General Information

Chemicals and Solvents

Chemicals and solvents were purchased from Merck (Merck group, Germany), TCI (Tokyo chemical industry CO., LTD., Japan) and Acros Organics (Thermo Fisher scientific, USA) and used without further purification. Dry solvents were purchased from Acros Organics (Thermo Fisher scientific, USA). Adcetris was obtained from Takeda. Brentuximab was produced by Evitria (evitria AG, Switzterland). PEG_{24} was purchased from BiochemPEG (Pure Chemistry Scientific Inc., United States)

Preparative and Semi Preparative HPLC

Preparative HPLC was performed on a Gilson PLC 2020 system (Gilson Inc, WI, Middleton, USA) using a VP 250/32 Macherey-Nagel Nucleodur C18 HTec Spum column (Macherey-Nagel GmbH & Co. Kg, Germany) and a flowrate of 30 mL/min. Semi Preparative HPLC was performed on the same system using a VP 250/21Macherey-Nagel Nucleodur C18 HTec Spum column (Macherey-Nagel GmbH & Co. Kg, Germany) with a flowrate of 16 mL/min.

NMR

NMR spectra were recorded with a Bruker Ultrashield 300 MHz spectrometer and a Bruker Avance III 600 MHz spectrometer (Bruker Corp., USA) at ambient temperature. Chemical shifts δ are reported in ppm relative to residual solvent peak ($CDCl_3$: 7.26 [ppm]; DMSO-d6: 2.50 [ppm] for ^{1}H-spectra and $CDCl_3$: 77.16 [ppm]; DMSO-d6: 39.52 [ppm] for ^{13}C-spectra. Coupling constants *J* are stated in Hz. Signal multiplicities are abbreviated as follows: s: singlet; d: doublet; t: triplet; q: quartet; m: multiplet.

HR-MS

High resolution ESI-MS spectra were recorded on a Waters H-class instrument equipped with a quaternary solvent manager, a Waters sample manager-FTN, a Waters PDA detector and a Waters column manager with an Acquity UPLC protein BEH C18 column (1.7 µm, 2.1 mm x 50 mm). Samples were eluted with a flow rate of 0.3 mL/min. The following gradient was used: A: 0.01% FA in H_2O; B: 0.01% FA in MeCN. 5% B: 0-1 min; 5 to 95% B: 1-7min; 95% B: 7 to 8.5 min. Mass analysis was conducted with a Waters XEVO G2-XS QTof analyzer.

Intact Protein MS

Intact proteins were analyzed using Waters H-class instrument equipped with a quaternary solvent manager, a Waters sample manager-FTN, a Waters PDA detector and a Waters column manager with an Acquity UPLC protein BEH C4 column (300 Å, 1.7 µm, 2.1 mm x 50 mm). Proteins were eluted with a flow rate of 0.3 mL/min. The following gradient was used: A: 0.01% FA in H_2O; B: 0.01% FA in MeCN. 5-95% B 0-6 min. Mass analysis was conducted with a Waters XEVO G2-XS QTof analyzer. Raw data was analyzed with MaxEnt 1.

Preparative Size-Exclusion Chromatography

Protein purification by size-exclusion chromatography was conducted with an ÄKTA FPLC system (GE Healthcare, United States) equipped with a P-920 pump system, a UPC-900 detector, and a FRAC-950 fraction collector. Desalting after small scale antibody modification was done on a 5 ml HiTrap® desalting column, with a flow rate of 1.5 mL/min. ADCs were purified using a 25 mL Superose™ 6 Increase 10/300GL column with a flowrate of 0.8 mL/min.

Protein Concentration Determination

Protein concentration was determined in a 96-well plate with a Pierce™ Rapid Gold BCA Protein Assay Kit (Thermo Fisher Scientific, USA) and a Bradford reagent B6916 (Merck, Germany) with pre-diluted protein assay standards of bovine gamma globulin (Thermo Fisher Scientific, USA). Results of both Assays were arithmetically averaged.

Analytical Size-exclusion Chromatography

Analytical size-exclusion chromatography (A-SEC) of the ADCs was conducted on a Vanquish Flex UHPLC System with a DAD detector, Split Sampler FT (4°C), Column Compartment H (25°C) and binary pump F (Thermo Fisher Scientific, USA) using a MAbPac SEC-1 300 Å, 4 x 300 mm column (Thermo Fisher Scientific, USA) with a flow rate of 0.15 mL/min. Separation of different ADC/mAb populations has been achieved during a 30 minute isocratic gradient using a phosphate buffer at pH 7 (20 mM Na_2HPO_4/NaH_2PO_4, 300 mM NaCl, 5% v/v isopropyl alcohol as a mobile phase. 8 µg ADC/mAb where loaded onto the column for A-SEC analysis. UV chromatograms were recorded at 220 and 280 nm. Quantification of monomer and HMWS was achieved after integration of the peak area at 220 nm.

Analytical Hydrophobic Interaction Chromatography

The measurements were conducted on a Vanquish Flex UHPLC System (2.9) with a MabPac HIC Butyl 4.6 x 100 mm column (Thermo Fischer Scientific, USA). Separation of different ADCs/antibodies have been achieved with the following gradient: A: 1 M $(NH_4)_2SO_4$, 500 mM NaCl, 100 mM NaH_2PO_4 pH 7.4 B: 20 mM NaH_2PO_4, 20% (v/v) Isopropyl alcohol, pH 7.4. 0% B: 0-1 min, 0-95% B: 1-15 min, 95% B: 15-20 min, 95-0% B: 20-23 min, 0% B: 23-25 min, with a flow of 700 uL/min. 15 µg sample where loaded onto the column for each analysis. UV chromatograms were recorded at 220 and 280 nm.

FAR and DAR determination of brentuximab conjugates by intact protein MS

Purified brentuximab conjugates in PBS were diluted to approximately 1 mg/mL. 10 µL of this solution was treated with 0.5 µL PNGase-F solution (Pomega, Germany, Recombinant, cloned from Elizabethkingia miricola 10 U/µL) and incubated at 37 °C for 2 hours. Disulfide bridges were reduced by addition of 1 µL DTT solution (70 mM in H_2O) and incubation at 37°C for 30 min. Samples were diluted to 0.1 mg/mL with PBS and subjected to intact protein MS injecting 5 µL for each sample. After deconvolution of the crude spectra, the DAR was determined with the following formula, were I corresponds to the mass intensities of the respective species: LC (light chain), LC_{mod}^{*1} (single modified light chain), HC (heavy chain), HC_{mod}^{*1} (single modified heavy chain), HC_{mod}^{*2} (double modified heavy chain), HC_{mod}^{*3} (triple modified heavy chain).

$$DAR = 2 * \frac{I\left(LC_{mod}^{*1}\right)}{I(LC) + I\left(LC_{mod}^{*1}\right)} + 2 * \frac{I\left(HC_{mod}^{*1}\right) + 2 * I\left(HC_{mod}^{*2}\right) + 3 * I(HC_{mod}^{*3})}{I(HC) + I\left(HC_{mod}^{1}\right)I\left(HC_{mod}^{2}\right) + I(HC_{mod}^{3})}$$

Experimental Procedures

Solubility Assay

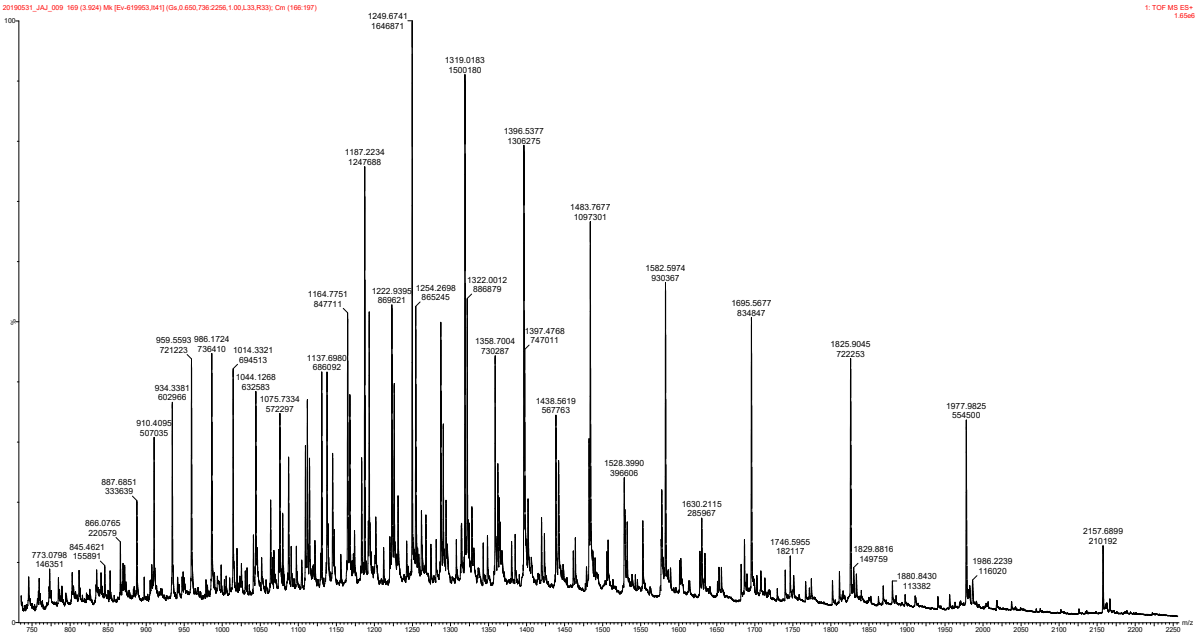
The aqueous solubility of compounds **5a-d**[1] was determined in a shake flask solubility assay. Saturated solutions of the compounds in reaction buffer with 5% DMSO were prepared in triplicates by adding 10 µL of compound (1.2M in DMSO) to 190 µL 50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl,

pH 8.5. The samples were incubated at 1000 rpm shaking and 25°C for 2h and subsequently subjected to high-speed centrifugation (10 minutes, 16873 rcf). The supernatant was analyzed by UV/Vis and the concentration was determined via Cy5 absorbance at 646 nm and the molar extinction coefficient of Cy5 ($\varepsilon(Cy5)_{646nm}$ = 250.000).

