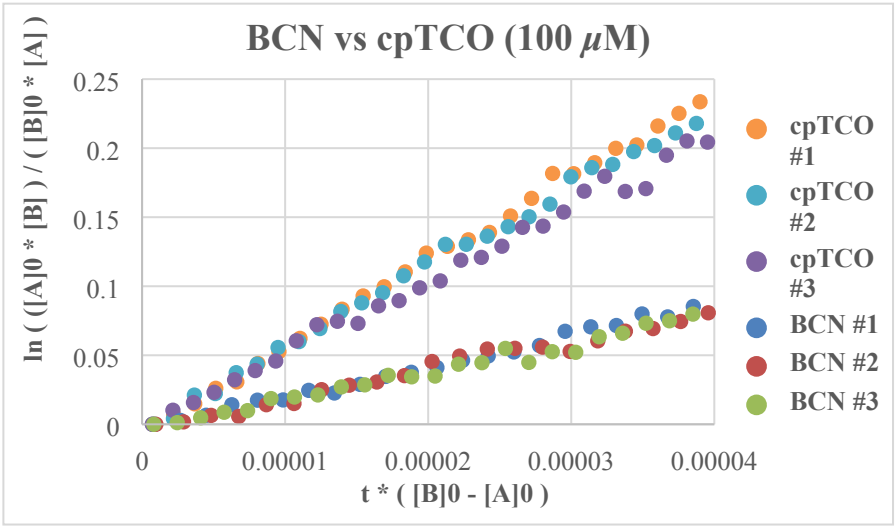
k	**50 µM**	
	BCN	**cpTCO**
1	1120	3010
2	1110	2910
3	1105	2780
AVG	1112	2900
SD	7.5	115.482



100 µM quinone concentration.

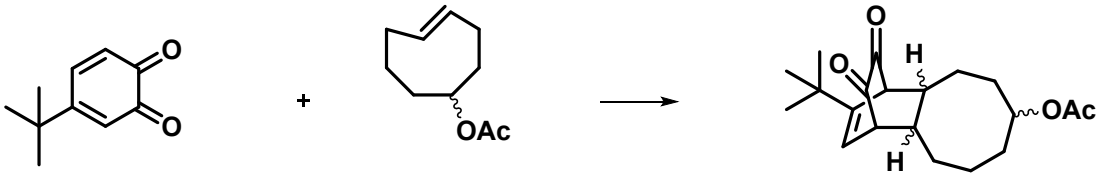
Table S7. Kinetic data from 100 µM quinone and 190 µM strained alkene/alkyne.

k	**100 µM**	
	BCN	**cpTCO**
1	2276	5792
2	2222	5697
3	2107	5092
AVG	2192	5527
SD	119.3	379.8



Conclusion: cpTCO has a 2.5–2.6 fold higher reaction rate than BCN when performing a Diels-Alder reaction with *tert*-butyl quinone.

TCO-OAc kinetics



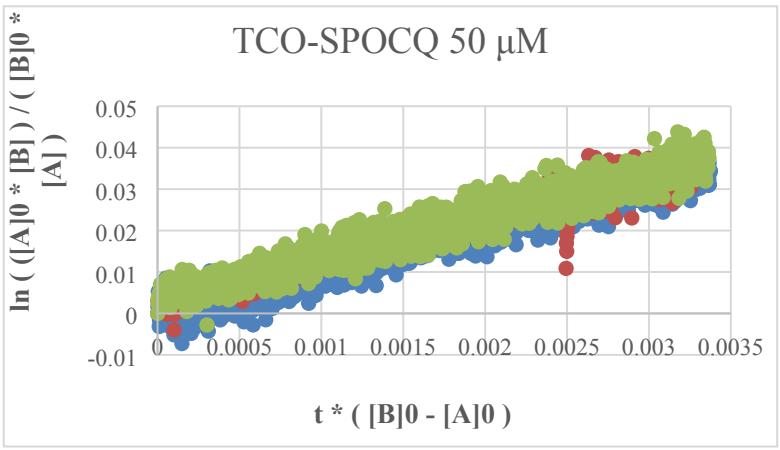
Scheme S3. TCO-SPOCQ as performed for kinetic studies.

Kinetics were performed as with BCN-SPOCQ and cpTCO-SPOCQ

50 µM quinone concentration.

