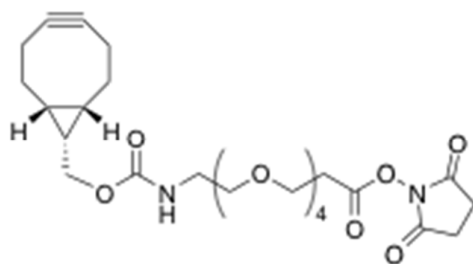


General Method and Materials

Solvents were purchased from Sigma-Aldrich or Fisher Scientific and used as received. Thin layer chromatography was performed on silica gel-coated plates (Kieselgel 60 F254, Merck, Germany) with the indicated solvent mixture, spots were detected by KMnO_4 staining (1.5 g KMnO_4 , 10 g K_2CO_3 , 2.5 mL 5% NaOH-solution, 150 mL H_2O), *p*-anisaldehyde staining (9.2 mL *p*-anisaldehyde, 321 mL EtOH, 17 mL H_2O , 3.75 mL AcOH, 12.7 mL H_2SO_4), and UV-detection. NMR spectra were recorded on a Bruker Biospin 400 (400 MHz) and a Bruker DMX300 (300 MHz). Protein mass spectra (HRMS) were recorded on a JEOL AccuTOF JMS-T100CS (Electrospray Ionization (ESI) time-of-flight) or a JEOL AccuTOF JMS-100GCv (Electron Ionization (EI), Chemical Ionization (CI)). Low-resolution mass spectra (LRMS) were recorded on a ThermoScientific Advantage LCQ Linear ion-trap electrospray and a Waters LCMS consisting of a 2767 Sample manager, 2525 pump, 2996 UV-detector and a Micromass ZQ with an Xbridge™ C18 3.5 μm column (ESI).

Synthesis of BCN-PEG₄-C(O)OSu



To a solution of amino-PEG₄-acid (1.23 g, 4.23 mmol) in anhydrous DMF (30 mL) were subsequently added BCN-OSu carbonate (1.02 g, 3.85 mmol) and triethylamine (1.60 mL, 11.53 mmol). The reaction mixture was stirred for 3 h at r.t., after which $\text{EDCI} \cdot \text{HCl}$ (0.884 g, 4.61 mmol) and NHS (88 mg, 0.77 mmol) were added. The resulting solution was stirred overnight at r.t. and poured into 100 mL NaHCO_3 (sat.) and 150 mL EtOAc. The layers were separated and the organic phase was washed with sat. NaHCO_3 (90 mL) and H_2O (75 mL). The organic phase was dried (Na_2SO_4), filtered and concentrated *in vacuo*. Gradient flash chromatography ($\text{MeCN} \rightarrow \text{MeCN}:\text{H}_2\text{O}$ 30:1) afforded BCN-PEG₄-C(O)OSu as colorless oil (800 mg, 1.48 mmol, 40%). ^1H NMR (300 MHz, CDCl_3): δ (ppm) 4.13 (d, $J = 8.1$ Hz, 2H), 3.84 (t, $J = 6.3$ Hz, 2H), 3.68-3.58 (m, 12 H), 3.54 (t, $J = 5.1$ Hz, 2H), 3.35 (q, $J = 5.1$ Hz, 2H), 2.89 (t, $J = 6.3$ Hz, 2H), 2.82 (s, 4H), 2.35-2.13 (m, 6H), 1.67-1.46 (m, 2H), 1.35 (d, $J = 8.4$ Hz, 1H), 1.00-0.86 (m, 2H). LRMS (ESI+) calcd for $\text{C}_{34}\text{H}_{54}\text{N}_2\text{O}_{14}$ ($M + \text{Na}^+$) 539.60, found 539.2.

Synthesis of BCN-PEG₄-pyrene (7a)

To a solution of BCN-PEG₄-C(O)OSu (50 mg, 0.095 mmol) in DCM (10 mL) was added 2-(aminomethyl)pyrene.HCl (30 mg, 0.11 mmol) and Et_3N (17 μL , 0.12 mmol). After stirring overnight at r.t., the reaction mixture was concentrated under reduced pressure. Subsequent purification via flash

column chromatography (DCM \rightarrow DCM:MeOH 9:1) yielded product **7a** (38 mg, 61%). ^1H NMR (400 MHz, CDCl_3) δ (ppm) 8.29-8.26 (m, 2H), 8.19-8.11 (m, 4H), 8.06-7.97 (m, 4H), 7.04 (br. s, 1H), 5.24 (br. s, 1H), 5.16 (d, 2H, $J = 4$ Hz), 4.06 (d, 2H, $J = 4$ Hz), 3.76 (t, 2H, $J = 5.6$ Hz), 3.50 (m, 2H), 3.39-3.22 (m, 14H), 2.56-2.53 (m, 2H), 2.27-2.13 (m, 7H), 1.29-1.25 (m, 2H), 0.87-0.83 (m, 2H).

Synthesis of BCN-PEG₈-C(O)OSu

To a solution of amino-PEG₈-acid (217 mg, 0.492 mmol) in anhydrous DMF (3 mL) were subsequently added BCN-OSu carbonate (143 mg, 0.492 mmol) and Et_3N (204 μL , 1.47 mmol). The reaction mixture stirred for 3 h at r.t., after which EDCI.HCl (0.88 g, 4.61 mmol) and NHS (88 mg, 0.77 mmol) were added. The resulting solution was stirred overnight at r.t. and poured into 50 mL NaHCO_3 (sat.) and 50 mL EtOAc. The layers were separated and the organic phase was washed with sat. NaHCO_3 (50 mL) and H_2O (30 mL). The organic phase was dried (Na_2SO_4), filtered and concentrated *in vacuo*. Gradient flash chromatography (MeCN \rightarrow MeCN: H_2O 30:1) afforded BCN-PEG₈-C(O)OSu as colorless oil (212 mg, 0.30 mmol, 60%). ^1H NMR (300 MHz, CDCl_3): δ (ppm) 4.13 (d, $J = 8.1$ Hz, 2H), 3.84 (t, $J = 6.3$ Hz, 2H), 3.68-3.59 (m, 28 H), 3.54 (t, $J = 5.1$ Hz, 2H), 3.36 (q, $J = 5.4$ Hz, 2H), 2.89 (t, $J = 6.3$ Hz, 2H), 2.82 (s, 4H), 2.35-2.15 (m, 6H), 1.68-1.48 (m, 2H), 1.44-1.23 (m, 1H), 1.00-0.86 (m, 2H). LRMS (ESI+) m/z calcd for $\text{C}_{34}\text{H}_{54}\text{N}_2\text{O}_{14}$ ($M+\text{Na}^+$) = 737.8; found 737.3.

BCN-PEG₈-pyrene (7b)

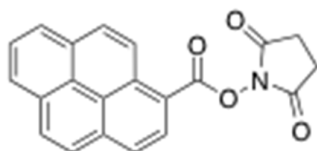
To a solution of BCN-PEG₈-C(O)OSu (100 mg, 0.14 mmol) in DCM (15 mL) was added 2-aminomethylpyrene.HCl (50 mg, 0.19 mmol) and Et_3N (47 μL , 0.25 mmol). After stirring for 3 h, the reaction mixture was concentrated under reduced pressure. Subsequent purification via flash column chromatography (DCM \rightarrow DCM:MeOH 95:5) yielded product **7b** (35 mg, 30%). ^1H NMR (400 MHz, CDCl_3) δ (ppm) 8.30-8.28 (m, 1H), 8.21-8.13 (m, 4H), 8.05-7.99 (m, 4H), 7.22 (bs, 1H), 5.16 (d, 2H, $J = 5.2$ Hz), 4.12 (d, 2H, $J = 8.0$ Hz), 3.76 (t, 2H, $J = 6.0$ Hz), 3.65-3.49 (m, 21H), 3.45-3.42 (m, 2H), 3.36-3.32 (m, 4H), 2.59-2.54 (m, 4H), 2.28-2.20 (m, 4H), 1.59-1.54 (m, 2H), 1.38-1.25 (m, 2H), 0.93-0.91 (m, 2H).

Synthesis of BCN-PEG₁₂-pyrene (7c)

BCN OSu carbonate (25 mg, 0.09 mmol) was dissolved in DMF (1 mL) followed by the addition of amino-PEG₁₂-carboxylic acid (50 mg, 0.08 mmol) and Et_3N (39 μL , 0.23 mmol). After stirring for 2h additional BCN OSu carbonate (5 mg, 0.015 mmol) was added and the reaction mixture was stirred for an additional 1 h. After complete conversion (based on TLC-analysis), NHS (4 mg, 0.03 mmol) and EDCI.HCl (44 mg, 0.22 mmol) were added and the reaction was stirred for 2h.. To the reaction mixture were added 2-(aminomethyl)pyrene.HCl (35 mg, 0.12 mmol) and Et_3N (40 μL , 0.29 mmol) and after stirring o.n. the reaction mixture was concentrated under reduced pressure.

Purification via gradient flash column chromatography (DCM \rightarrow DCM:MeOH 9:1) yielded product **7c** (12 mg, 15%). ^1H NMR (400 MHz, CDCl_3) δ (ppm) 8.31-8.29 (m, 2H), 8.21-8.13 (m, 4H), 8.05-7.99 (m, 4H), 7.10 (bs, 1H), 5.16 (d, 2H, $J = 5.6$ Hz), 4.12 (d, 2H, $J = 8$ Hz), 3.75 (t, 2H, $J = 6.0$ Hz), 3.84-3.65 (m, 37H), 3.44-3.42 (m, 2H), 3.36-3.31 (m, 6H), 2.55 (t, 2H, $J = 5.6$ Hz), 2.28-2.21 (m, 6H), 1.58-1.55 (m, 2H), 0.95-0.92 (m, 2H).

