Supporting information

CBTF: New Amine-to-Thiol Coupling Reagent for Preparation of Antibody Conjugates with Increased Plasma Stability

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General Methods

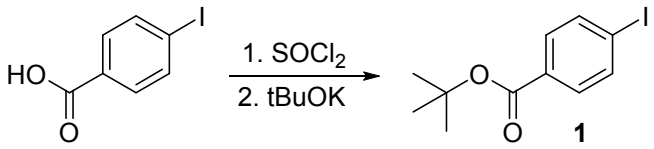
General experimental procedures: Unless otherwise indicated, reactions were carried out under an atmosphere of argon in flame-dried glassware with magnetic stirring. Air and/or moisture-sensitive liquids were transferred via syringe. When required, solutions were degassed by bubbling of argon through a needle. Organic solutions were concentrated by rotary evaporation at 25-60 °C at 15-30 torr. Analytical thin layer chromatography (TLC) was performed using plates cut from glass sheets (silica gel 60F-254 from Merck). Visualization was achieved under a 254 or 365 nm UV light and by immersion in an appropriate revelation solution. Column chromatography was carried out as "Flash Chromatography" using silica gel G-25 (40-63 μm) from Macherey-Nagel.

Materials: All reagents were obtained from commercial sources and used without prior purifications. Dry solvents were obtained from Sigma-Aldrich.

Instrumentation: ^{1}H and ^{13}C NMR spectra were recorded at 23°C on Bruker 400 and 500 spectrometers. Recorded shifts are reported in parts per million (δ) and calibrated using residual non-deuterated solvent. Data are represented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, br = broad), coupling constant (*J*, Hz) and integration. High resolution mass spectra (HRMS) were obtained using a Agilent Q-TOF (time of flight) 6520 and low resolution mass spectra using a Agilent MSD 1200 SL (ESI/APCI) with a Agilent HPLC1200 SL. Antibody MS experiments were performed on an electrospray time-of-flight mass spectrometer MS (LCT, Waters, Manchester) coupled to an automated chip-based nanoelectrospray device (Triversa Nanomate, Advion Biosciences, Ithaca, U.S.A.) operating in the positive ion mode.

Synthesis of CBTF

***tert*-Butyl 4-iodobenzoate (1):**



4-iodobenzoic acid (1 eq., 100 g, 0.403 mol) was added to $SOCl_2$ (8 eq., 383 g, 233 mL, 3.23 mol). Three drops of DMF were added and the suspension was stirred under reflux for 1 hour. After completion of the

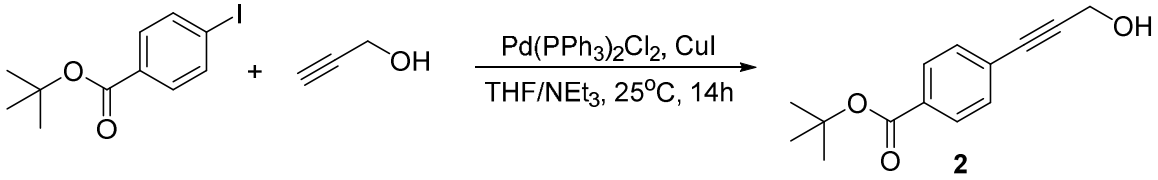
reaction the excess of thionyl chloride was removed in vacuum. The residual yellowish solid was dissolved in dry THF (500 mL) and cooled to 0°C. To this solution was added slowly over the period of 1 hour and under constant stirring a solution of potassium *tert*-butylate (1.1 eq., 49.8 g, 0.444 mol) in dry THF (755 mL). During the addition the temperature was not allowed to exceed 5°C. The suspension was stirred for 1 hour and carefully quenched with water (250 mL). The resulting solution was concentrated under vacuum and the residue diluted with water. The aqueous phase was extracted with diethyl ether. The organic phase was washed with 5% NaOH solution and with brine, then dried over $MgSO_4$ and evaporated to give *tert*-butyl 4-iodobenzoate (111 g, 366 mmol, 91 %) in form of yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 7.69 (d, *J* = 8.6 Hz, 2H), 7.61 (d, *J* = 8.6 Hz, 2H), 1.51 (s, 9H).