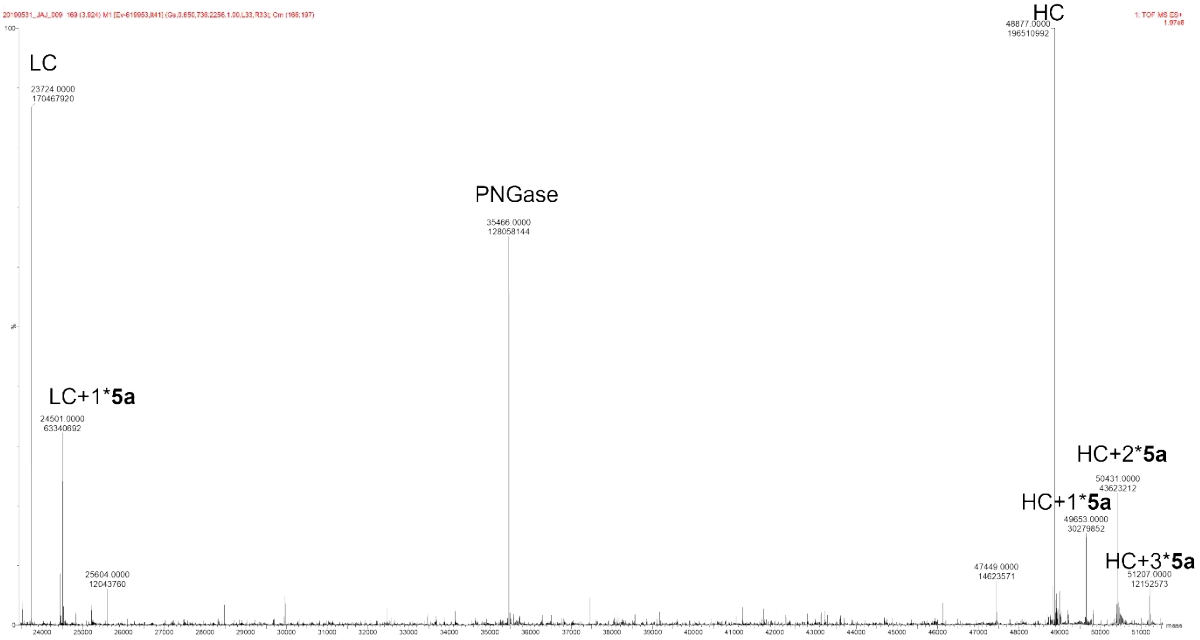
Antibody modification with Cy5-phosphonamidates

Brentuximab was expressed and purified as previously published[2] with an additional final purification by gel filtration on a Superdex 200 Increase 10/300 from GE with PBS and flow rate of 0.75 ml/min. 190 µL brentuximab (35.8 µM) were treated with 200 equivalents DTT in 50 mM sodium borate in PBS (pH 8.0) at 37°C for 40 minutes. The antibody solution was split in 47.5 µL batches and excess DTT was removed using 0.5 mL Zeba™ Spin Desalting Columns with 7K MWCO (Thermo Fisher Scientific, USA) that were pre equilibrated with the reaction buffer (50mM Tris-HCl, 1mM EDTA, 100 mM NaCl, pH 8.5). The reduced antibody was treated with 2.5 µL phosphonamidates **5 a-d**[1] (0.33 mM, 10 eq. in DMSO). The mixtures were briefly vortexed and incubated at 850rpm and 14°C for 16 hours. For modification with 100 equivalents of **5b** the same procedure was followed using 2.5 µL of a 3.3 mM solution in DMSO. Excess reagent was removed via size exclusion chromatography and FAR was determined as described.

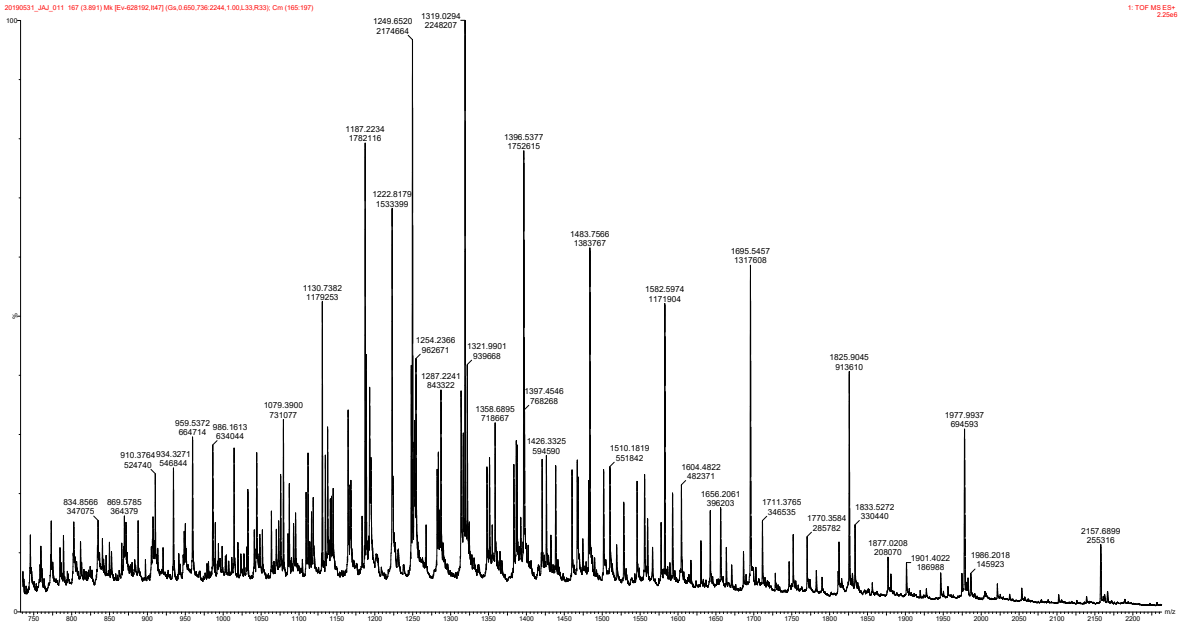
Raw mass spectrum of brentuximab-**5a** FAR 1.63



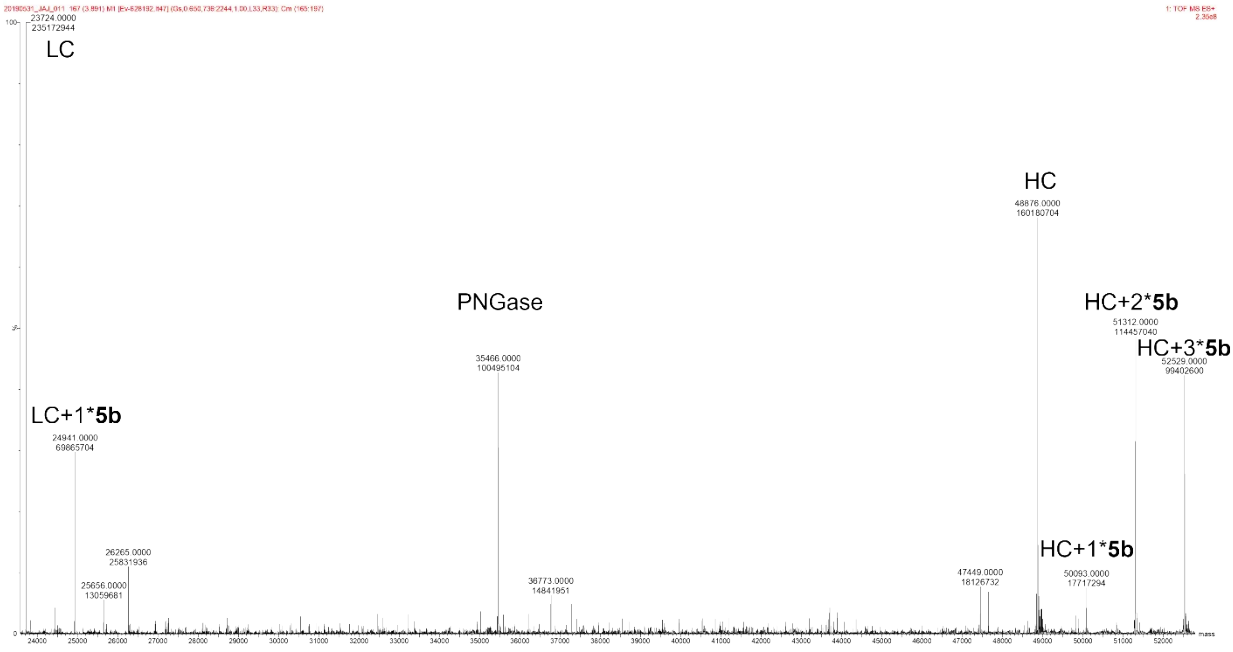
Deconvoluted mass spectrum of brentuximab-**5a** FAR 1.63