1-Pyrenecarboxylic acid OSu ester



To a solution of 1-pyrenecarboxylic acid (65 mg, 0.24 mmol) in DCM/DMF (2 mL each) was added NHS (34 mg, 0.29 mmol) and EDCI.HCl (70 mg, 0.36 mmol). The reaction was stirred for 2 h and subsequent diluted with DCM (10 mL), washed with aqueous citric acid (10%, 5 mL) and saturated NaHCO_3 (3×5 mL), dried over Na_2SO_4 , filtrated and concentrated in vacuo to give crude 1-pyrenecarboxylic acid OSu ester.

DBCO-PEG₄-pyrene (8a)

Crude 1-pyrenecarboxylic acid OSu ester (75 mg, 0.22 mmol) was dissolved in DCM (3 mL) followed by the addition of amino-PEG₄-carboxylic acid (53 mg, 0.20 mmol) and Et_3N (83 μL , 0.6 mmol). After stirring overnight additional 1-pyrenecarboxylic acid OSu ester (25 mg, 0.07 mmol) was added and the reaction mixture was stirred for an additional 1 h. After complete conversion (based on TLC-analysis), NHS (5 mg, 0.04 mmol) and EDCI.HCl (70 mg, 0.36 mmol) were added and the reaction was stirred overnight at r.t.. To the reaction mixture were added aminopropionyl-DBCO (60 mg, 0.2 mmol, commercially available from ClickChemistryTools) and Et_3N (50 μL , 0.36 mmol) and after 1 h the reaction mixture was concentrated under reduced pressure. Purification via gradient flash column chromatography (DCM \rightarrow DCM:MeOH 9:1) yielded product **8a** (12 mg, 8%). ^1H NMR (400 MHz, CDCl_3) δ (ppm) 8.61 (d, 1H, $J = 9.2$ Hz), 8.23-8.21 (m, 2H), 8.17-8.10 (m, 4H), 8.07-8.02 (m, 3H), 7.59 (d, 1H, $J = 7.2$ Hz), 7.38-7.18 (m, 7H), 7.06 (bs, 1H), 6.45 (m, 1H), 5.00 (d, 1H, $J = 13.6$), 3.86-3.79 (m, 3H), 3.70-3.19 (m, 14H), 2.42-2.38 (m, 1H), 2.19-2.15 (m, 2H), 1.89-1.85 (m, 1H).

DBCO-PEG₈-pyrene (8b)

Compound **8b** was prepared analogously to compound **8a**. Purification via flash column chromatography (DCM \rightarrow DCM:MeOH 93:7) yielded **8b** (8 mg, 14%). ^1H NMR (400 MHz, CDCl_3) δ (ppm) 8.55 (d, 1H, $J = 9.2$ Hz), 8.19-7.86 (m, 7H), 7.59 (d, 1H, $J = 6.8$ Hz), 7.33-7.18 (m, 8H), 6.99

(m, 1H), 6.45 (m, 1H), 5.03 (d, 1H, $J = 14.0$), 3.78-3.74 (m, 3H), 3.65-3.29 (m, 29H), 2.43-2.37 (m, 1H) 2.25-2.21 (m, 2H), 1.91-1.84 (m, 1H).

DBCO-PEG₁₂-pyrene (8c)

Compound **8c** was prepared analogously to compound **8a**. Purification via flash column chromatography (DCM \rightarrow DCM:MeOH 9:1) yielded **8b** (12 mg, 22%). ¹H NMR (400 MHz, CDCl₃) δ (ppm) 8.61 (d, 1H, $J = 9.6$ Hz), 8.28-8.03 (m, 9H), 7.66 (d, 1H, $J = 7.2$ Hz), 7.52-7.25 (m, 7H), 7.14 (bs, 1H), 6.60 (m, 1H), 5.12 (d, 1H, $J = 14$), 3.86-3.82 (m, 4H), 3.73-3.70 (m, 2H), 3.66-3.46 (m, 44H).

Synthesis of biotin-alkyne (6)

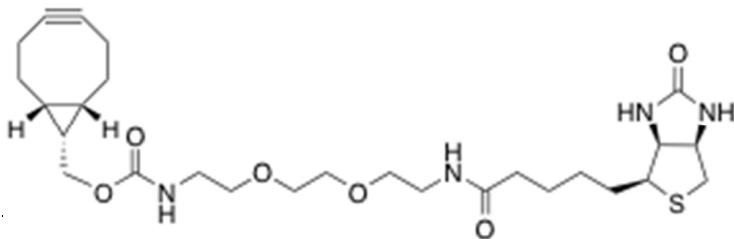
To a solution of biotin-PEG₂-NH₂ in DCM (6 mL) were added Et₃N (54 μ L, 39 mg, 0.39 mmol) and pent-4-ynoic acid succinimidyl ester (25 mg, 0.13 mmol). After 19 h, the reaction mixture was concentrated and the residue was purified *via* column chromatography (5 \rightarrow 20% MeOH in DCM). The purified product was dissolved in DCM, filtered and concentrated, which yielded the desired product as a white solid (52 mg, 0.11 mmol, 85%).

Conjugation of 6 to trastuzumab(GalNAz)₂ (III-2)

To a solution of trastuzumab(GalNAz)₂ (10 μ L, 9.6 mg/mL, 0.64 nmol) in PBS buffer was added a solution of biotin-alkyne **6** (45, 1 μ L, 10 mM, 10 nmol) and a stock premix (1 μ L, 10 mM CuSO₄, 10 mM Na-ascorbate, 10 mM tris((1-((O-ethyl)carboxymethyl)-(1,2,3-triazole-4-yl))methylamine in 20% MeCN in milliQ). The reaction was rotated end-over-end overnight, after which time AccuTOF analysis showed complete formation of the desired product (observed mass 50195 Da).

Detailed mass spectrometric analysis of trastuzumab and derivatives

Native trastuzumab, trastuzumab(GalNAz)₂, **III-2**, biotin conjugate (prepared from **III-2** by copper-catalyzed click conjugation with **6**) or trastuzumab conjugate obtained by strain-promoted click chemistry with BCN-PEG₂-biotin (structure below) were subjected to detailed mass spectral analysis analysis of intact protein species (nanoLC-MS) using nanoLC coupled to ultra high resolution QTOF MS (maXis 4G).



Intact protein analysis

Sample preparation

Protein reduction was performed for 30 minutes in 10 mM DTT at 56 °C. Samples were diluted 1:1 in 2% formic acid prior to analysis.

Liquid chromatography – mass spectrometry

Protein separations were performed using a UHPLC nanoflow liquid chromatography (Bruker Daltonics nano advance) coupled online to an ultra high resolution quadrupole time-of-flight mass spectrometer (Bruker Daltonics maXis 4G ETD) via an axial desolvation vacuum assisted electrospray source (Bruker Daltonics captive sprayer). Proteins were loaded on the trap column (Dionex PepSwift, 0.2 × 5 mm) in 3 minutes at a flowrate of 5000 nL/min using 0.1% formic acid. Proteins were separated on a 0.2 × 150 mm monolithic particle column (Michrom 8µm 4000Å PLRP-S) at 50°C using a linear gradient of 20 to 50% acetonitrile and 0.1% formic acid at a flowrate of 1000 nL/min. Desolvation and ionization of peptides eluting from the column was performed using 6 L/min nitrogen gas at 180 °C and 1600 V capillary voltage. The mass spectrometer was calibrated externally using Agilent tuning mix (G1969-85000) and used lockmass calibration at 1221.9906 m/z (Agilent G1982-85001). The mass spectrometer was programmed to acquire spectra in the range of 500-4200 m/z at 1 Hz with the following settings: 400 Vpp Funnel RF, 10 eV isCID, 400 Vpp Multipole RF, 8 eV Quadrupole ion energy, 510 m/z Low mass, 10 eV Collision cell energy, 3500 Vpp Collision RF, 110 µs Transfer time, 450 Vpp Ion cooler RF and 18 µs Pre pulse storage.

Data processing

All data were processed in Data Analysis software. After lock mass calibration, both the light- and heavy-chain MS spectra were averaged over each chromatographic peak, respectively. Averaged spectra were deconvoluted using the maximum entropy algorithm in combination with the SNAP peak picking algorithm for the light chain spectrum or Sum peak picking for the heavy chain spectrum.

Proteolytic peptide analysis

Sample preparation

From each sample, five µg of protein was subjected to in-solution tryptic digestion. Briefly, reduction was performed in 10 mM DTT for 30 minutes at 56 °C. Alkylation of reduced cysteines (carbamidomethylation) was performed using 50 mM chloroacetamide. Protein digestion was first performed by addition of 0.5 µg LysC peptidase and incubation for 3 hours at 37 °C. Next, 800 ng trypsin was added to the sample and incubated O/N at 37 °C. Resulting proteolytical peptides were concentrated and desalted using stop-and-go elution tips.

Liquid Chromatography – tandem mass spectrometry

Peptide separations were performed using a UHPLC nanoflow liquid chromatograph (Bruker Daltonics nano advance) coupled online to a high capacity ion trap (Bruker Daltonics amaZon speed ETD) via an axial desolvation vacuum assisted electrospray source (Bruker Daltonics captive sprayer). Peptides were loaded onto the trap column (Dionex PepSwift, 0.2×5 mm) in 3 minutes at a flowrate of 5000 nL/min using 0.1% formic acid. Peptides were separated on a 0.1×250 mm monolithic column (Dionex PepSwift) at 60°C using a linear gradient of 5 to 25% acetonitrile and 0.1% formic acid at a flowrate of 800 nL/min. Desolvation and ionization of peptides eluting from the column was performed using 3 L/min nitrogen gas at 150 °C and 1300 V capillary voltage. The mass spectrometer was programmed to acquire a single survey spectrum (MS) with subsequent data dependent fragmentation analysis (MS/MS) of the top 6 most abundant ions. Survey spectra were acquired at enhanced resolution mode and used the following instrument settings: 50 ms maximum accumulation time, 500.000 ICC target, tune at 1000 m/z, 5 spectra averages. Fragmentation spectra were acquired in extreme scan mode with autoselect fragmentation mode enabled which switches between collision induced dissociation (CID) and electron transfer dissociation (ETD) fragmentation methods based on the mass-to-charge ratio and charge state of the precursor ion. The following instrument settings were used for fragmentation scans: 500.000 ICC target, 200 ms maximum accumulation time, SPS enabled, 70% CID energy and 0.2 min dynamic exclusion.