^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 165.23, 137.51, 131.54, 130.96, 100.01, 81.48, 28.18.

MS (ESI) *m/z*: 305.1 $[M+H]^+$.

***tert*-Butyl 4-(3-hydroxyprop-1-yn-1-yl)benzoate (2):**



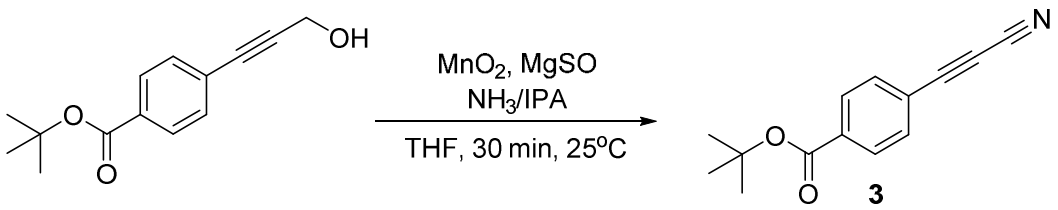
A solution of *tert*-butyl 4-iodobenzoate (1 eq., 111 g, 366 mmol) and 2-propyn-1-ol (2 eq., 41.1 g, 43.4 mL, 733 mmol) in THF (659 mL) and NEt_3 (549 mL) was degassed and purged with argon. To the resulting solution was added dichlorobis(triphenylphosphine)palladium (1 %, 2.58 g, 3.67 mmol) and CuI (2 %, 1.4 g, 7.34 mmol) and the solution was degassed again. The reaction mixture was stirred at 25°C under argon for 14 hours. The mixture was diluted with DCM (2500 mL), washed with sat. NH_4Cl (2000 mL), and DI water (2500 mL). The solvent was removed under reduced pressure to give *tert*-butyl 4-(3-hydroxyprop-1-yn-1-yl)benzoate **2** (83.5 g, 359 mmol, 98 %) as a yellow oil.

^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 7.93 (d, *J* = 8.10 Hz, 2H), 7.47 (d, *J* = 8.10 Hz, 2H), 4.53 (s, 2H), 1.60 (s, 9H).

^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 165.1, 131.7, 131.4, 129.3, 126.6, 89.8, 85.1, 81.4, 51.6, 28.1.

MS (ESI) *m/z*: 233.2 $[M+H]^+$.

***tert*-butyl 4-(cyanoethynyl)benzoate (3):**



To the solution of *tert*-butyl 4-(3-hydroxyprop-1-yn-1-yl)benzoate (1 eq., 83.5 g, 359 mmol) in THF (1620 mL) was added $MgSO_4$ (15 eq., 649 g, 5394 mmol), NH_3 solution in isopropanol (4 eq., 2M, 719 mL, 1438 mmol) and MnO_2 activated (15 eq., 468 g, 5394 mmol). After stirring the mixture at 25°C for

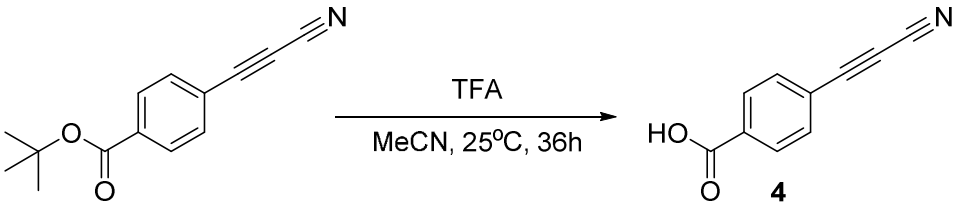
30 min, TLC (EtOAc/Cy : 1/9) showed only traces of the starting compound. The mixture was filtered through Celite, evaporated and purified by flash chromatography (EtOAc/Cyclohexane gradient: 0/100 to 30/70) to give *tert*-butyl 4-(cyanoethynyl)benzoate (39.2 g, 172 mmol, 48 %) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.04 (d, *J* = 8.30 Hz, 2H), 7.68 (d, *J* = 8.30 Hz, 2H), 1.62 (s, 9H).