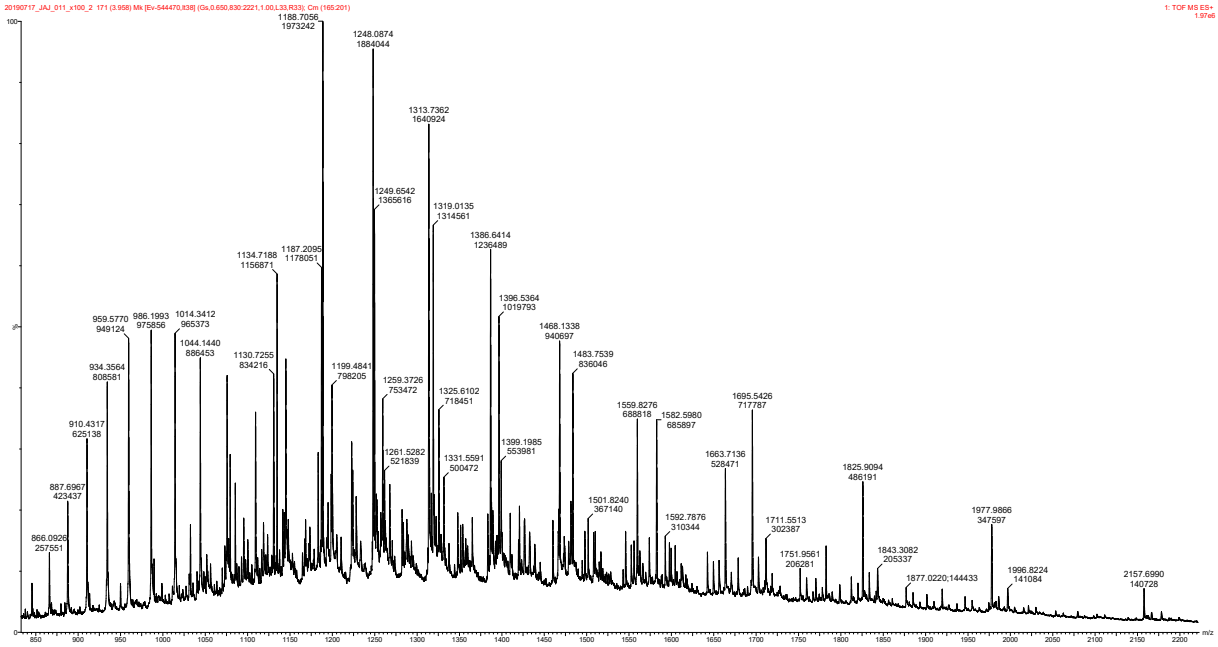
Raw mass spectrum of brentuximab-**5b** FAR 3.23



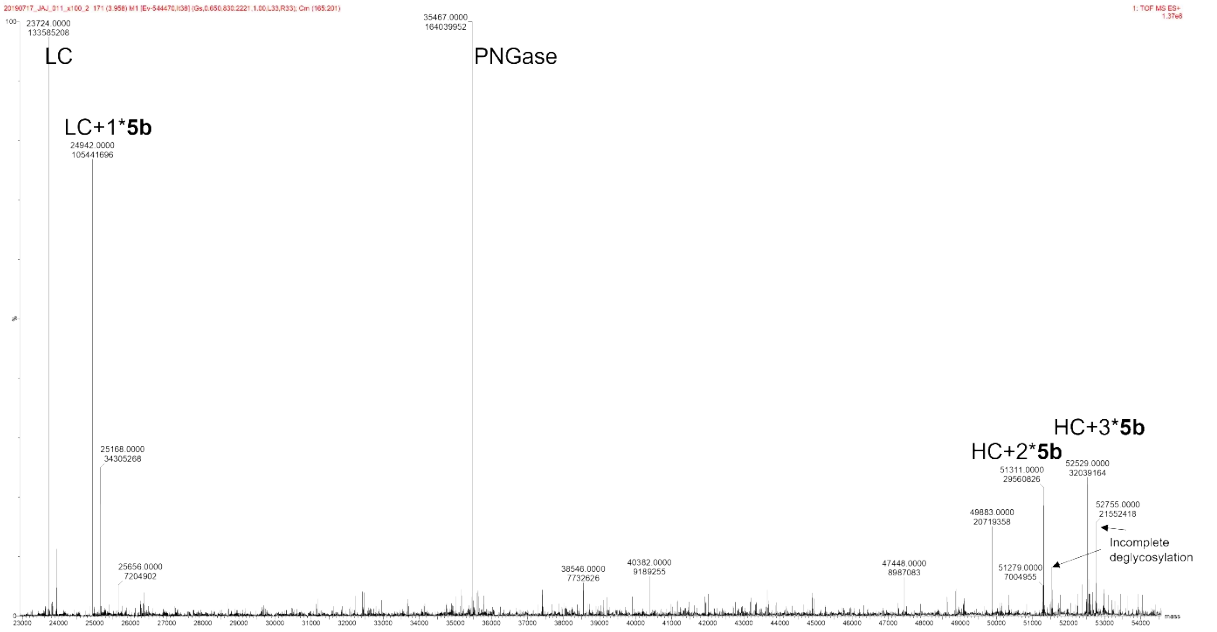
Deconvoluted mass spectrum of brentuximab-**5b** FAR 3.23



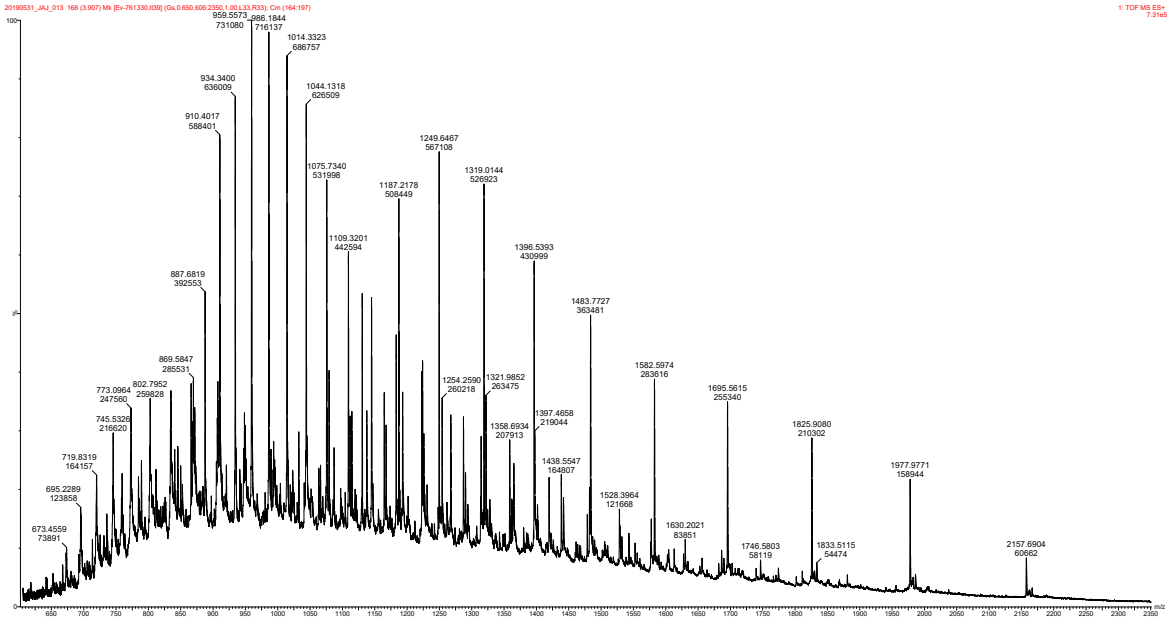
Raw mass spectrum of brentuximab-**5b** FAR 5.9