Database searches

Acquired raw mass spectrometry data were processed by Data Analysis software (Bruker Daltonics, v4.1) to generate Mascot compatible input files (GMF format). GMF files were loaded into ProteinScape software (Bruker Daltonics, V3.1) and searched against a sequence database using the Mascot database search software. The fasta sequence database contained the trastuzumab light- and heavy-chain protein sequences with added contaminant protein sequences (e.g. LysC, Trypsin and keratins). Searches were performed using the following settings: 0.35 Da precursor mass tolerance, 0.35 Da fragment ion mass tolerance, tryptic specificity with a maximum of 2 missed cleavages, carbamidomethylation (C) as fixed modification. The following variable modifications were specified: oxidation (MHW), deamidation (NQ), carbamylation (K + N-terminus) and acetylation (protein N-terminus). Peptides with Mascot ion scores above identity score threshold were accepted as valid identifications (peptide false discovery rate < 1%).

Relative quantitation

For the trastuzumab heavy chain protein, a single peptide with histidine oxidation (sequence K.FNWYVDGVEVH*NAK.T, with oxidized H11) was exclusively identified in trastuzumab-conjugate obtained by copper-catalyzed conjugation of **6**, but none was detected in the other samples. Extracted ion current (EIC) chromatograms for the peptide with and without oxidized histidine (EIC: 847.46 ± 0.5 m/z and EIC: 839.97 ± 0.5 m/z, respectively) were generated in DataAnalysis software.

In addition, EIC chromatograms for the following two peptides were used for normalization purposes: -.EVQLVESGGGLVQPGGSLR.L (EIC: 941.6 \pm 0.5 m/z) and K.GPSVFPLAPSSK.S (EIC: 593.95 \pm 0.5 m/z). Integrated peak areas from three replicate measurements were averaged and expressed relative to native trastuzumab, indicating that the above fragment with oxidized histidine amounts to 69% of total of that particular fragment (31% unmodified fragment).

For the trastuzumab light chain protein, a single peptide with methionine oxidation (sequence DIQM*TQSPSSLSASVGDR.V, with oxidized M4) was exclusively identified in trastuzumab-conjugate obtained by copper-catalyzed conjugation of **6**, but none was detected in the other samples.

*Conjugation of **7a-c** or **8a-c** to trastuzumab(GalNAz)₂ (**III-2**)*

To a solution of trastuzumab(GalNAz)₂ (8.8 μ L, 0.2 mg, 22.7 mg/mL in Tris buffer pH 7.5 10 mM) was added Tris buffer pH 7.5 10 mM (4 μ L) and substrate **7a-c** or **8a-c** (2 μ L, 2 mM solution in MilliQ + 5% DMA). The reaction was incubated at rt and samples (2 μ L) were taken at pre-set time points. These samples were incubated with DTT (2 μ L 0.2 M), diluted with MilliQ (30 μ L) and subjected to MS analysis (AccuTof) to determine the conjugation efficiency (see Table S1).

*Table S1. Conversion of trastuzumab(GalNAz)₂ **III-2** upon treatment with **7a-c** or **8a-c** as judged by mass spectral analysis.*

III-2 + compound	conversion (%)				
	30'	60'	90'	120'	960'
7a	0	10	20	30	80
7b	20	20	30	60	80
7c	30	40	60	80	100
8a	20	25	30	30	30
8b	30	40	60	65	65
8c	10	10	10	10	20

*Conjugation of **III-2** with BCN-linker-payload constructs provided in Figure S1 and Table 1 according to the general procedure provided in the Experimental Procedures*

A set of ADCs was prepared from trastuzumab(GalNAz)₂ **III-2** according to the general protocols provided in the Experimental Procedures. After reduction with DTT, mass spectral analysis was performed to determine the conversion (Table S2).

Figure S1. Structures of Linker-Payloads

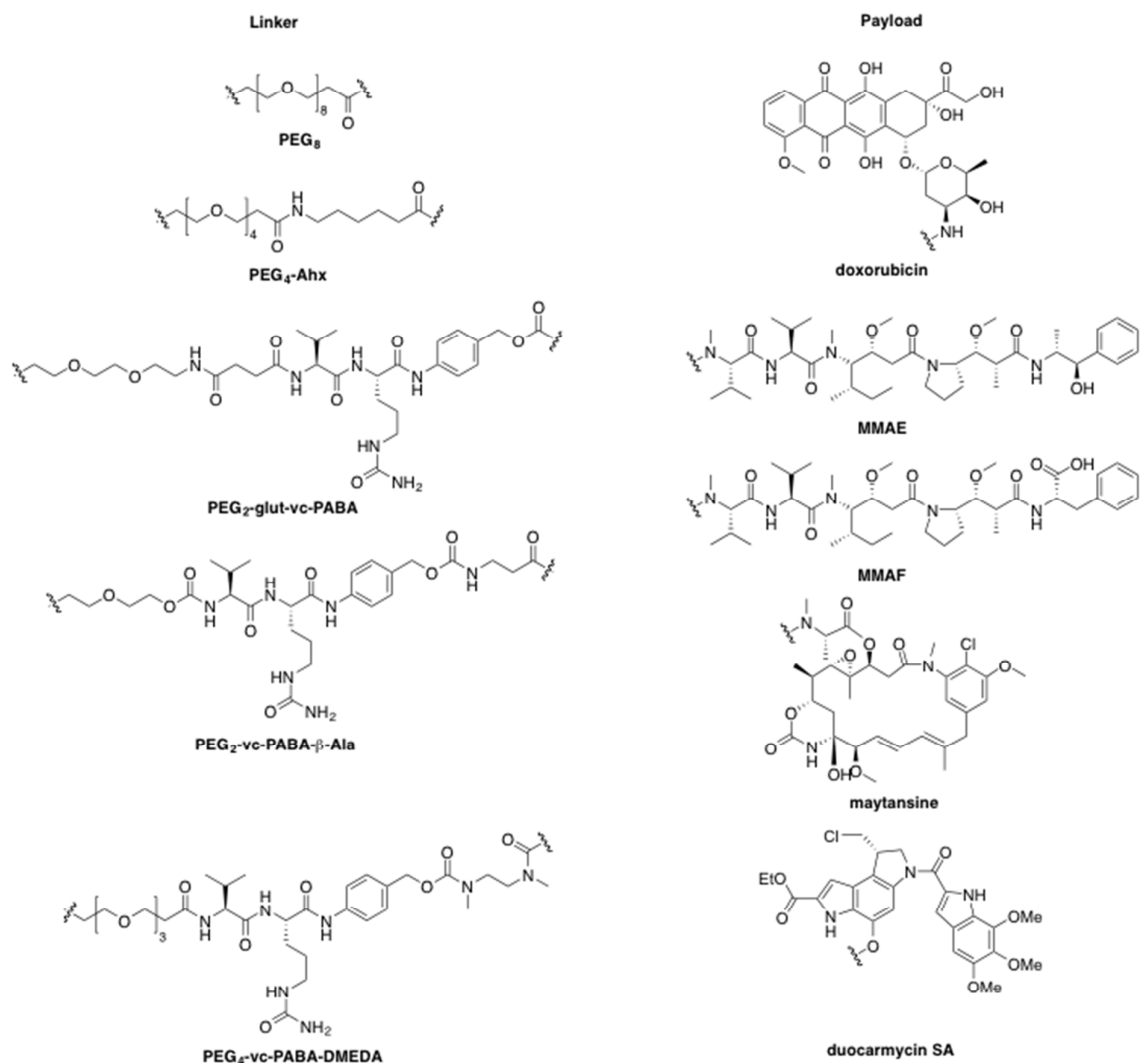


Table S2. Mass spectral characterization of ADCs prepared from **III-2**.

Linker	Payload	Co-solvent	Amount (%)	Equiv	Conv (%)	Mass spectral data of ADC
PEG ₈	doxorubicin	DMF	5	2	>95	50883
PEG ₂ -glut-vc-PABA	MMAE			5	>95	51283
PEG ₂ -glut-vc-PABA	MMAF			1.5	>95	51295
PEG ₄ -Ahx	maytansine			2	90	50924
PEG ₂ -vc-PABA-- β -Ala	maytansine		25	2.5	60	51174
PEG ₄ -vc-PABA-DMEDA	duocarmycin SA		12.5	1.5	85	51201

Stability study in plasma of trastuzumab(GalNAz-MMAF)₂

Human plasma was centrifuged at 200 g for 10 minutes at 4 °C and the supernatant was incubated with protein A agarose (Kem-En-Tec) for 1 hour at 4 °C to deplete for IgG.

The depleted plasma was filter sterilized using a 0.22 μ m filter (Whatman). Trastuzumab(GalNAz-MMAF)₂ was added to the sterile human plasma to a final concentration of 100 μ g/mL and incubated at 37 °C in a CO₂ incubator to keep plasma pH levels close to the physiological pH of 7.2. Samples were taken at 0, 24, 48 and 144 hours and stored at -80°C. The trastuzumab conjugate was purified with protein A agarose followed by MS analysis. Human plasma samples were incubated with protein A agarose for 1 hour at 4 °C, washed three times with phosphate-buffered saline (PBS) and the trastuzumab conjugate was eluted with 100 mM glycine-HCl pH 2.7 followed by neutralization with 1 M Tris-HCl pH 8.0. MS analysis showed that the peak corresponding to the trastuzumab(GalNAz-MMAF)₂ conjugate (50706 Da) did not decrease in time. In addition, no peaks corresponding to degradation products could be detected, proving that the conjugate is stable for at least 144 hours in human plasma (Figure S2).