^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 164.3, 134.8, 133.3, 129.7, 121.3, 105.2, 82.2, 81.9, 64.8, 28.1.

MS (ESI) *m/z*: 228.1 $[M+H]^+$.

4-(cyanoethynyl)benzoic acid (4):



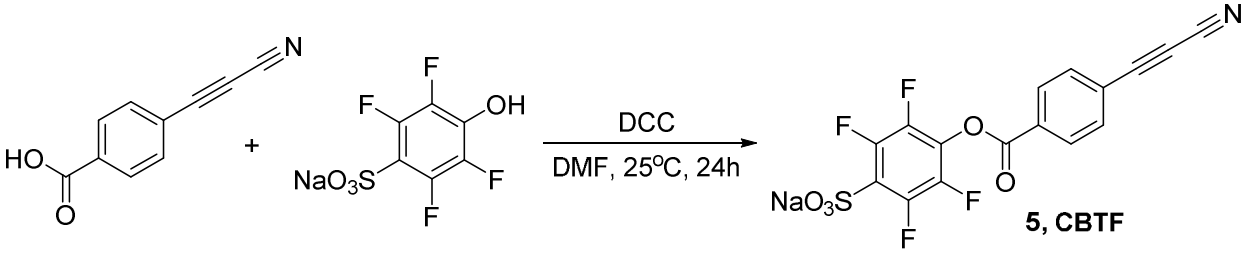
To the solution of tert-butyl 4-(2-cyanoeth-1-yn-1-yl)benzoate (1 eq., 39.2 g, 0.172 mol) in MeCN (1568 mL) was added TFA (30.6 eq., 601 g, 392 mL, 5.28 mol) . The mixture was stirred for 36h at r.t. until no starting compound was observed on TLC. The precipitate was filtered, washed with Et_2O and dried to give 4-(2-cyanoeth-1-yn-1-yl)benzoic acid (27.2 g, 158 mmol, 92 %) as a white solid.

^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 13.48 (br. s., 1H), 8.03 (d, *J* = 8.40 Hz, 2H), 7.92 (d, *J* = 8.40 Hz, 2H).

^{13}C NMR (100 MHz, DMSO-d_6, δ ppm): 166.1, 134.0, 133.9, 129.7, 120.3, 105.1, 82.5, 63.9.

MS (ESI) *m/z*: 172.0 $[M+H]^+$.

Sodium 4-((4-(cyanoethynyl)benzoyl)oxy)-2,3,5,6-tetrafluorobenzenesulfonate (5):



To the solution of sodium 2,3,5,6-tetrafluoro-4-hydroxybenzene-1-sulfonate (1 eq., 42.6 g, 158 mmol) and 4-(2-cyanoeth-1-yn-1-yl)benzoic acid (1 eq., 27.2 g, 158 mmol) in dry DMF (397 mL) was added DCC (1 eq., 32.8 g, 158 mmol). The resulting mixture was stirred at 25°C for 24 h, then cooled to 0°C, stirred for 1h, filtered and washed with 25 mL of dry DMF. The filtrate was diluted with 1600 mL of EtOAc, stirred for 15 min to allow crystallization and the precipitate was filtered to give sodium 4-((4-(cyanoethynyl)benzoyl)oxy)-2,3,5,6-tetrafluorobenzenesulfonate (58.2 g, 138 mmol, 87 %) as a white solid.

^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 8.31 (d, *J* = 6.30 Hz, 2H), 8.09 (br. d, *J* = 6.30 Hz, 2H).

HRMS (+ESI) *m/z*: calc. for $C_{16}H_4F_4NNaO_5S$: 420.96440; found: 420.96487.

HRMS (-ESI) *m/z*: calc. for $C_{16}H_5F_4NO_5S$: 398.98246; found: 398.98133.