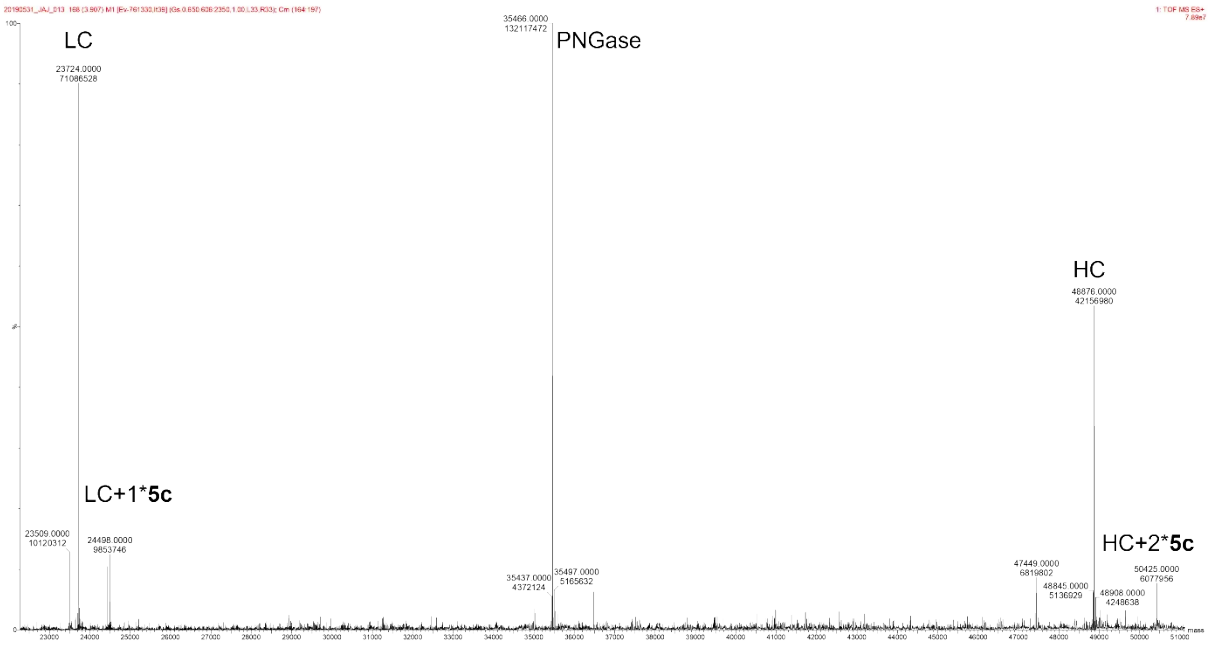
Deconvoluted mass spectrum of brentuximab-**5b** FAR 5.9



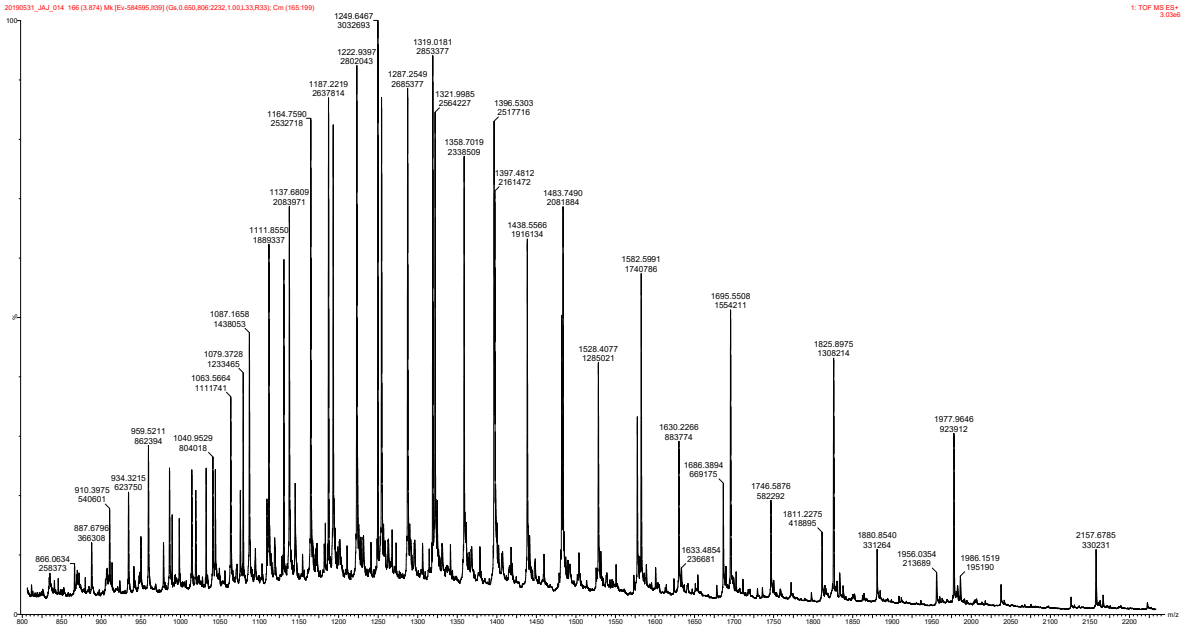
Raw mass spectrum of brentuximab-**5c** FAR 0.74



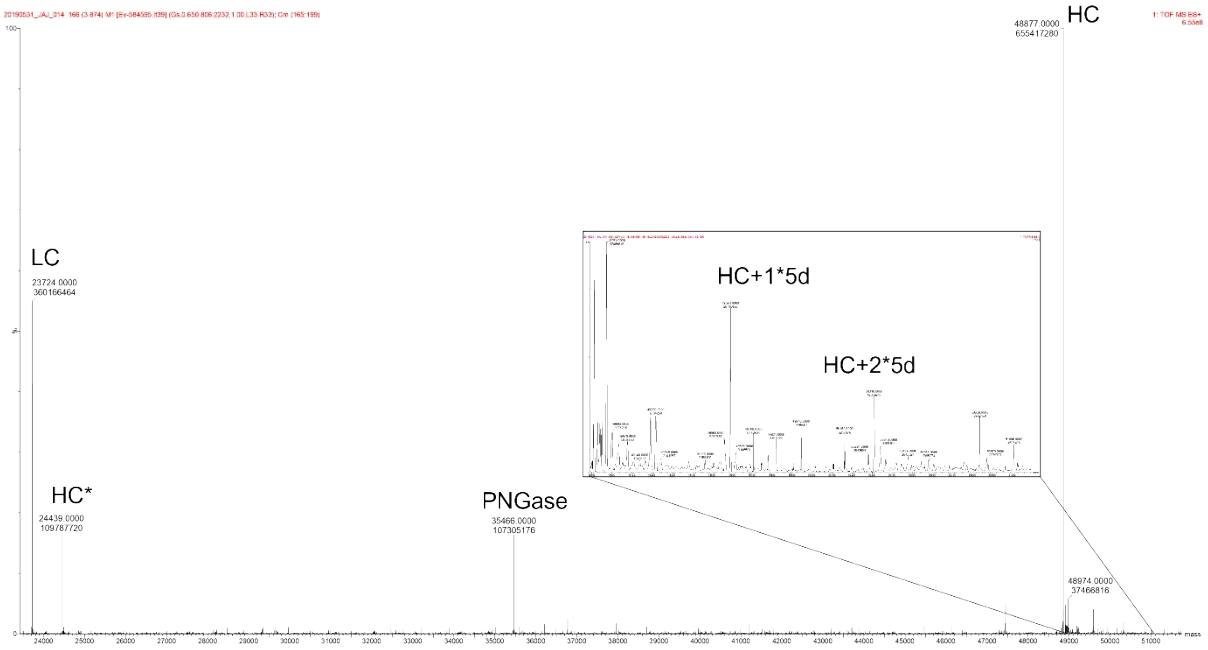
Deconvoluted mass spectrum of brentuximab-**5c** FAR 0.74



Raw mass spectrum of brentuximab-**5d** FAR 0.14

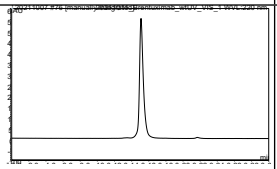
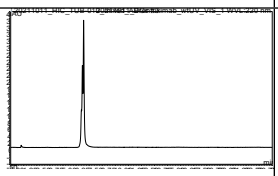
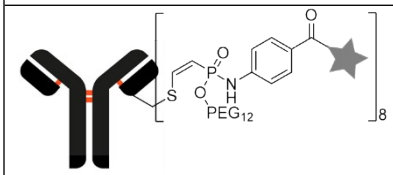
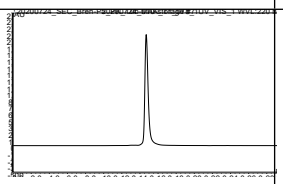
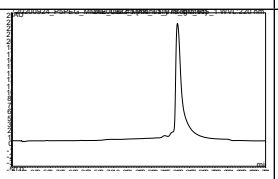
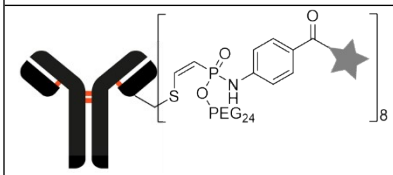
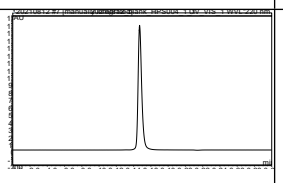
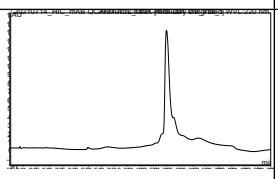


Deconvoluted mass spectrum of brentuximab-**5d** FAR 0.14



Preparative synthesis of DAR 8 ADCs for Brentuximab with 7 and 8

50 µl of the antibody solution of 10.0 mg/ml in conjugation buffer (50 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 8.3 at RT) were mixed with 3.33 µl of a 10 mM TCEP solution in P5-conjugation buffer. Directly afterwards, 1.67 µl of a 40 mM solution of the P5-construct dissolved in DMSO were added. The mixture was shaken at 350 rpm and 25°C for 16 hours. The reaction mixtures were purified by preparative size-exclusion chromatography with a 25 ml Superdex™ 200 Increase 10/300GL (Cytiva, Sweden) and a flow of 0.8 ml/min eluting with sterile PBS (Merck, Germany). The antibody containing fractions were pooled and concentrated by spin-filtration (Amicon® Ultra- 2mL MWCO: 30 kDa, Merck, Germany).