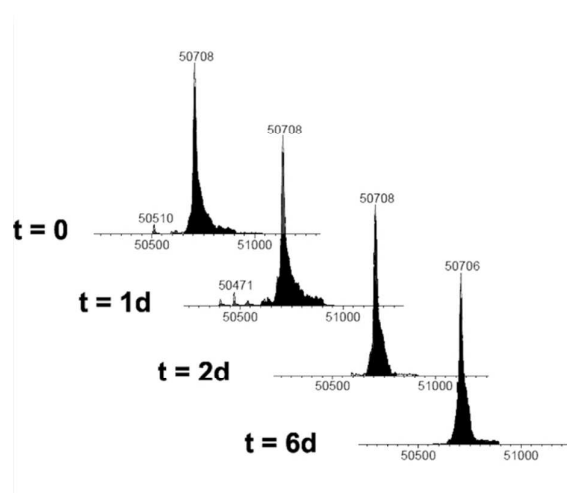


Figure S2. Mass spectral analysis after reduction showing the heavy chain fragment trastuzumab(GalNAz-MMAF)₂ after incubation in human plasma for 0, 1, 2 and 6 days.

Aggregation study of DBCO- and BCN-containing ADCs

Trastuzumab(F₂-GalNAz-DBCO-PEG₄-Ahx-May)₂ and trastuzumab(F₂-GalNAz-BCN-PEG₄-Ahx-May)₂ were incubated at a concentration of 1 mg/mL in 25 mM Na-citrate pH 5.0 and 150 mM NaCl for 2 weeks at 40 °C. After 0, 2, 7, 10 and 14 days the percentage of aggregate formation was determined. After 2 weeks the DBCO-containing ADC showed 37.5% of aggregates while the BCN-containing ADC showed only 28.6% aggregation (Figure S3A). A similar experiment was performed at physiological pH by incubation in PBS pH 7.4. Under these conditions both ADCs showed less than 1% aggregation after 2 weeks at 40 °C, although the DBCO-containing ADC still showed a higher levels of aggregation than the BCN-containing ADC (Figure S3B).

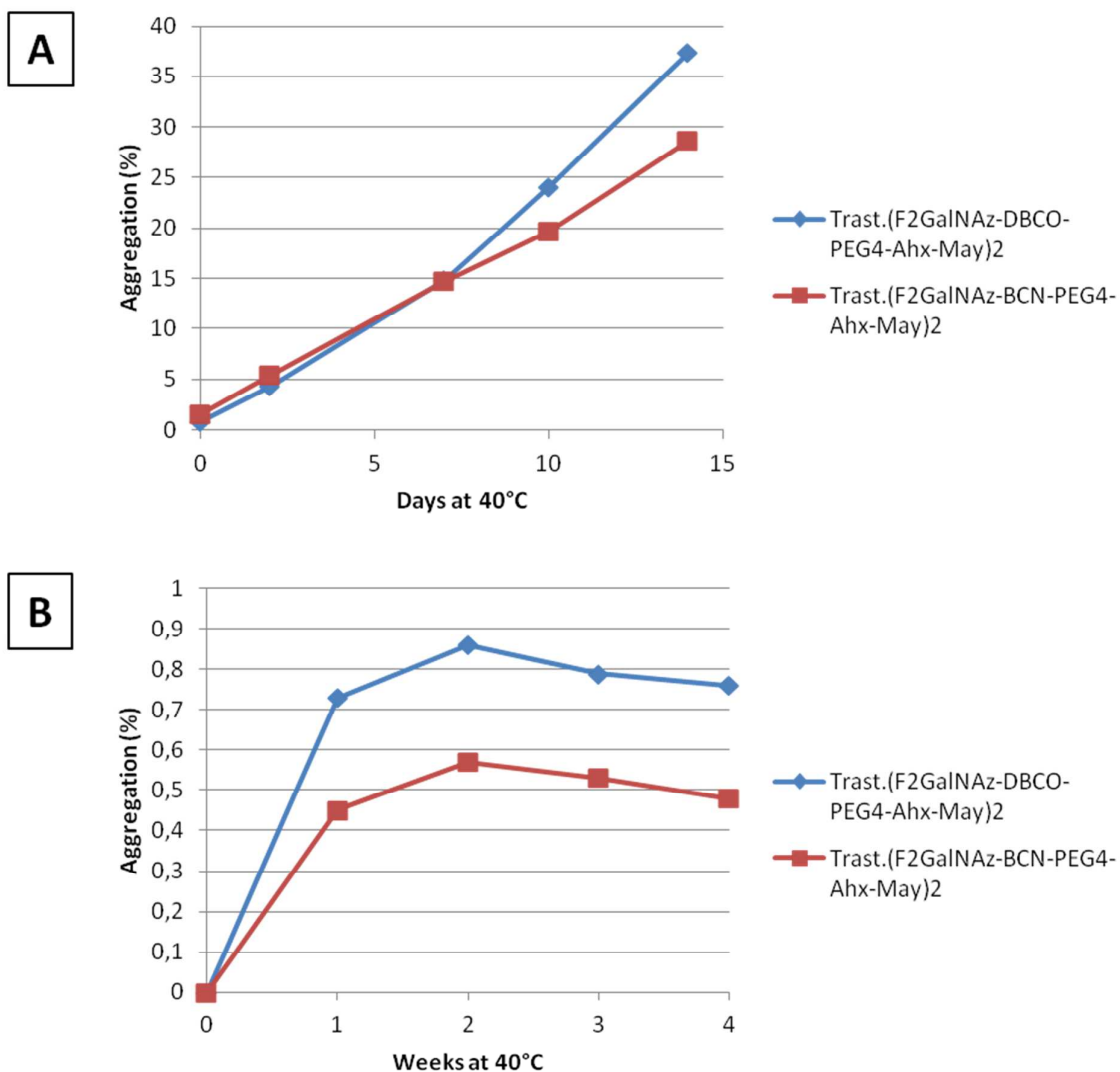


Figure S3. Aggregation behaviour compared between a DBCO- and BCN-containing ADC by incubation at a concentration of 1 mg/mL in (A) Na-citrate pH 5.0, 150 mM NaCl and (B) PBS pH 7.4. After 0, 2, 7, 10 and 14 days samples were analyzed using a superdex200 pc 3.2/30 (GE Healthcare) on an AKTA Purifier-10 (GE Healthcare). The percentage of aggregate peaks compared to the total peak area was quantified using the absorbance at 215 nm.

Large scale preparation

Trimming of rituximab

Rituximab (707 mL, 9.4 mg/mL, 44 μ m) was trimmed with endo S (3.7 mL, 28 mg/mL) in Tris-HCl (25 mM, pH 8.0) for 16 hours at 37 °C. AccuTOF measurements after DTT showed complete conversion (mass heavy chain 49408 Da).

GalNAz-transfer to rituximab

The trimmed rituximab (530 mL, 9.4 mg/mL, 33 μ mol) was incubated with UDP-GalNAz (4.9 mL, 0.1 M) and β 1,4-GalT(Y289L) (21 mL, 2 mg/mL) in 10 mM MnCl₂ and 25 mM Tris-HCl pH 8.0 for 16 hours at 30 °C. Purification with ProtA gave rituximab(GalNAz)₂ (4.77 g, 10.42 mg/mL). AccuTOF measurements showed after DTT showed complete conversion of the reduced azide (mass heavy chain 49626).

Conjugation with doxorubicin

To a solution of rituximab(GalNAz)₂ (4.77 g, 10.42 mg/mL, 31.8 μ mol) was added BCN-doxorubin (0.1 M in DMF, 0.19 mmol). The solution was slowly stirred overnight and subsequent AccuTOF analysis after DTT showed complete conversion (mass heavy chain 50795 Da).

In vitro efficacy

SK-Br-3 (Her2+) and MDA-MB-231 (Her2-) cells were plated in 96-well plates (5000 cells/well) in RPMI 1640 GlutaMAX (Invitrogen) supplemented with 10% fetal calf serum (FCS) (Invitrogen, 200 μ L/well) and incubated overnight at 37 °C and 5% CO₂. A three-fold dilution series (ranging from \pm 0.002 to 100 nM) of the sterile-filtered compounds was prepared in RPMI 1640 GlutaMAX supplemented with 10% FCS. After removal of the culture medium, the concentration series were added in quadruplo and incubated for three days at 37°C and 5% CO₂. The culture medium was replaced by 0.01 mg/mL resazurin (Sigma Aldrich) in RPMI 1640 GlutaMAX supplemented with 10% FCS. After 4 to 6 hours at 37 °C and 5% CO₂ fluorescence was detected with a fluorescence plate reader (Tecan Infinite 200) at 540 nm excitation and 590 nm emission. The relative fluorescent units (RFU) were normalized to cell viability percentage by setting wells without cells at 0% viability and wells with lowest dose of compound at 100% viability. For each conditions the average cell viability percentage \pm sem is shown.

The in vitro cytotoxicity of trastuzumab-(vc-PABA-MMAF)₂ (entry 3 in Table 1) and trastuzumab-(vc-PABA-maytansine)₂ (entry 5 in Table 1) were compared to Kadcyla as a positive control and trastuzumab as a negative control. All trastuzumab-based ADCs affect the viability of the Her2-positive cell line SK-Br-3, but not of the Her2-negative cell line MDA-MB-231, which shows that these ADCs specifically target Her2-positive cells.

DTPA conjugation, radio-labeling and determination of IRF

Trastuzumab(vc-PABA-MMAF)₂ (Entry 3 from Table 1) and trastuzumab(vc-PABA-maytansine)₂ (Entry 5 from Table 1), trimmed trastuzumab (obtained from treatment of trastuzumab and endo S) and native trastuzumab were conjugated with isothiocyanate-DTPA (Macrocyclics, Houston, TX) and labeled with ¹¹¹In (Covidien, Petten). For the animal experiments, all constructs were labeled at specific activity between 2.7 and 3.4 MBq/ μ g.