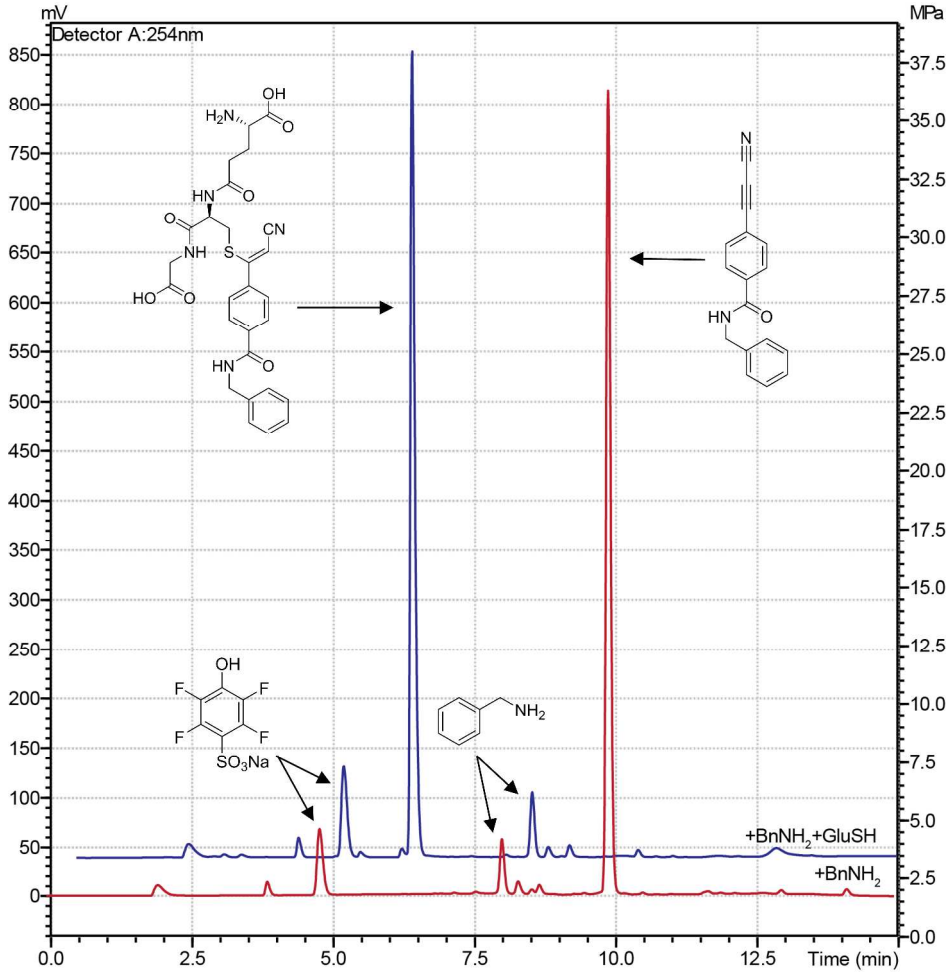
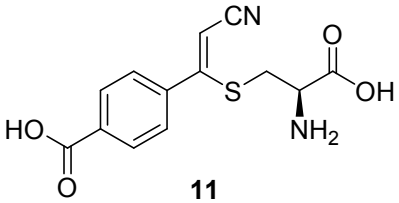


Figure S1. Reaction of CBTF with benzyl amine and glutathione monitored by HPLC.

Linker toxicity test



11

Preparation of the model linker. To the solution of the acid **4** (1 eq., 10 mg, 0.12 mmol) in DMF (1 mL) was added a solution of L-cysteine (1.5 eq., 21 mg, 0.18 mmol) in water (0.2 mL) followed by the addition of TEA (3 eq., 49 µL, 0.35 mmol). After incubation at 25°C for 15 minutes the mixture was purified by semi-preparative HPLC to give product **11** (22 mg, 0.08 mmol, 65 %) as a white solid.

^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 7.88 - 8.04 (m, *J* = 8.28 Hz, 2H), 7.48 - 7.69 (m, *J* = 8.28 Hz, 2H), 6.10 (s, 1H), 3.88 (t, *J* = 5.65 Hz, 1H), 3.08 (dd, *J* = 5.60, 13.90 Hz, 1H), 2.98 (dd, *J* = 5.65, 13.93 Hz, 1H).

MS (ESI) *m/z*: 292.1 $[M+H]^+$.