Table S8. Kinetic data from 50 µM quinone and 95 µM TCO-OAc

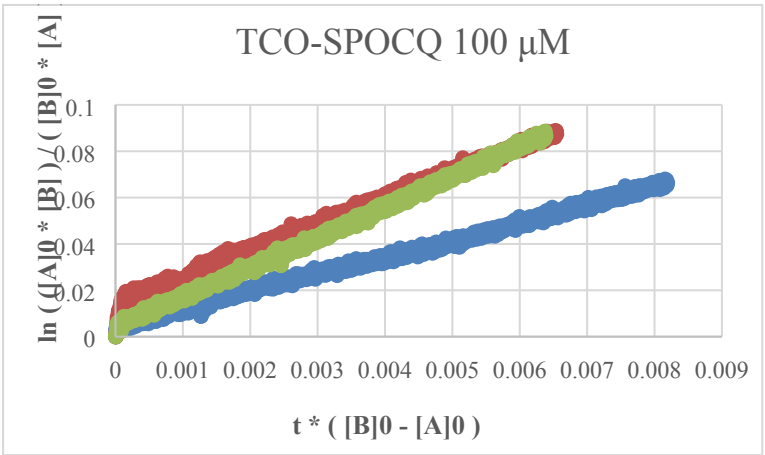
k	**50 µM TCO**
1	10.4
2	10.3
3	10.0
AVG	10.3
SD	0.2



100 µM quinone concentration.

Table S9. Kinetic data from 100 µM quinone and 190 µM TCO-OAc

k	**100 µM TCO**
1	7.52
2	11.3
3	13.0
AVG	10.6
SD	13.8



As the averages of 50 µM quinone and 100 µM quinone concentration are nearly the same. Combining the two leads to an average of 10.4 ± 1.8 M^{-1} s^{-1} for TCO-OAc. About 300–600 times slower than cpTCO-SPOCQ