Antibody/ADC	Analytical SEC	Analytical HIC	MS analysis of the fully conjugated DAR 8
 Brentuximab			LC: calcd.: 23724 found: 23724 HC: calcd.: 48878 found: 48877
 Brentuximab-P5(PEG12)-VC-PAB-MMAE			DARav:8.0 LC: calcd.: 25582 found: 25583 HC: calcd.: 54452 found: 54454
 Brentuximab-P5(PEG24)-VC-PAB-MMAE			DARav: 8.0 LC: calcd.: 26111 found: 26110 HC: calcd.: 56039 found: 56040

Preparative synthesis of DAR 4 ADCs for Brentuximab with 7 and 8

50 µl of the antibody solution of 10.0 mg/ml in conjugation buffer (50 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 8.3 at RT) were mixed with 1.16 µl of a 10 mM TCEP (3.5 eq.) solution in P5-conjugation buffer. Directly afterwards, 0.84 µl of a 40 mM solution of the P5-construct dissolved in DMSO were added (10 eq.). The mixture was shaken at 350 rpm and 25°C for 16 hours. The reaction was stopped via the addition of 5 µl of 100 mM Cysteine in conjugation buffer. After further 30 minutes, the reaction mixture was purified by preparative size-exclusion chromatography with a 25 ml Superdex™ 200 Increase 10/300GL (Cytiva, Sweden) and a flow of 0.8 ml/min eluting with sterile PBS (Merck,

Germany). The antibody containing fractions were pooled and concentrated by spin-filtration (Amicon® Ultra- 2mL MWCO: 30 kDa, Merck, Germany).

***In vitro* cytotoxicity assay**

Respective cell lines were cultured in DMEM/F12 supplemented with 10% FCS and 1% Penicillin-Streptomycin. Cells were seeded at a density of 5*10^3 cells/well in 96-well cell culture microplates. 1:1 serial dilutions of ADCs or antibodies were performed in cell culture medium starting at 3 µg/mL final concentration and transferred in duplicates to respective wells on the microplate. Plates were incubated for 4 days at 37°C, 5 % CO_2. Subsequently, resazurin was added to a final concentration of 50 µM followed by incubation for 3 – 4 h at 37°C, 5% CO_2. Metabolic conversion of resazurin to resorufin is quantified by the fluorescent signal of resorufin (λ_{EX} = 560 nm and λ_{EM} = 590 nm) on a Tecan Infinite M1000 micro plate reader. Mean and standard deviation was calculated from duplicates, normalized to untreated control and plotted against antibody concentration. Data analysis was performed with MatLab R2017b software.

***In vitro* bystander assay**

To analyze bystander activity of ADCs on target-negative cells, 20.000 target-positive cells (SKBR-3 for trastuzumab ADCs) were incubated with increasing concentrations of ADCs (0-3 µg/ml). After 5 days, half of the cell culture supernatant volumes was transferred to 5.000 target-negative cells (MDA-MB-468 for trastuzumab ADCs) and incubated for another 5 days. Killing was analyzed by a resazurin-based viability measurement as described above.

***In vivo* efficacy study**

In vivo efficacy evaluations of ADCs were performed at EPO GmbH. All animal experiments were conducted in accordance with German animal welfare law and approved by local authorities. In brief, $1x10^7$ Karpas-299 cells were subcutaneously injected to CB17-Scid mice. Treatment was initiated when tumours reached a mean tumour volume of 0.114 cm^3 9 days after implantation. 0.5 mg/kg of brentuximab-**7** DAR 4, brentuximab-**7** DAR 8, Adcetris or vehicle (PBS) were administered as intravenous injection on day 0, 3, 6 and 9 after randomisation into treatment and control groups. Tumour volumes, body weights and general health conditions were recorded throughout the whole study.

***In vivo* PK study**

Female Sprague-Dawley rats were treated intravenously via the tail-vein with 5 mg ADC per kilogram bodyweight (bolus) with the respective ADC. Approximately 1 mL of blood was collected after 0.5h, 1h, 4h, 24h, 48h, 96h, 168h, 336h, and 504h. Blood samples were analyzed via ELISA as follows.

Analysis of the in vivo samples by ELISA for total antibody

To evaluate the PK of the ADCs in vivo, the total antibody concentration was measured at different time points in serum of ADC-treated SD rats. Total humanized anti-CD30 antibody was analyzed in rat serum over the range 2000 – 15,6 ng/ml. Nunc 96-well plate with (100 µl/well) were coated with Recombinant Human CD30/TNFRSF8 diluted in PBS (required concentration: 0,25 µg/ml) and sealed with PCR Foil. Plates were incubated in a fridge to maintain a temperature between 2-8°C overnight. The coated plates were washed 3x with 300 µl PBST. 200 µl/well of blocking solution (2 % Albumin in PBST) was added, the plate was sealed and an incubated at room temperature for 1 hour. The coated plates were washed 3x with 300 µl PBST. 100 µl/well of prepared standards (2000 – 15,6 ng/ml of the respective ADCs, QCs and test samples were added, the plates were sealed and incubated at room

temperature for 1 hour. The plates were washed 3x with 300 µl PBST. 100 µl/well Anti-Human IgG (γ-chain specific)-Peroxidase antibody (dilution 1:60000 in PBS) was added and incubated for 1h at rt. The plates were washed 3x with 300 µl PBST. 50 µl/well TMB was added, the plates were sealed and incubated at room temperature for 15 min. 50 µl/well of 1 M Sulfuric Acid was added. Using a Tecan Plate Reader, the absorbance at a wavelength of 450 nm was measured.

Analysis of the in vivo samples by ELISA for intact ADC

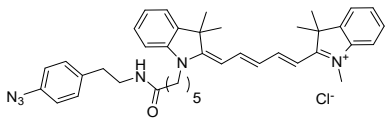
To evaluate the stability of the ADCs in vivo, the intact ADC concentration was measured at different time points in serum of ADC-treated SD rats. Intact ADC was analyzed in rat serum over the range 2000 – 15,6 ng/ml. Nunc 96-well plate with (100 µl/well) were coated with rabbit anti-vc-PAB-MMAE pAb diluted in PBS (required concentration: 1 µg/ml) and sealed with PCR Foil. Plates were incubated in a fridge to maintain a temperature between 2-8°C overnight. The coated plates were washed 3x with 300 µl PBST. 200 µl/well of blocking solution (2 % Albumin in PBST) was added, the plate was sealed and an incubated at room temperature for 1 hour. The coated plates were washed 3x with 300 µl PBST. 100 µl/well of prepared standards (2000 – 15,6 ng/ml of the respective ADCs, QCs and test samples were added, the plates were sealed and incubated at room temperature for 1 hour. The plates were washed 3x with 300 µl PBST. 100 µl/well Goat Anti-Human IgG (H+L) Preabsorbed (dilution 1:25000 in PBS was added and incubated for 1h at rt. The plates were washed 3x with 300 µl PBST. 100 µl/well TMB was added, the plates were sealed and incubated at room temperature for 10 min. 100 µl/well of 1 M Sulfuric Acid was added. Using a Tecan Plate Reader, the absorbance at a wavelength of 450 nm was measured.

Chemical Synthesis

General procedure for the synthesis of *O*-substituted ethynylphosphonites

A 25-ml Schlenk flask was charged with 267 mg bis(diisopropylamino)chlorophosphine (1.00 mmol, 1.00 eq.) under an argon atmosphere, cooled to 0 °C and 2.20 mL ethynylmagnesium bromide solution (0.5 M in THF, 1.10 mmol, 1.10 eq.) was added drop wise. The yellowish solution was allowed to warm to room temperature and stirred for further 30 minutes. The desired alcohol, dissolved in 5.56 mL 1H tetrazole solution (0.45 M in MeCN, 2.50 mmol, 2.50 eq.) was added and the white suspension was stirred until completion (2-3h) at room temperature. The crude phosphonites were directly used without further purification in a subsequent Staudinger-Phosphonite reaction. It should be noted, that mass analysis of phosphonites failed for all of the tested compounds, possibly due to decomposition under ESI-conditions.