MTS cell toxicity assay. Huh-7 hepatocarcinoma were grown in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) and HeLa cervix adenocarcinoma cells were grown in MEM (Thermo Fisher

Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum, Penicillin (100 units/mL), and Streptomycin (100 µg/mL). Cell lines were maintained in a 5% CO_2 humidified atmosphere at 37°C. The day before experiment, cells were seeded in 96-well plates at 3000 cells/well in 100 µL fresh complete medium. Cells were incubated with product **11** during 96 h. The CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was used to determine the cell viability according to manufacturer's instructions. Absorbances were measured at 490 nm using a 96-well plate reader (Flx-Xenius XM, Safas, Monaco).

No significant toxicity of the model linker **11** was observed at up to 100 µM concentration according to MTS assays carried out on Huh-7 and HeLa cells (Figure S2).

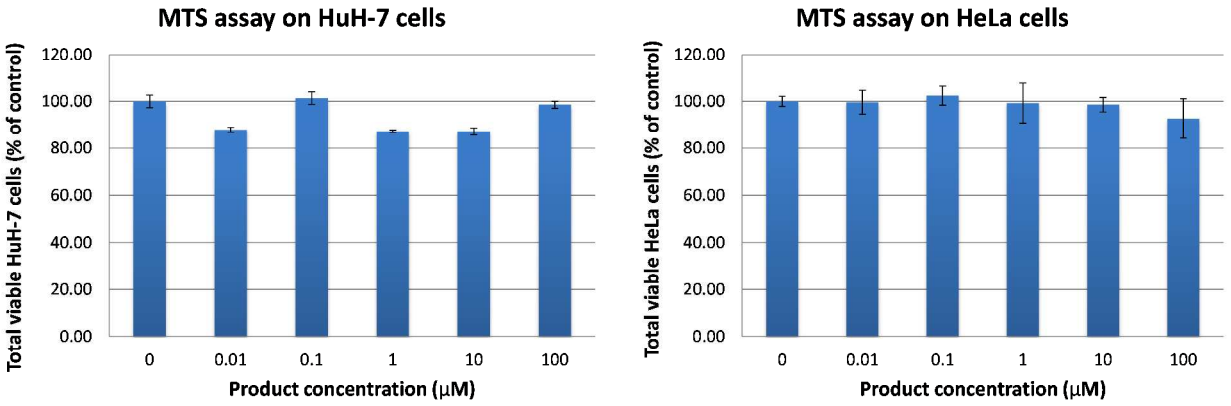


Figure S2. Toxicity tests of the model linker 11 on HuH-7 and HeLa cells using MTS assay.

Preparation of antibody conjugates

1. Reduction of the antibody

Trastuzumab was dissolved in 50mM PBS buffer (pH 6.8) containing EDTA (2mM). To this solution was added a solution of TCEP (1.1 eq. or 2.2 eq., 10 mM in H_2O). The mixture was incubated at 37°C for 2 h and used in the step 3 without further purification.

2. Conjugation with amines

To the solution of CBTF (100 µL, 10 mM in DMSO) was added a solution of TAMRA-NH_2 (150 µL, 10 mM in DMSO) followed by the solution of triethylamine (100 µL, 100mM in DMSO). The mixture was incubated at 25°C for 30 mins and used in the step 3 without further purification.

For the preparation of MCC-TAMRA conjugate the same protocol was used with the commercial SMCC instead of CBTF.

3. Conjugation with cysteines

To 100 µL of the solution of the reduced Trastuzumab was added the solution resulting from the step 2 (12 eq., 7.21µL). The resulting mixture was incubated at 25°C for 12h and then purified using Bio-Spin P-30 Columns (Bio-Rad, Hercules, U.S.A.) equilibrated with Tris buffer. Conjugates were analyzed by SDS-PAGE and mass spectrometry.