Pharmacokinetics studies

BALB/c nude mice (n=15) were inoculated subcutaneously with 5×10^6 SK-OV-3 cells (injection volume: 200 μ l) in the right flank. At 14 days after tumor induction, groups of five mice were injected i.v. with 15-20 MBq of ^{111}In -labeled antibody, labeled at a protein dose of 25 μ g. To assess the in vivo stability and pharmacokinetics of the conjugates, 50 μ l blood samples were taken via submandibular bleeding at 0.5, 1, 30 min, 1, 2 and 4 hours, and 1, 2, 5, 7, 9, 13, 16 and 21 days after injection of the radiolabeled antibody preparations.

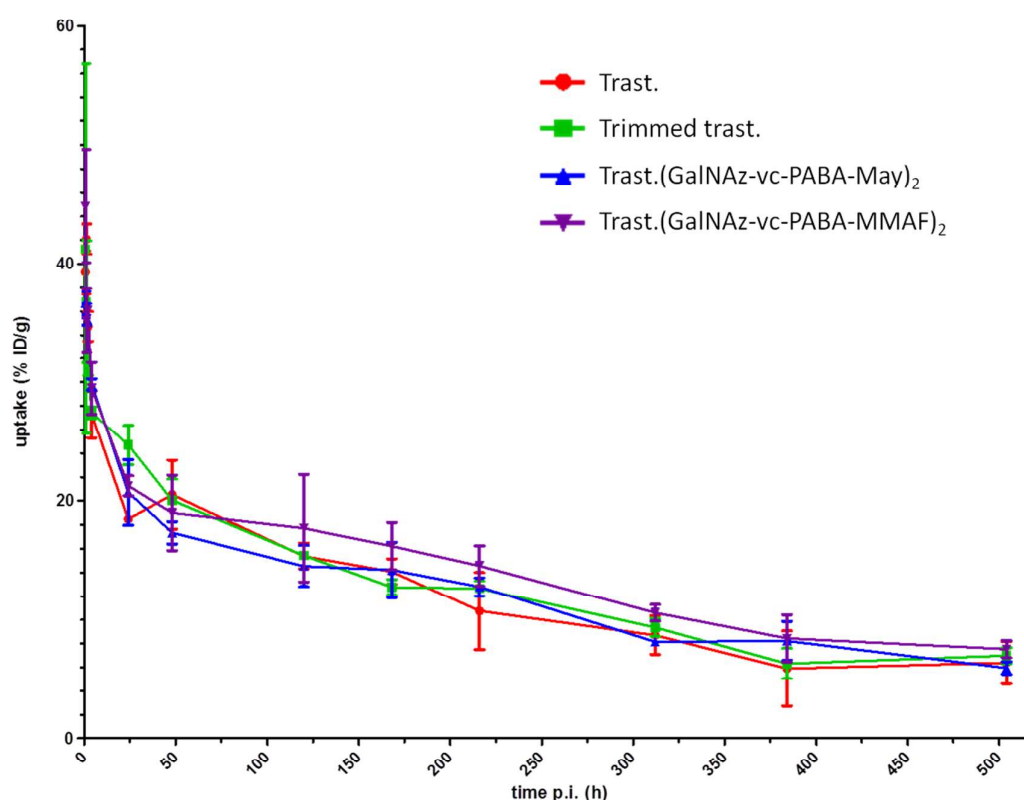


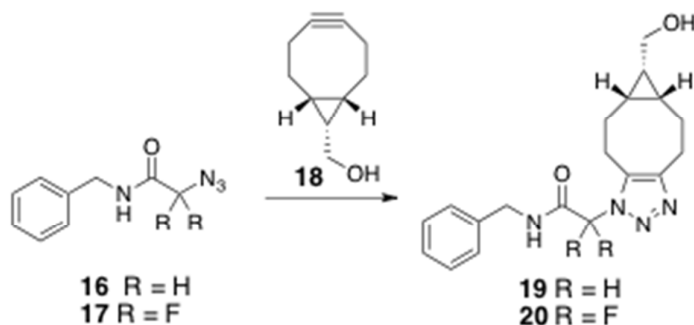
Figure S4. The total level of Indium-111-labeled antibody or antibody conjugate in blood samples was determined up to 3 weeks after injections for trastuzumab (red line), Endo S-trimmed trastuzumab (green line), trastuzumab(GalNAz-vc-PABA-maytansine)₂ (blue line) and trastuzumab(GalNAz-vc-PABA-MMAF)₂ (purple line).

In vivo efficacy studies

Female SHO mice (CrI:SHO-Prkdcscid Hrhr, 6- to 9-week-old at the beginning of the experimental phase, obtained from Charles River Laboratories, L'Arbresles, France) were anaesthetized with

ketamine/xylazine, the skin was aseptized with a chlorhexidine solution, incised at the level of the interscapular region, a 20 mm³ tumor fragment 20 (HBCx-13B breast cancer patient-derived xenograft model) was placed in the subcutaneous tissue and the skin was closed with clips. When the tumor volume was in the range of 60 to 200 mm³, groups of four mice were injected i.v. with either vehicle, trastuzumab-(vc-PABA-maytansine)₂ (entry 5 from Table 1, at 3 mg/kg and 9 mg/kg), trastuzumab-(vc-PABA-MMAF)₂ (entry 3 from Table 1, at 3 mg/kg and 9 mg/kg), and Kadcyra (9 mg/kg) and tumors were measured twice weekly.

Competition experiment between 2-azido-N-benzylacetamide (16) and 2-azido-N-benzyl-2,2-difluoroacetamide (17) with BCN-OH (18).



Ethyl 2-azido-2,2-difluoroacetate

To a solution of ethyl 2-bromo-2,2-difluoroacetate (950 mg, 4.68 mmol) in dry DMSO (5 mL) was added sodium azide (365 mg, 5.62 mmol). After stirring overnight at room temperature, the reaction mixture was poured out into water (150 mL). The layers were separated, dichloromethane was added to the organic layer and the layer was dried over sodium sulfate (Na₂SO₄). After filtration, the solvent was removed under reduced pressure (300 mbar) at 35 °C affording the crude product (250 mg, 1.51 mmol, 32%). ¹H-NMR (300 MHz, CDCl₃): δ 4.41 (q, *J* = 7.2 Hz, 2H), 1.38 (t, *J* = 6.9 Hz, 3H).

2-Azido-N-benzylacetamide (16)

Azidoacetic acid succinimidyl ester¹ (67 mg, 0.338 mmol) was dissolved in dichloromethane (4 mL) and benzylamine (44 μL, 0.406 mmol) and Et₃N (70 μL, 0.507 mmol) were added. The reaction was allowed to stir overnight and the solvent was removed under reduced pressure. Flash chromatography (1:1 -> 9:1 EtOAc:pentane) afforded **16**. ¹H NMR (300 MHz, CDCl₃): δ 7.37-7.24 (m, 5H), 4.44 (d, *J* = 5.7 Hz, 2H), 3.97 (s, 2H).

2-Azido-2,2-difluoro-N-benzylacetamide (17)

Ethyl 2-azido-2,2-difluoroacetate (48 mg, 0.291 mmol) was dissolved in dichloromethane (4 mL) and benzylamine (32 μL, 0.291 mmol) and Et₃N (60 μL, 0.436 mmol) were added. The reaction was

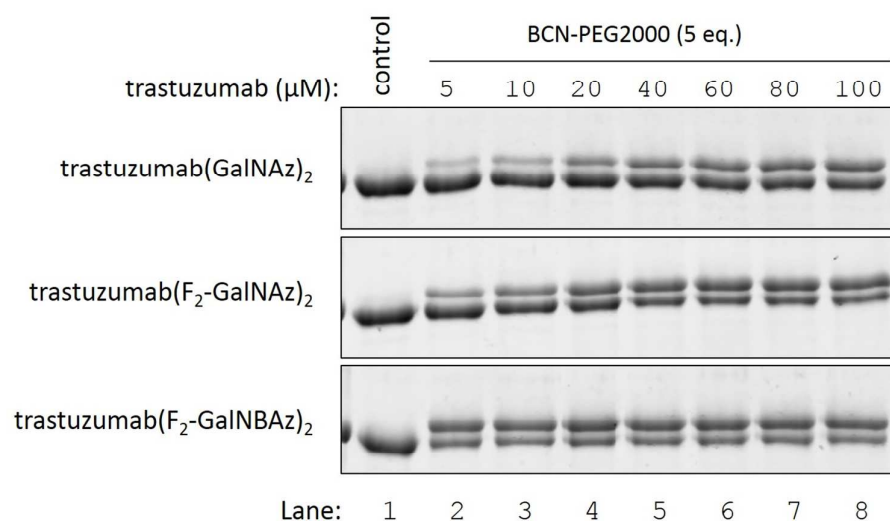
allowed to stir overnight and the solvent was removed under reduced pressure. Flash chromatography (15:1 pentane:EtOAc) afforded **17**. ^1H NMR (300 MHz, CDCl_3): δ 7.40-7.25 (m, 5H), 4.50 (d, J = 5.7 Hz, 2H).

*Competition experiment between **16** and **17** with BCN-OH (**18**).*

First, 2-azido-*N*-benzylacetamide (**16**) (10.0 mg, 0.044 mmol) and 2-azido-*N*-benzyl-2,2-difluoroacetamide (**17**) (8.4 mg, 0.044 mmol) were dissolved in CD_3CN (815 μL) and D_2O (407 μL) was added. Next, BCN-OH (**18**) (1.33 mg, 0.0088 mmol, 0.2 equiv. with respect to each azide) was dissolved in $\text{CD}_3\text{CN}:\text{D}_2\text{O}$ 2:1 and added to the reaction mixture. After 2 h, the solvent was removed under reduced pressure, the crude product was dissolved in CDCl_3 and an ^1H NMR spectrum was measured to determine the ratio of the formed products **19** and **20** as 1:2.3. ^1H NMR (300 MHz, CDCl_3): δ 7.42-7.23 (m, 11H), 7.18-7.14 (m, 0.11H), 6.69 (br s, 1H), 6.61 (br s, 1H), 4.95 (s, 0.10H), 4.62 (d, J = 6.0 Hz, 0.25 H), 4.50 (d, J = 5.7 Hz, 2H), 4.46 (d, J = 6.0 Hz, 2H), 4.39 (dd, J = 6.0, 3.3 Hz, 0.1H), 4.02 (s, 2H), 3.78-3.65 (m), 3.24-2.82 (m), 2.35-2.19 (m), 1.78-1.52 (m), 1.40-1.05 (m), 1.04-0.79 (m).