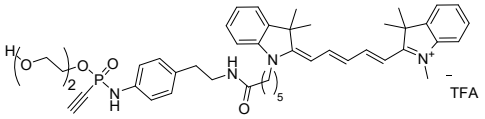
(4-azidophenethyl)amido Cy5 (4)



A dried Schlenk flask was charged with 18 mg of Cy5[3] (0.030 mmol , 1.1 eq) and 23 mg (0.059 mmol, 2.2 eq.) HATU. The solids were dissolved in DMF and 20 µL DIPEA (0.118 mmol, 4.4 eq.) were added. 2-(4-Azidophenyl)-ethylamine hydrochloride[1] (7.5 mg, 0.027 mmol, 1.0 eq.) was added and the solution was stirred for 2 hours at room temperature. The reaction progress was monitored via UPLC-MS/UV and all volatiles were removed under reduced pressure after full conversion of the azide. 10 mL

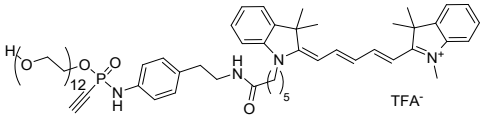
of water were added and extracted 3 times with EtOAc. The combined organic fractions were washed twice with water, dried over $MgSO_4$ and concentrated under reduced pressure. The product was obtained by flash column-chromatography on silica gel (10% methanol in dichloromethane) as a blue solid (20.3 mg, 0.0265 mmol, 98%). ^{1}H-NMR (300 MHz, Chloroform-*d*) δ 7.86 (t, *J* = 12.7 Hz, 2H), 7.46 – 7.32 (m, 4H), 7.30 – 7.17 (m, 4H), 7.11 (d, *J* = 7.9 Hz, 2H), 6.98 – 6.87 (m, 2H), 6.77 (t, *J* = 12.4 Hz, 1H), 6.35 – 6.15 (m, 3H), 5.32 (s, 1H), 3.98 (t, *J* = 7.5 Hz, 2H), 3.58 (s, 3H), 3.45 (q, *J* = 6.8 Hz, 2H), 2.81 (dd, *J* = 8.6, 6.5 Hz, 2H), 2.31 – 2.13 (m, 2H), 1.83 (d, *J* = 6.4 Hz, 0H), 1.58 – 1.39 (m, 2H), 1.27 (s, 3H), 0.96 – 0.80 (m, 1H), 0.85 (s, 1H). ^{13}C-NMR (75 MHz, Chloroform-*d*) δ 173.30, 173.07, 173.00, 153.20, 152.94, 142.71, 141.89, 141.10, 140.81, 137.78, 136.26, 130.27, 128.79, 128.70, 126.48, 125.35, 125.16, 122.23, 122.15, 118.98, 110.71, 110.33, 103.90, 103.78, 49.37, 49.13, 44.31, 40.78, 36.16, 35.08, 31.21, 27.98, 27.93, 27.11, 26.39, 25.20. HRMS: $C_{40}H_{47}N_6O^+$ calc.: 627.3806 $[M]^+$ exp.: 627.3807.

Cy5-*O*-diethylene glycol-*P*-ethynyl-phosphonamidate (5a)



100 mg Di-(diethylene glycol)ethynylphosphonite[4] (376 µmol, 1.00 eq.) and 235 mg (4-azidophenethyl)amido Cy5 (**4**) (376 µmol, 1.00 eq.) were stirred in 5 ml DMF overnight. The organic solvent was removed under reduced pressure and the blue oil was purified via semi-preparative HPLC with a gradient of 30-80% MeCN + 0.1% TFA in H_2O + 0.1% TFA over 40 min. The TFA salt was isolated and dried via lyophilization to afford 36 mg of the title compound (41.4 µmol, 11%). ^{1}H NMR (600 MHz, MeCN-d_3) δ 8.10 (td, *J* = 13.1, 3.6 Hz, 2H), 7.50 (d, *J* = 7.4 Hz, 2H), 7.43 (q, *J* = 7.7 Hz, 2H), 7.33 – 7.24 (m, 4H), 7.15 – 7.02 (m, 3H), 6.64 (d, *J* = 8.3 Hz, 1H), 6.60 – 6.49 (m, 2H), 6.24 (d, *J* = 13.9 Hz, 1H), 6.19 (d, *J* = 13.8 Hz, 1H), 4.30 – 4.19 (m, 2H), 4.01 (dd, *J* = 9.5, 5.7 Hz, 2H), 3.75 – 3.59 (m, 4H), 3.59 – 3.49 (m, 5H), 3.42 – 3.36 (m, 1H), 3.34 (q, *J* = 6.9 Hz, 2H), 3.30 (s, 6H), 2.71 – 2.63 (m, 2H), 2.13 – 2.07 (m, 2H), 1.79 (p, *J* = 7.8, 7.2 Hz, 3H), 1.72 – 1.66 (m, 10H), 1.66 – 1.57 (m, 2H), 1.41 (tt, *J* = 9.8, 6.1 Hz, 2H), 1.34 – 1.29 (m, 2H). ^{13}C NMR (151 MHz, MeCN-d_3) δ 173.95, 173.30, 153.82, 153.77, 143.07, 142.32, 141.41, 141.28, 133.59, 129.60, 129.57, 128.54, 128.52, 124.97, 124.52, 122.24, 122.13, 118.53, 118.49, 118.29, 117.36, 111.02, 110.82, 103.05, 102.97, 89.13, 88.82, 72.45, 72.41, 69.35, 69.31, 65.64, 65.60, 60.83, 49.20, 49.15, 48.89, 43.87, 40.37, 35.50, 34.63, 30.97, 26.78, 26.74, 26.61, 25.95, 25.01.. ^{31}P NMR (243 MHz, MeCN-d_3) δ -9.00. HRMS $C_{46}H_{58}N_4O_5P^+$ calc.: 777.4139 $[M]^+$, exp.: 777.4157.

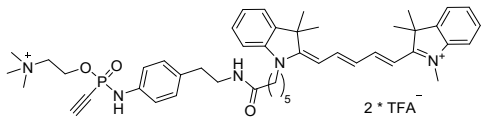
Cy5-*O*-dodecaethylene glycol-*P*-ethynyl-phosphonamidate (5b)



Phosphonite **3d** was synthesized according to the general procedure for the synthesis of *O*-substituted ethynylphosphonites from 19.5 mg bis(diisopropylamino)chlorophosphine (73 µmol, 1.00 eq.), 146 µL ethynylmagnesium bromide solution (0.5 M in THF, 73 µmol, 1 eq.), 100 mg of dodecaethylene glycol (183 µmol, 2.50 eq) and 400 µL 1H-tetrazole solution (0.45 M in MeCN, 183 µmol). Completion of the phosphonite formation was checked by ^{31}P-NMR (122 MHz, THF/MeCN, δ 130.12) before the crude reaction was transferred to an argon charged 50 mL falcon tube and centrifuged at 8000 rpm for 10

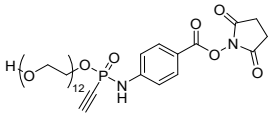
min to remove precipitated salts. The supernatant was used without further purification for the subsequent reaction and added to 45 mg of Cy5 azide **9** (73 µmol, 1.00 eq) in 1mL DMF and allowed to stir over night at room temperature. The solvent was removed under reduced pressure and the blue oil was purified via semi-preparative HPLC with a gradient of 30-80% MeCN + 0.1% TFA in H2O + 0.1% TFA over 40 min. The TFA salt was isolated after lyophilization to afford 7.8 mg of the title compound. (6.4 µmol, 8.8%). 1H NMR (600 MHz, DMSO-d6) δ 8.37 – 8.28 (m, 2H), 8.23 – 8.03 (m, 2H), 7.91 – 7.78 (m, 2H), 7.61 (d, J = 7.5 Hz, 2H), 7.46 – 7.36 (m, 3H), 7.34 – 7.18 (m, 4H), 7.09 – 6.98 (m, 4H), 6.67 – 6.53 (m, 1H), 6.42 – 6.19 (m, 2H), 4.50 (t, J = 4.7 Hz, 1H), 4.35 (d, J = 12.6 Hz, 3H), 4.13 – 4.05 (m, 3H), 3.76 – 3.60 (m, 3H), 3.59 (m, 2H), 3.56 – 3.46 (m, 28H), 3.42 (m, 2H), 3.24 (d, J = 8.3 Hz, 1H), 3.18 (m, 2H), 2.67 (t, J = 7.5 Hz, 1H), 2.62 – 2.52 (m, 2H), 2.10 – 2.01 (m, 2H), 1.68 (m, 10H), 1.61 – 1.50 (m, 2H), 1.42 – 1.29 (m, 2H), 1.29 – 1.21 (m, 2H), 1.11 – 0.73 (m, 6H). ^{31}P NMR (243 MHz, DMSO-d6) δ -9.01. . HRMS $C_{66}H_{99}N_4O_{15}P^{2+}$ calc.: 609.3417 $[M+H]^{2+}$, exp.: 609.3458.