ESI-MS spectra of antibody conjugates

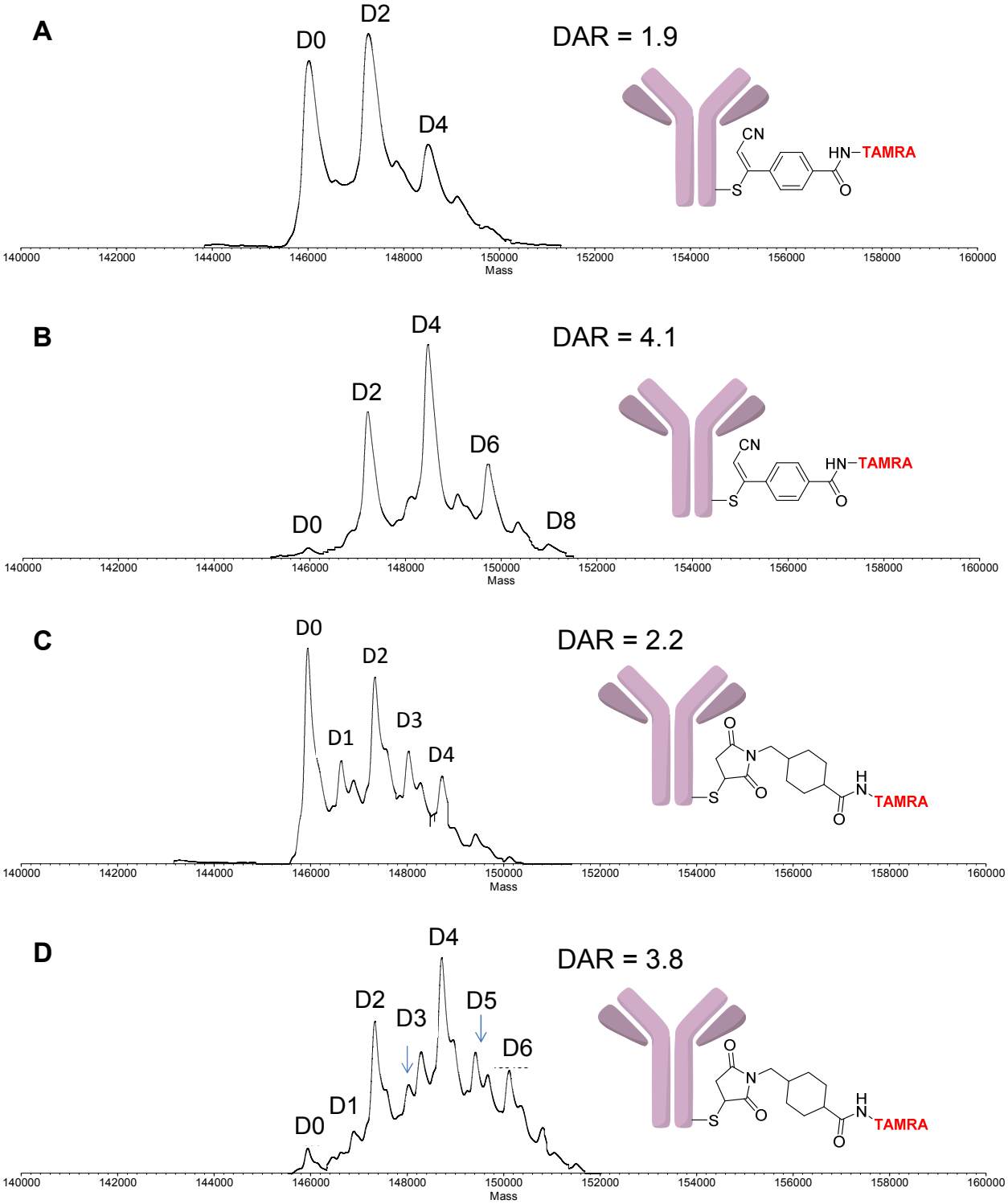


Figure S3. Deconvoluted mass spectra of the deglycosylated antibody conjugates. **A.** Conjugate prepared using CBTF and 1.1 eq. of TCEP. **B.** Conjugate prepared CBTF and 2.2 eq. of TCEP. **C.** Conjugate prepared using SMCC and 1.1 eq. of TCEP. **D.** Conjugate prepared SMCC and 2.2 eq. of TCEP.

Prior to native MS experiments, ADCs were desalted against 150 mM ammonium acetate solution buffered at pH 7.4 using six cycles of concentration/dilution on micro-concentrators (Vivaspin, 10 kD cutoff, Sartorius, Göttingen, Germany). Protein concentration was determined by UV absorbance using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Illkirch, France). ADC deglycosylation was achieved by incubating (37°C - 30 minutes) 0.4 units of Remove-iT® Endo S (New England Biolabs, Ipswich, U.S.A.) per microgram of ADC prior to buffer exchange desalting step.