*Conjugation of trastuzumab(*GalNAz*)₂ **III-2**, trastuzumab(*F*₂-*GalNAz*)₂ **III-4** and trastuzumab(*F*₂-*GalNBAz*)₂ **III-5** with BCN-PEG₂₀₀₀ at variable IgG concentrations*

A dilution series of trastuzumab(*GalNAz*)₂ (**III-2**), trastuzumab(*F*₂-*GalNAz*)₂ (**III-4**) or trastuzumab(*F*₂-*GalNBAz*)₂ (**III-5**) (5, 10, 20, 40 60, 80 and 100 μM) in PBS was incubated overnight at room temperature with 5 equivalents of BCN-PEG₂₀₀₀ (25, 50, 100, 200, 300, 400 and 500 μM). Reaction products were analyzed through reduced SDS-PAGE followed by coomassie staining.

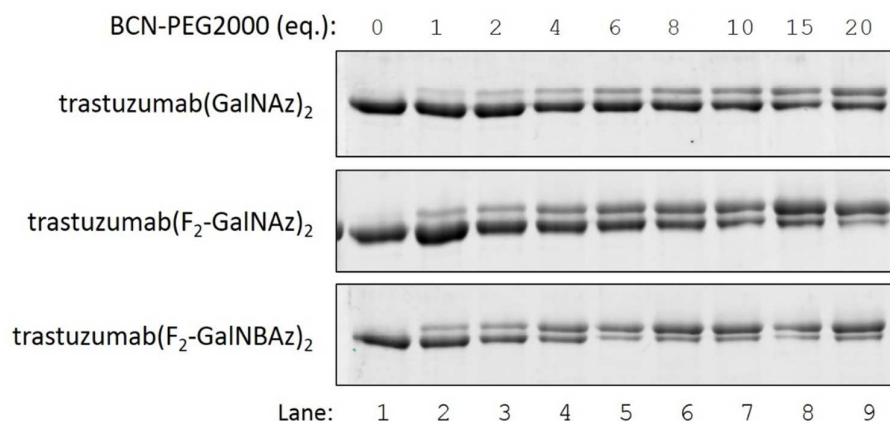


*Figure S5. SDS-PAGE of the heavy chain of trastuzumab(*GalNAz*)₂ **III-2**, trastuzumab(*F*₂-*GalNAz*)₂ **III-4** and trastuzumab(*F*₂-*GalNBAz*)₂ **III-5** before conjugation to BCN-PEG₂₀₀₀ (lower band) and*

after conjugation to BCN-PEG₂₀₀₀ (upper band). Trastuzumab which was not incubated with BCN-PEG₂₀₀₀ was used as a negative control (lane 1). Trastuzumab(GalNAz)₂ **III-2** shows approximately 50% conversion at an IgG concentration of 100 μ M (upper panel, lane 8) while trastuzumab(F₂-GalNAz)₂ **III-4** shows approximately 50% conversion at an IgG concentration of 40 μ M (middle panel, lane 5). Trastuzumab(F₂-GalNBaz)₂ **III-5** shows approximately 50% conversion at an IgG concentration of 5 μ M IgG (lower panel, lane 2).

*Conjugation of of trastuzumab(GalNAz)₂ **III-2** and trastuzumab(F₂-GalNAz)₂ **III-4** with BCN-PEG₂₀₀₀ at variable concentrations of BCN-PEG₂₀₀₀*

Trastuzumab(GalNAz)₂ **III-2**, trastuzumab(F₂-GalNAz)₂ **III-4** or trastuzumab(F₂-GalNBaz)₂ **III-5** (10 μ M IgG) in PBS was incubated overnight at room temperature with 0 to 20 equivalents of BCN-PEG₂₀₀₀ (0 to 200 μ M). Reaction products were separated by reducing SDS-PAGE followed by coomassie staining.



*Figure S6. SDS-PAGE of the heavy chain of trastuzumab(GalNAz)₂ **III-2**, trastuzumab(F₂-GalNAz)₂ **III-4** and trastuzumab(F₂-GalNBaz)₂ **III-5** before conjugation to BCN-PEG₂₀₀₀ (lower band) and after conjugation to BCN-PEG₂₀₀₀ (upper band). Trastuzumab(GalNAz)₂ **III-2** shows less than 50% conversion when incubated with 20 equivalents BCN-PEG₂₀₀₀ (upper panel, lane 9), while trastuzumab(F₂-GalNAz)₂ **III-4** shows approximately 50% conversion when incubated with only 8 equivalents BCN-PEG₂₀₀₀ (middle panel, lane 6). Trastuzumab(F₂-GalNBaz)₂ **III-5** shows approximately 50% conversion using only 4 equivalents BCN-PEG₂₀₀₀ (lower panel, lane 4).*

*Conjugation of **III-4** or **III-5** with BCN-linker-payload constructs according to the general procedure provided in the Experimental Procedures*

A set of ADCs was prepared from trastuzumab(F₂-GalNAz)₂ **III-4** or trastuzumab(F₂-GalBAz)₂ **III-5** according to the general protocols provided in the Experimental Procedures. After reduction with DTT, mass spectral analysis was performed to determine the conjugation efficiency for

ADCs prepared from trastuzumab(F₂-GalNAz)₂ **III-4** (Table S3) or trastuzumab(F₂-GalBAz)₂ **III-5** (Table S4).

Table S3. Mass spectral characterization of ADCs prepared from III-4.

Linker	Payload	Co-solvent	Amount (%)	Equiv	Conv (%)	Mass spectral data of ADC
PEG ₂ -glut-vc-PABA	MMAF	DMSO	5	4	>95	51335
PEG ₄ -Ahx	maytansine	DMF	5	3	>95	50932
PEG ₂ -vc-PABA-β-Ala	maytansine	DMA		3	>95	51218
PEG ₄ -vc-PABA-DMEDA	duocarmycin SA		2.5	3	>95	51237

Table S4. Mass spectral characterization of ADCs prepared from III-5.

Linker	Payload	Co-solvent	Amount (%)	Equiv	Conv (%)	Mass spectral data of ADC
PEG ₄ -Ahx	maytansine	DMA	5	3	>95	51028
PEG ₂ -vc-PABA-Ahx	maytansine			3	>95	51360
PEG ₂ -glut-vc-PABA	MMAF	DMSO	5	4	>95	51397

Preparation of antibody conjugates from monoclonal antibodies infliximab, palivizumab, panitumumab, natalizumab upon trimming, treatment with UDP-F₂-GalNAz (4) and conjugation with maytansine.

The remodeling of all four IgGs was performed according to the general protocols provided in the Experimental Procedures. Mass spectral analysis of the heavy chain fragments are shown at the various stages of the remodelling process for infliximab (Figure S7), palivizumab (Figure S8), panitumumab (Figure S9) and natalizumab (Figure S10).

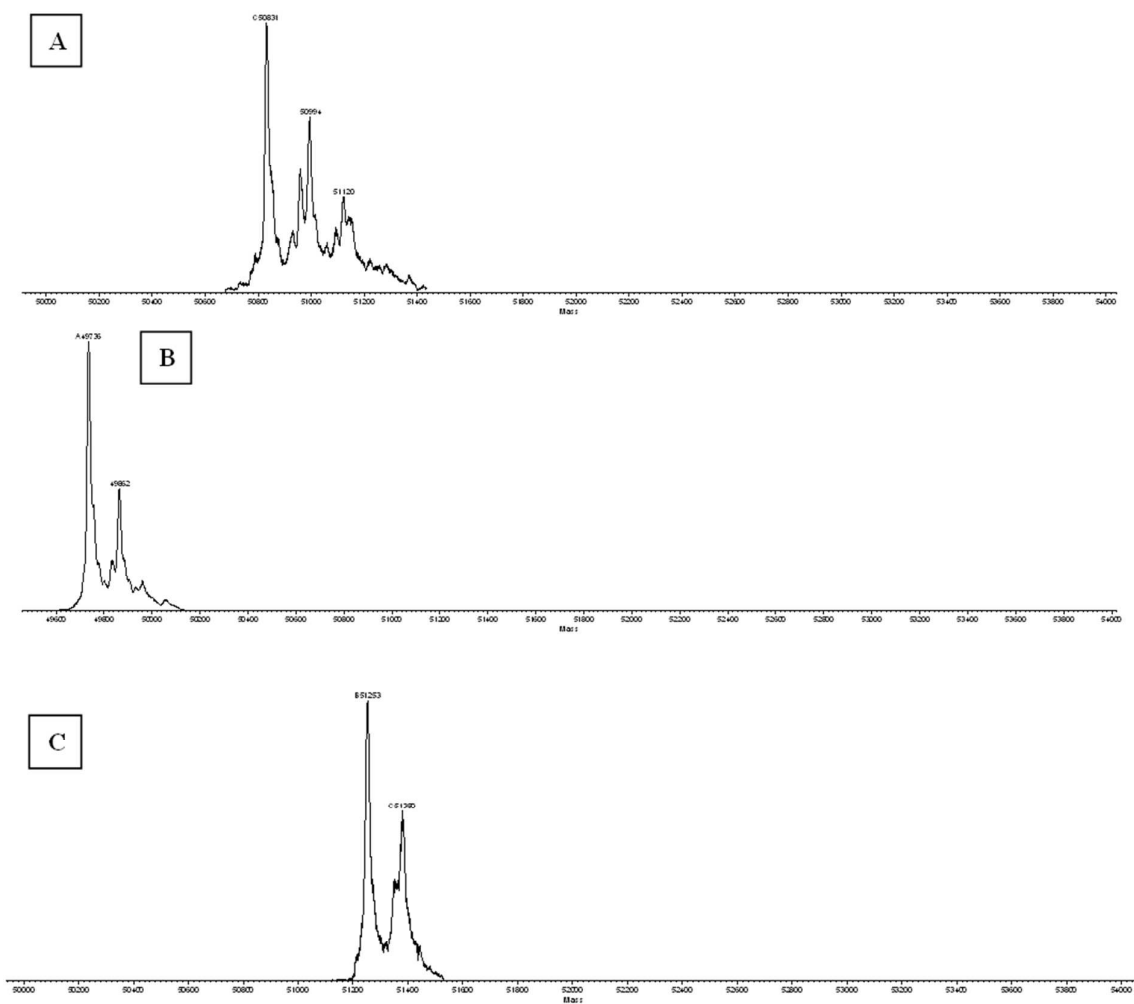


Figure S7. Glycan-remodeling-conjugation of infliximab showing the mass spectral profile of the heavy chain region of (A) native IgG (main peaks of 50831, 50994 and 51120 Da), (B) trimmed IgG (main peaks of 49736 and 49862 Da) and (C) maytansine-conjugated IgG (main peaks of 51253 and 51380 Da).