Raw absorbance data

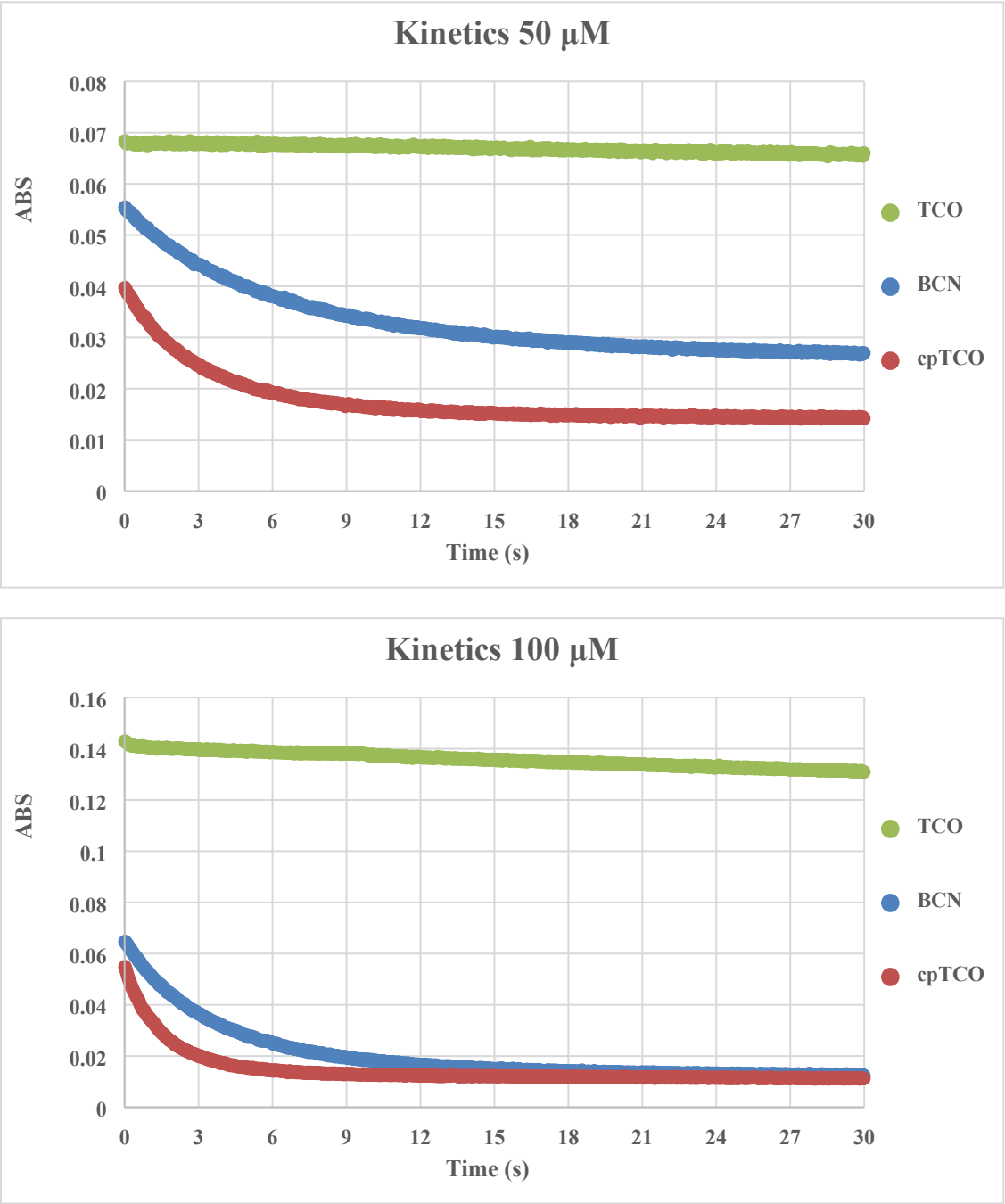


Figure S30. Change in absorbance at 395 nm as function of time. Note that BCN-SPOCQ product has a 4-fold higher absorption at 395 nm for its products than cpTCO-SPOCQ (132 L mol^{-1} cm^{-1} versus 30.3 L mol^{-1} cm^{-1}). Average values over N=3 experiments are plotted.

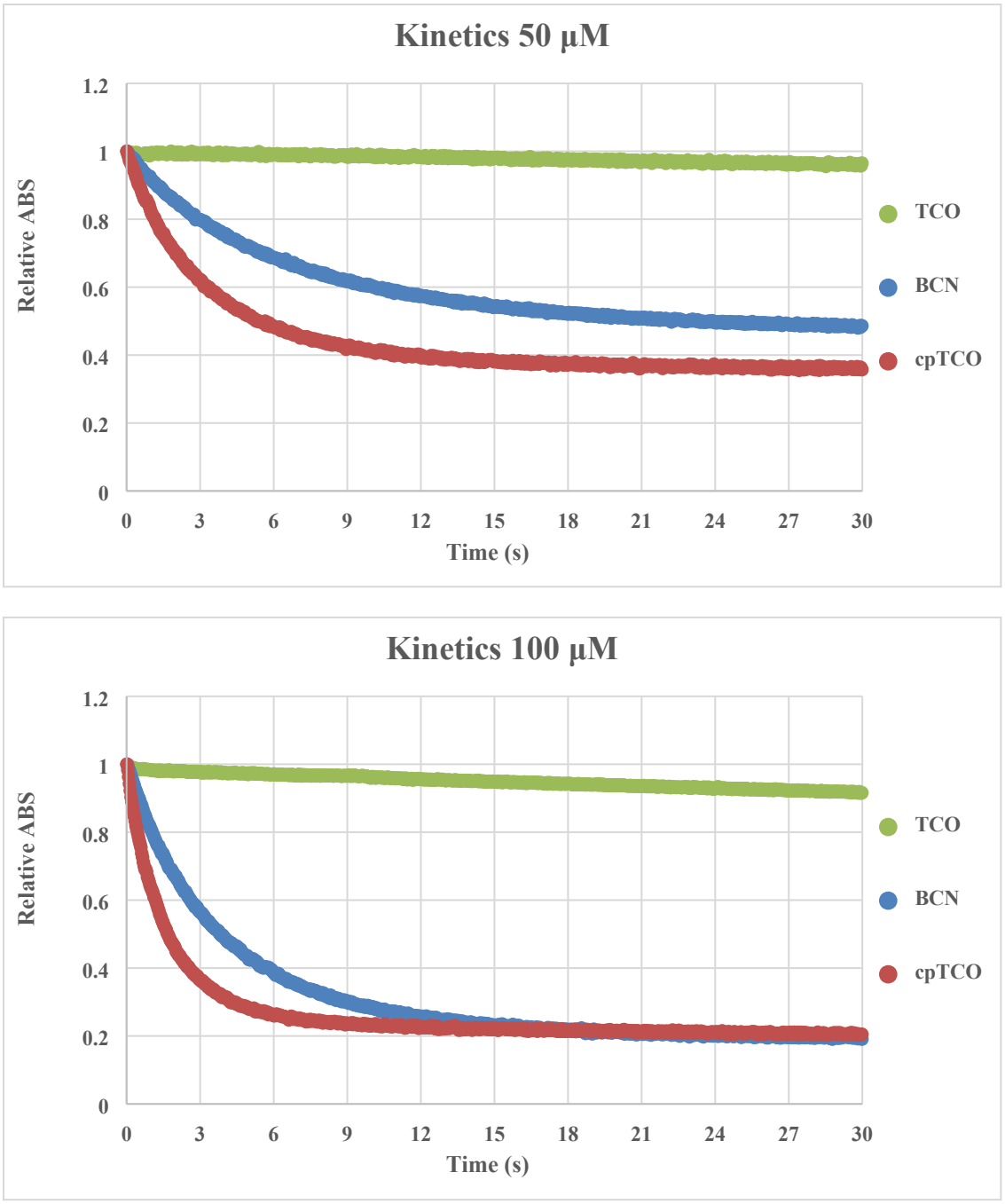


Figure S31. Change in absorbance at 395 nm as function of time, normalized against the absorbance at t=0 sec. Average values over N=3 experiments are plotted.

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