MS experiments were performed on an electrospray time-of-flight mass spectrometer MS (LCT, Waters, Manchester) coupled to an automated chip-based nanoelectrospray device (Triversa Nanomate, Advion Biosciences, Ithaca, U.S.A.) operating in the positive ion mode. For native MS experiments, external calibration of the ESI-TOF instrument was performed using singly charged ions produced by a 2 mg/mL solution of cesium iodide in 2-propanol/water (1v/1v). Tuning parameters of the mass spectrometer were carefully optimized to improve desolvation and ion transfer as well as maintaining weak interactions. Particularly, the sample cone voltage Vc was set to 120 V and the backing pressure Pi was increased to 6 mbar to improve ion collisional cooling and maintain non-covalent interaction for averaging DAR calculation. Native MS data interpretation was performed using MassLynx 4.1 (Waters, Manchester, UK.).

Average DAR values from native MS were calculated from the relative peak intensities measured from the raw mass spectrum (taking into account 21+ to 26+ charge states). Average DAR value was obtained by summing up the weighted peak percentage from all observed species and dividing the sum by 100, as follows: DAR = Σ(relative peak intensity × number of loaded drugs)/100 (number of drug load ranging from 0 to 8).

LC-MS characterization of reduced antibody conjugates.

Prior to LC-MS analysis, 120µg of EndoS deglycosylated antibodies were reduced through a Na_2HPO_4 50mM, NaCl 150mM, guanidine 3.5M, TCEP 50mM, pH 6.6 buffer dilution. Samples were next incubated at 37°C during 1h, before being quenched by acidification (trifluoroacetic acid 1%). 800pmol of each antibody were then injected on a HPLC system (Alliance 2695, Waters, Manchester, UK) coupled to an electrospray Q-TOF mass spectrometer (MicrOTOF-Q, Bruker Daltonics, Bremen, DE). Liquid chromatography was performed at 50°C on a C4 reversed phase PLRP-S 1000Å, 8µm, 150 x 2.1mm column (Agilent Technology) according to a flow rate of $0.25mL.min^{-1}$. Solvents A and B were respectively composed of acidified water and acetonitrile (0.1% TFA). The gradient was set as follows: 5 min at 25% of B; 50 min with increasing B from 25 to 55 %; 1 min with increasing B from 55 to 85%; and 1 min of isocratic elution at 85% of B. Signal acquisition was realized by both UV absorbance measurement at 214 and 280nm, and mass spectrometry. MS spectra were recorded on a 200-3000 m/z range with a scan time of 1 s. Instrumental parameters included a V positive ion mode, a capillary voltage of 4500 V, a nebulizer pressure of 0.4 bar, a dry gas flow of $4L.min^{-1}$ and a source temperature of 200°C. Calibration was performed using the singly charged ions produced by a tuning mix solution (Agilent technology). Data processing was achieved on Data Analysis 4.0 software (Bruker Daltonics, Bremen, DE).