Cy5-*O*-choline-*P*-ethynyl-phosphonamidate (5c)



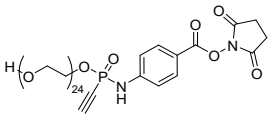
Phosphonite **4c** was synthesized according to the general procedure for the synthesis of *O*-substituted ethynylphosphonites from 26.7 mg bis(diisopropylamino)chlorophosphine (0.1 mmol, 1.00 eq.), 220 µL ethynylmagnesium bromide solution (0.5 M in THF, 0.11 mmol, 1.10 eq.), 35.2 mg of choline * PF6 (0.15 mmol, 3.00 eq) and 330 µL 1H-tetrazole solution (0.45 M in MeCN, 0.15 mmol). Completion of the phosphonite formation was checked by ^{31}P-NMR (122 MHz, THF/MeCN, δ 132.12) before the crude reaction was transferred to an argon charged 50 mL falcon tube and centrifuged at 8000 rpm for 10 min to remove precipitated salts. The supernatant was used without further purification for the subsequent reaction and added to 62.7 mg of Cy5 azide **9** (0.1 mmol, 1.00 eq) in 1mL DMF and allowed to stir over night at room temperature. The solvent was removed under reduced pressure and the blue oil was purified via semi-preparative HPLC with a gradient of 30-80% MeCN + 0.1% TFA in H_2O + 0.1% TFA over 40 min. The TFA salt was isolated after lyophilization to afford 15 mg of the title compound. (0.015 mmol, 15%). ^{1}H NMR (600 MHz, DMSO-d6) δ 8.22 (t, J = 13.3 Hz, 2H), 7.72 – 7.45 (m, 2H), 7.39 (q, J = 8.1 Hz, 2H), 7.31 (dd, J = 11.7, 8.4 Hz, 2H), 7.23 (dt, J = 11.6, 7.4 Hz, 2H), 7.08 (d, J = 8.2 Hz, 2H), 7.03 – 6.95 (m, 2H), 6.57 (t, J = 12.3 Hz, 1H), 6.23 (dd, J = 26.6, 13.7 Hz, 2H), 4.57 – 4.30 (m, 3H), 4.03 (t, J = 7.2 Hz, 2H), 3.67 (t, J = 4.8 Hz, 2H), 3.53 (s, 2H), 3.15 (p, J = 9.4, 8.2 Hz, 2H), 3.10 (s, 6H), 2.66 – 2.56 (m, 7H), 2.39 (t, J = 2.0 Hz, 2H), 2.03 (t, J = 7.2 Hz, 2H), 1.73 – 1.56 (m, 12H), 1.50 (p, J = 7.0 Hz, 2H), 1.28 (t, J = 7.9 Hz, 2H). ^{31}P-NMR (243 MHz, DMSO-d6) δ = -8.24. HRMS $C_{47}H_{62}N_5O_3P^{2+}$ calc.: 387.7290 $[M]^{+2}$, exp.: 387.7304.

4-benzoic-acid-N-hydroxysuccinimideester *O*-dodecaethylene glycol-*P*-ethynyl-phosphonamidate (6)

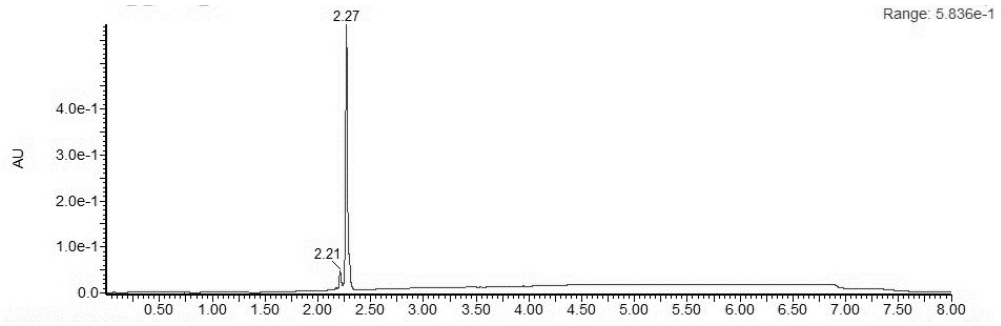


Phosphonite **3b** was synthesized according to the general procedure for the synthesis of *O*-substituted ethynylphosphonites from 19.5 mg bis(diisopropylamino)chlorophosphine (73 µmol, 1.00 eq.), 146 µL ethynylmagnesium bromide solution (0.5 M in THF, 73 µmol, 1 eq.), 100 mg of dodecaethylene glycol (183 µmol, 2.50 eq) and 400 µL 1H-tetrazole solution (0.45 M in MeCN, 183 µmol). Completion of the phosphonite formation was checked by ^{31}P-NMR (122 MHz, THF/MeCN, δ 130.12) before the crude reaction was transferred to an argon charged 50 mL falcon tube and centrifuged at 8000 rpm for 10 min to remove precipitated salts. The supernatant was used without further purification for the subsequent reaction and added to 19 mg 4-azidobenzoic-acid-*N*-hydroxysuccinimide ester (73 µmol, 1.00 eq.) in 1 ml of DMF. The reaction mixture was stirred overnight and all volatiles were removed under reduced pressure. The residue was purified by semi-preparative HPLC (10-90% MeCN + 0,1% TFA in H2O + 0.1% TFA over 60 min) and the title compound was obtained as colourless oil. (42.5 mg, 50 µmol, 68%). ^{1}H NMR (300 MHz, Acetonitrile-d3) δ 8.06 (d, J = 8.7 Hz, 2H), 7.32 (d, J = 8.8 Hz, 2H), 4.40 – 4.14 (m, 2H), 3.79 – 3.69 (m, 2H), 3.66 – 3.47 (m, 40H), 3.21 (d, J = 13.1 Hz, 1H), 2.86 (s, 4H), 1.30 (m, 2H), 1.13 – 0.79 (m, 2H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 169.77, 169.46, 161.66, 161.47, 152.75, 146.09, 132.90, 132.24, 117.82, 113.97, 113.29, 89.25, 88.92, 77.27, 77.06, 76.85, 74.69, 72.57, 71.19, 70.62, 70.54, 70.51, 70.47, 70.44, 70.36, 70.27, 70.20, 69.74, 69.70, 68.14, 65.77, 65.73, 61.63, 61.60, 40.72, 30.34, 25.68. ^{31}P NMR (122 MHz, Acetonitrile-d_3) δ -10.87. HRMS $C_{37}H_{60}N_2O_{19}P^+$ calc.: 851.3573 $[M+H]^+$, 851.3571.

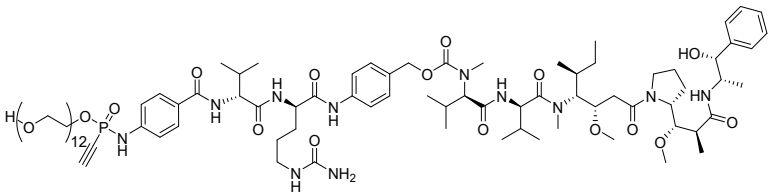
4-benzoic-acid-N-hydroxysuccinimideester *O*-PEG_{24}-*P*-ethynyl-phosphonamidate (S1)



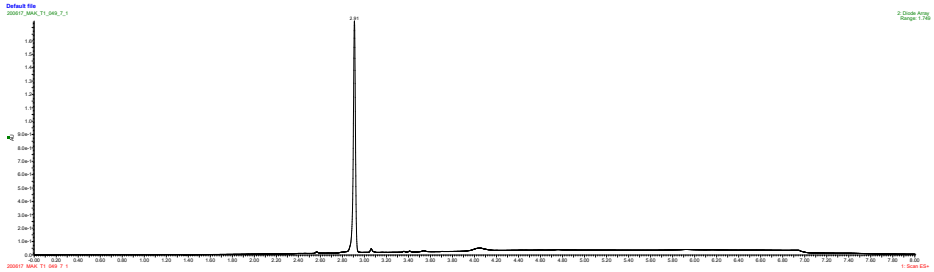
PEG_{24} phosphonite was synthesized according to the general procedure for the synthesis of *O*-substituted ethynylphosphonites from 41 mg bis(diisopropylamino)chlorophosphine (159 µmol, 1.00 eq.), 370 µL ethynylmagnesium bromide solution (0.5 M in THF, 185 µmol, 1.2 eq.), 450 mg of PEG_{24} (388 µmol, 2.50 eq) and 1.02 mL 1H-tetrazole solution (0.45 M in MeCN, 466 µmol, 3.0 eq.). After completion of phosphonite formation, the reaction mixtrure was used without further purification and added to 40 mg 4-azidobenzoic-acid-*N*-hydroxysuccinimide ester (155 µmol, 1.00 eq.). The product was obtained as colourless oil after preparative HPLC HPLC (10-90% MeCN + 0,1% TFA in H2O + 0.1% TFA over 60 min) and lyophilization. (79 mg, 57 µmol, 37%). MS for $C_{61}H_{109}N_2O_{30}P^{2+}$ $[M+2H]^{2+}$ calcd.: 690.3396, found 690.81.