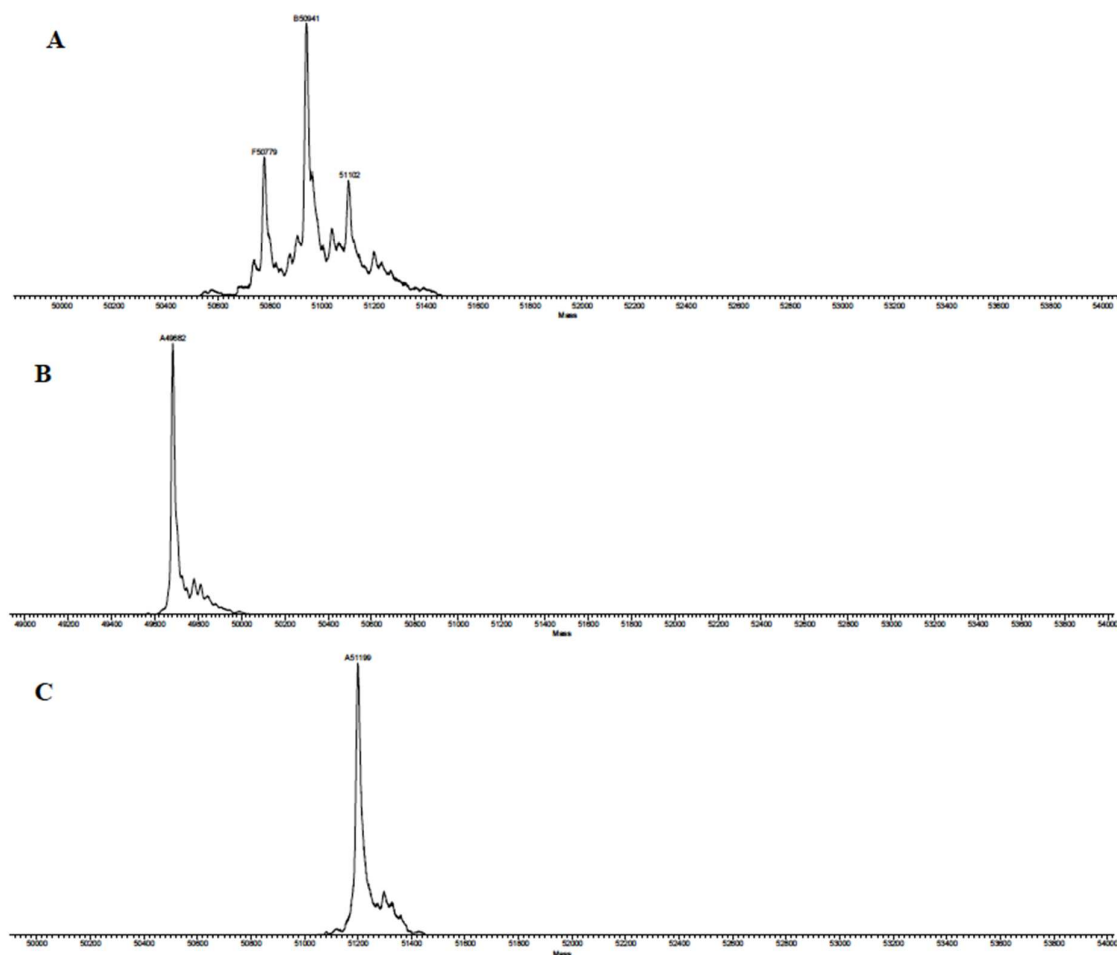


Figure S8. Glycan-remodeling–conjugation of showing the mass spectral profile of the heavy chain region of palivizumab (A) native IgG (main peaks of 50779, 50941 and 51102 Da), (B) trimmed IgG (main peak of 49682 Da) and (C) maytansine-conjugated IgG (main peak of 51199 Da).

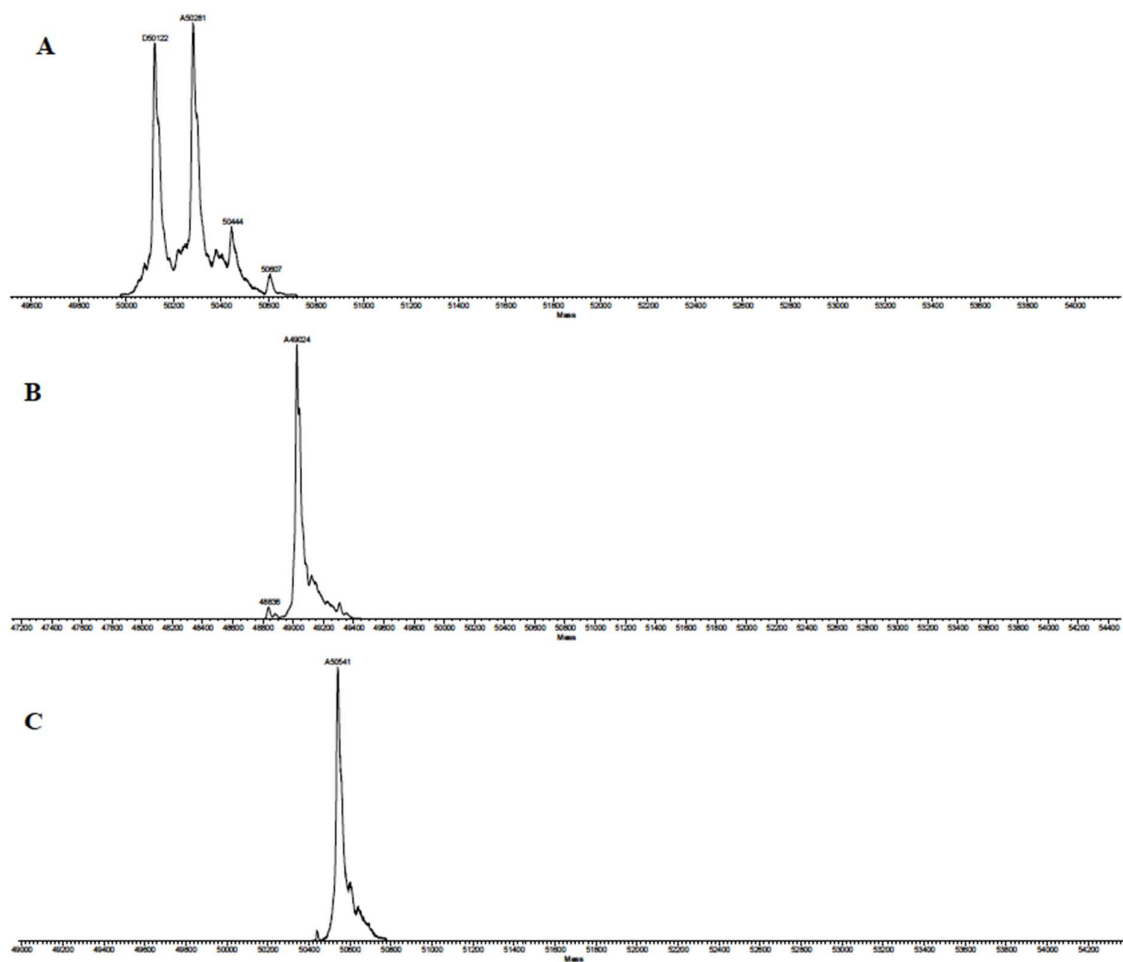


Figure S9. Glycan-remodeling–conjugation of showing the mass spectral profile of the heavy chain region of panitumumab (A) native IgG (main peaks of 50122 and 50281 Da), (B) trimmed IgG (main peak of 49024 Da) and (C) maytansine-conjugated IgG (main peak of 50541 Da).