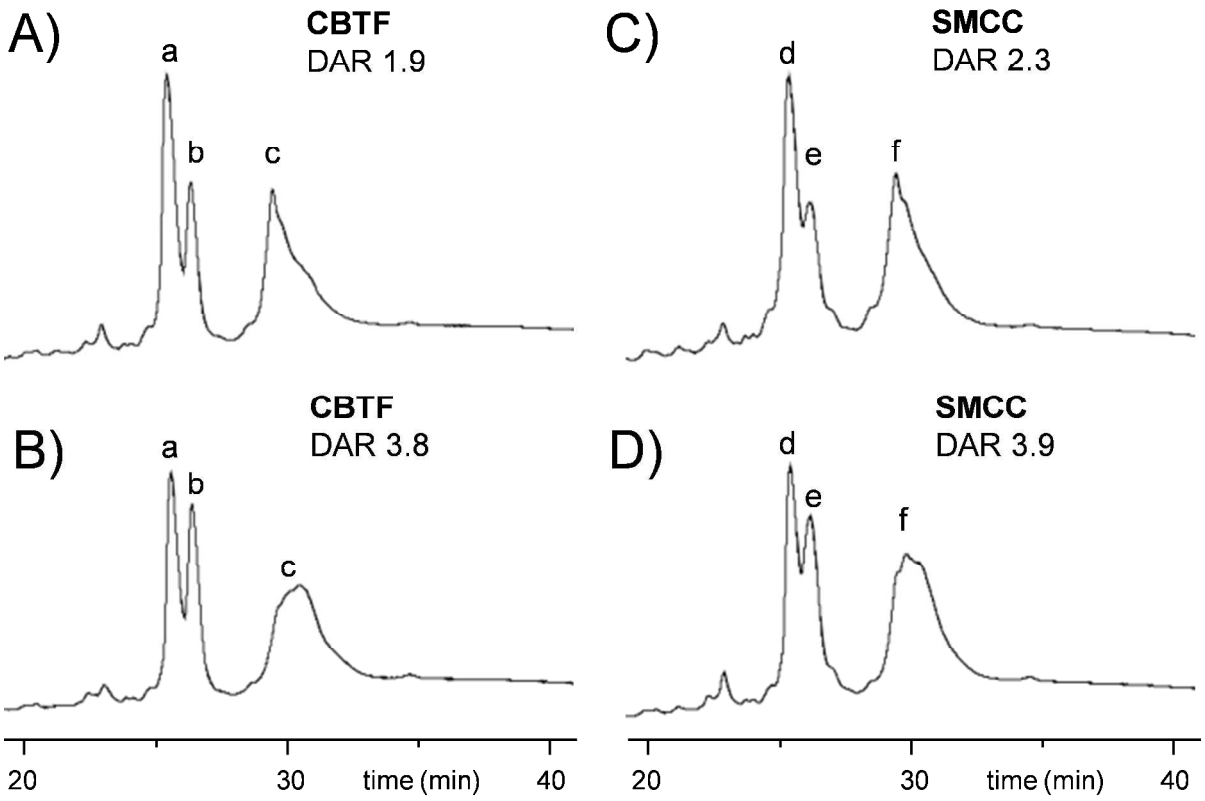


Figure S4. RPHPLC-UV analysis of CBTF- and SMCC-derived Trastuzumab conjugates from 2 conjugation protocols. Following the deglycosylation and reduction step, absorption profiles at 214nm of CBTF-derived conjugates (A, B) were compared as a function of coupling conditions: 1.1 eq. of TCEP (A) or 2.2 eq. of TCEP (B). Similarly, UV profiles of SMCC-derived conjugates (C, D) were obtained, with eq. of TCEP (C) or 2.2 eq. of TCEP (D). As depicted, 3 predominant signals of variable retention time and width are observed, depending on payload nature and conjugation conditions (a,b,c and d,e,f, respectively).

Table S1. RPLC-MS characterization of CBTF- and SMCC-derived Trastuzumab conjugates from 2 conjugation protocols. Mass assignation of eluted species allows to unambigously point out a payload distribution dependent on payload nature and conjugation conditions, on both light (peaks a,b,d and e) and heavy chains (peaks c and f). LC: Light Chain, HC: Heavy Chain.

peak	Masses (Da)	
	CBTF 1.1 eq. of TCEP	CBTF 2.2 eq. of TCEP
a	23442.7 ± 0.3 (free LC)	
b	24068.3 ± 0.4 (LC + 1 payload)	
c	49505.6 ± 2.6 (free HC) 50134.6 ± 1.6 (HC + 1 payload)	49503.6 ± 5.3 (free HC) 50162.6 ± 6.0 (HC + 1 payload) 50776.3 ± 30.1 (HC + 2 payloads)

peak	SMCC 1.1 eq. of TCEP	SMCC 2.2 eq. of TCEP
d	23442.4 ± 0.4 (free LC)	
e	24151.9 ± 0.4 24134.5 ± 0.7 (LC + 1 payload)	
f	49504.5 ± 2.1 (free HC) 50217.5 ± 14.2 (HC + 1 payload)	49502.7 ± 3.0 (free HC) 50218.0 ± 5.2 (HC + 1 payload) 50927.6 ± 14.9(HC + 2 payloads)