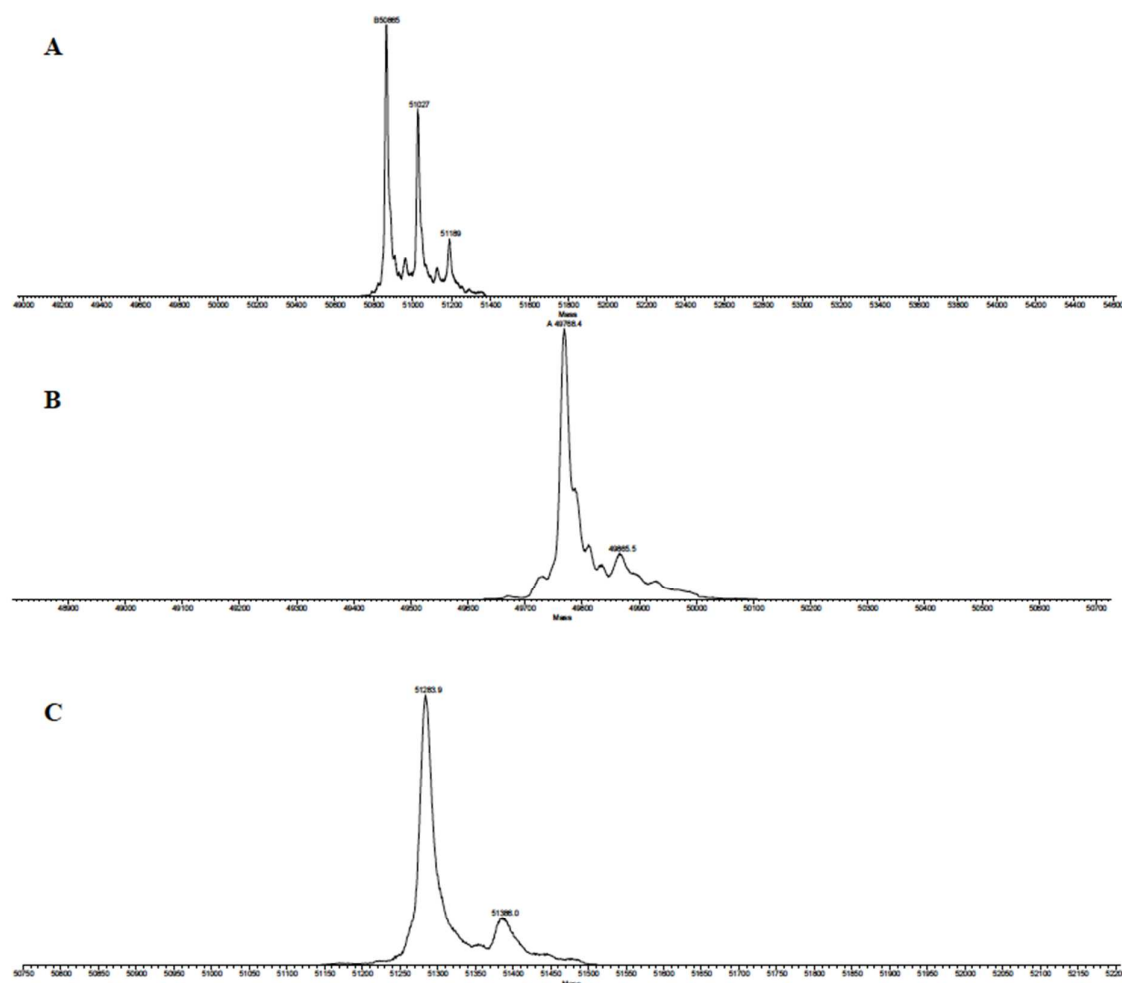


Figure S10. Glycan-remodeling–conjugation of showing the mass spectral profile of the heavy chain region of natalizumab (A) native IgG (main peaks of 50865 and 51027 Da), (B) trimmed IgG (main peak of 49768 Da) and (C) maytansine-conjugated IgG (main peak of 51284 Da).

Transient expression of a trastuzumab glycomutants

Both native trastuzumab and mutant antibodies were transiently expressed in CHO K1 cells by Evitria (Zurich, Switzerland) at 20-25 mL scale, purified using protein A sepharose and analyzed by mass spectrometry. Specific mutants of trastuzumab HC-L196N, HC-G164S and LC-R18N were derived from literature^{2,3} or de novo designed (HC-K291T, HC-V366T). In all cases, a second mutation involved the mutation of Asp-297 to Glu (N297Q) to remove the native glycosylation site.

General Protocol for trimming of IgG glycans with endo F3

Trimming of IgG glycans was performed using endo F3 from *Elizabethkingia meningosepticum* (commercially available from QA Bio). Thus, IgG (10 mg/mL) was incubated with endo F3 (25 mU/mg IgG) in 100 mM sodium citrate pH 4.5 for 16 hours at 37 °C. The deglycosylated IgG was

concentrated and washed with 25 mM Tris-HCl pH 8.0 using an Amicon Ultra-0.5, Ultracel-10 Membrane (Millipore).

Trimming of trastuzumab-mutant N300Q,L196N

Trastuzumab(N300Q, L196N) mutant with major base MS peaks at 50934, 51225 and 51516 Da, corresponding to G2F, G2FS and G2FS2 glycosylation isoforms, was subjected to the trimming protocol with endo F3 as described above. After deconvolution of peaks, the mass spectrum showed one peak of the light chain and one major peak of the heavy chain (49514 Da, 90% of total heavy chain), resulting from core GlcNAc(Fuc)-substituted trastuzumab-(N300Q, L196N) mutant. On the other hand, trastuzumab-(N300Q, L196N) mutant was completely inert to hydrolysis by the action of endo S.

Trimming of trastuzumab-mutant N300Q,G164S

Trastuzumab(N300Q, G164S) mutant with major base MS peaks at 50961, 51251 and 51542 Da, corresponding to G2F, G2FS and G2FS2 glycosylation isoforms, was subjected to the trimming protocol using endo F3 as described above. After deconvolution of peaks, the mass spectrum showed one peak of the light chain and one major peak of the heavy chain (49537 Da, 90% of total heavy chain), resulting from core GlcNAc(Fuc)-substituted trastuzumab-(N300Q, G164S).

Trimming of trastuzumab-mutant N300Q,V366T

Trastuzumab(N300Q, V366T) mutant with major base MS peaks at 50934, 51227 and 51517 Da, corresponding to G2F, G2FS and G2FS2 glycosylation isoforms, was subjected to the trimming protocol with endo F3 as described above. After deconvolution of peaks, the mass spectrum showed one peak of the light chain and one peak of the light chain and one major peak of the heavy chain (49515 Da, 90% of total heavy chain), resulting from core GlcNAc(Fuc)-substituted trastuzumab-(N300Q, V366T) mutant.

Trimming of trastuzumab-mutant N300Q,K291T

Trastuzumab(N300Q, K291T) mutant with major base MS peaks of the Fc/2 fragment at 25548, 25839 and 26131 Da, corresponding to G2F, G2FS and G2FS2 glycosylation isoforms, was subjected to the trimming protocol with endo F3 as described above. After deconvolution of peaks, the mass spectrum of the Fabricator-digested sample showed one major peak of the Fc/2 fragment (24127 Da, 90% of total Fc/2 fragment), resulting from core GlcNAc(Fuc)-substituted trastuzumab-(N300Q, K291T) mutant.

Trimming of trastuzumab mutant LC-R18N, HC-N300Q

Trastuzumab(LC-R18N, HC-N300Q) mutant with major base MS peaks at 25462 and 25754 Da for the light chain, was subjected to the trimming protocol with endo F3 as described above. After deconvolution of peaks, the mass spectrum of the reduced sample showed one major peak of the light chain (23750 Da, 90% of total light chain), resulting from core GlcNAc(Fuc)-substituted trastuzumab-(LC-R18N, HC-N300Q) mutant.

Glycosyltransfer of UDP-F₂-GalNAz (4) to deglycosylated trastuzumab mutants

Glycosyltransfer of **4** with GalT(Y289L) was executed according to the general protocol provided in the Experimental Procedures.

In vivo efficacy study of glycosylation mutants

Female SHO mice (CrI:SHO-Prkdcscid Hrhr, 6- to 9-week-old at the beginning of the experimental phase, obtained from Charles River Laboratories, L'Arbresles, France) were anaesthetized with ketamine/xylazine, the skin was aseptized with a chlorhexidine solution, incised at the level of the interscapular region, a 20 mm³ tumor fragment (HBCx-13B breast cancer patient-derived xenograft model) was placed in the subcutaneous tissue and the skin was closed with clips. When the tumor volume was in the range of 60 to 200 mm³, groups of ten mice were injected i.v. with either vehicle, with trastuzumab(F₂-GalNAz-Ahx-maytansine)₂ or with one of the glycosylation mutants trastuzumab(HC-G164S, HC-N300Q), trastuzumab(HC-L196N, HC-N300Q), trastuzumab(HC-K291T, HC-N300Q), trastuzumab(HC-N300Q, HC-V366T), trastuzumab(HC-L177N, HC-N300Q) or trastuzumab (LC-R18N, HC-N300Q), all dosed at 6 mg/kg. Tumors were measured on day 0, 4, 7, 11, 14, 18, 21, 25 and 28.

Cloning and expression of GalT(Y289M)

The GalT mutant gene was amplified from a construct containing the sequence encoding the catalytic domain of bovine β 1,4-galactosyltransferase I consisting of 130-402 aa residues, by the overlap extension PCR method. The first DNA fragment was amplified with a pair of primers: GalT_External_Fw (CAG CGA CAT ATG TCG CTG ACC GCA TGC CCT GAG GAG TCC) and GalT_Y289M_Rw (GAC ACC TCC AAA **CAT** CTG CAC GTA AGG TAG GCT AAA). The NdeI restriction site is underlined, while the mutation site is highlighted in bold. The second fragment was amplified with a pair of primers: GalT_External_Rw (CTG ATG GAT GGA TCC CTA GCT CGG CGT CCC GAT GTC CAC) and GalT_Y289N_Fw (CCT TAC GTG CAG **ATG** TTT GGA GGT GTC TCT GCT CTA). The BamHI restriction site is underlined, while the mutation site is highlighted in bold. The two fragments generated in the first round of PCR were fused in the second round using the GalT_External_Fw and GalT_External_Rw primers. After digestion with NdeI and BamHI. This fragment was ligated into the pET16b vector cleaved with the same restriction enzymes. The newly

constructed expression vector contained the gene encoding Y289M mutant and the sequence encoding for the His-tag from pET16b vector, which was confirmed by DNA sequencing results.

GalT mutants were expressed, isolated and refolded from inclusion bodies according to the reported procedure by Qasba et al.⁴ After refolding, the precipitate was removed and the soluble and folded protein was isolated using a Ni-NTA column (HisTrap HP 5 mL column, GE Healthcare). After elution with 25 mM Tris-HCl pH 8.0, 300 mM NaCl and 200 mM imidazole, the protein was dialyzed against 25 mM Tris-HCl pH 8.0 and concentrated to 2 mg/mL using a spinfilter (Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-10 membrane, Merck Millipore).

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