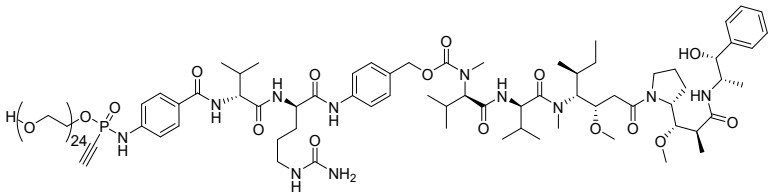
***O*-dodecaethylene glycol-*P*-ethynyl-phosphonamidate-VC-PAB-MMAE (7)**



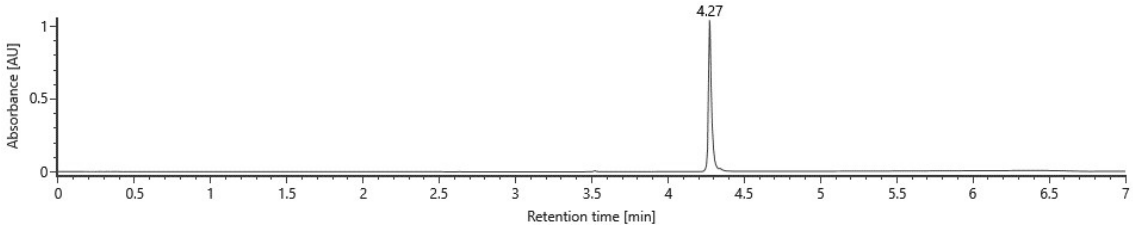
In a screw-cap-vial equipped with a stirring bar, 20.7 mg of H_2N-Val-Cit-PAB-MMAE [4] (18.4 µmol, 1.00 eq.) and 15.7 mg 4-benzoic-acid-*N*-hydroxysuccinimideester *O*-dodecaethylene glycol-*P*-ethynyl-phosphonamidate (S1) (18.4 µmol, 1 eq.) were dissolved in 200 µl DMSO. 12 µL DIPEA (73.6 µmol, 4.00 eq.) were added and the solution was stirred overnight at room temperature. The solution was diluted with 3 ml 30% MeCN in H_2O and subjected to semi-preparative HPLC purification (30-90% MeCN + 0,1% TFA in H2O + 0.1% TFA over 40 min). The desired compound was obtained as a white solid after lyophilization. (11.9 mg, 6.44 µmol, 35.2%). HRMS $C_{91}H_{150}N_{11}O_{27}P^{2+}$ calcd.: 930.0215 $[M+2H]^{2+}$, exp.: 930.0211.



***O*-PEG$_{24}$-*P*-ethynyl-phosphonamidate-VC-PAB-MMAE (8)**



The compound was synthesized analogously to compound 12 from 15 µL of a 200 mM solution of P5(PEG24)-OSu (S2) in dry DMSO (3 µmol, 1.00 eq.), 15 µL of a 250 mM solution of H2N-VC-PAB-MMAE [4] in dry DMSO (3.8 µmol, 1.25 eq.) and 5 µL of DIPEA. The product was obtained as colourless oil after preparative HPLC (Method C) and lyophilization. (1.31 mg, 0.55 µmol, 18%) HR-MS for $C_{115}H_{199}N_{11}O_{39}P^{3+}$ $[M+3H]^{3+}$ calcd.: 796.7894, found 796.7839



References

1. Kasper MA, Glanz M, Stengl A, Penkert M, Klenk S, Sauer T, Schumacher D, Helma J, Krause E, Cardoso MC, et al.: **Cysteine-Selective Phosphonamidate Electrophiles for Modular Protein Bioconjugations**. *Angew Chemie - Int Ed* 2019, **58**:11625–11630.

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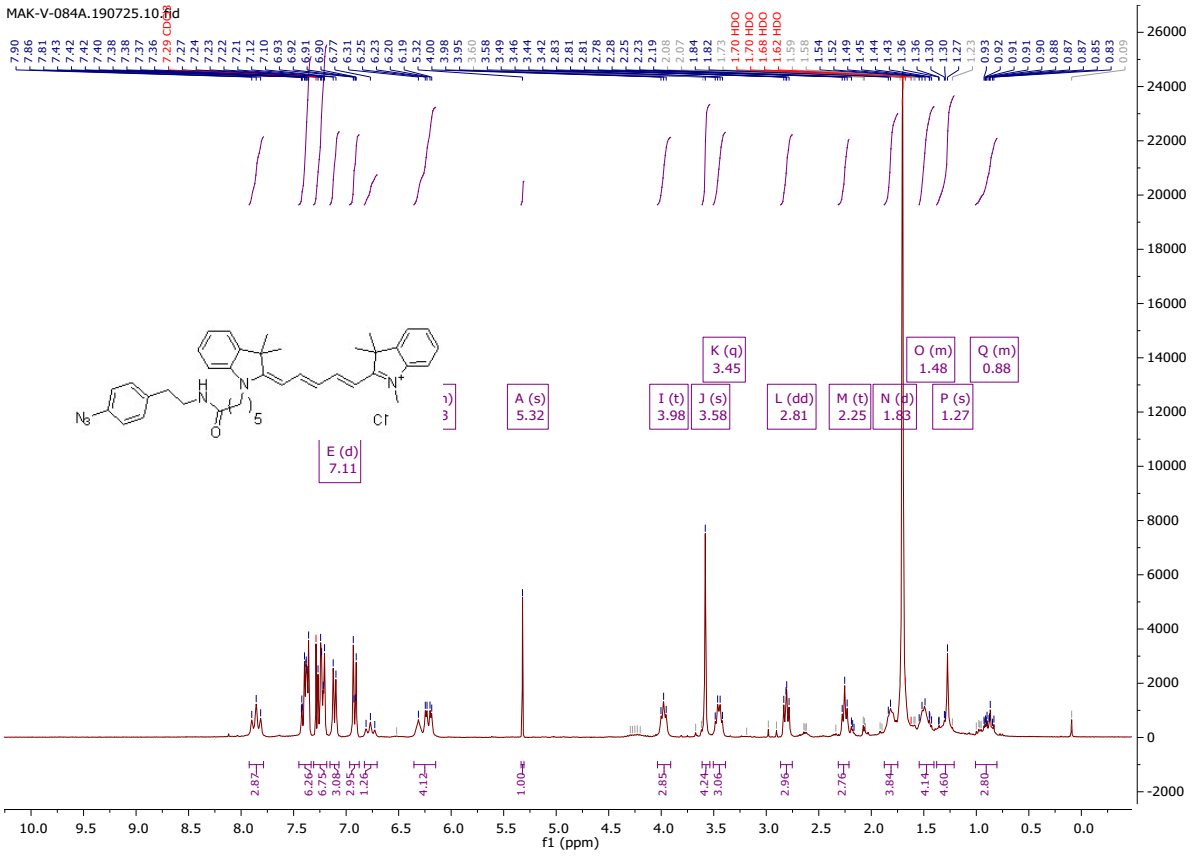
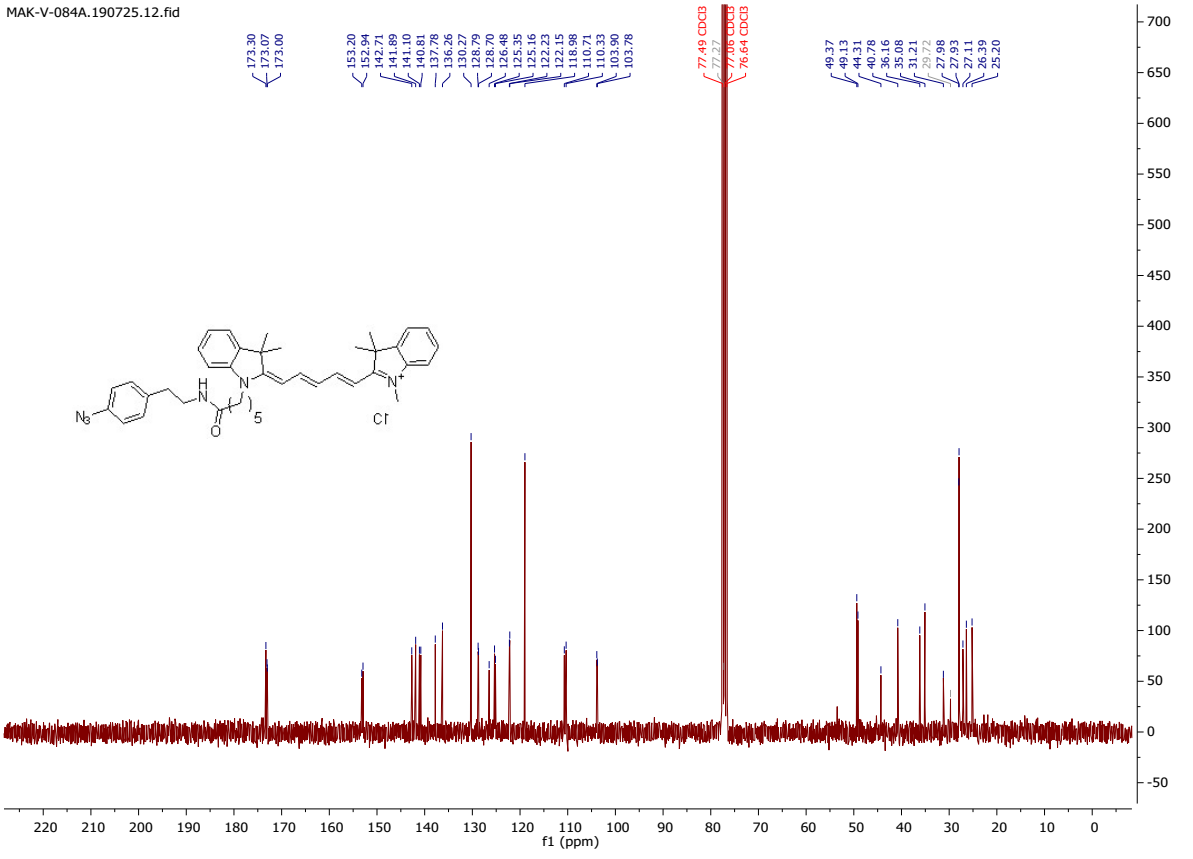
Appendix

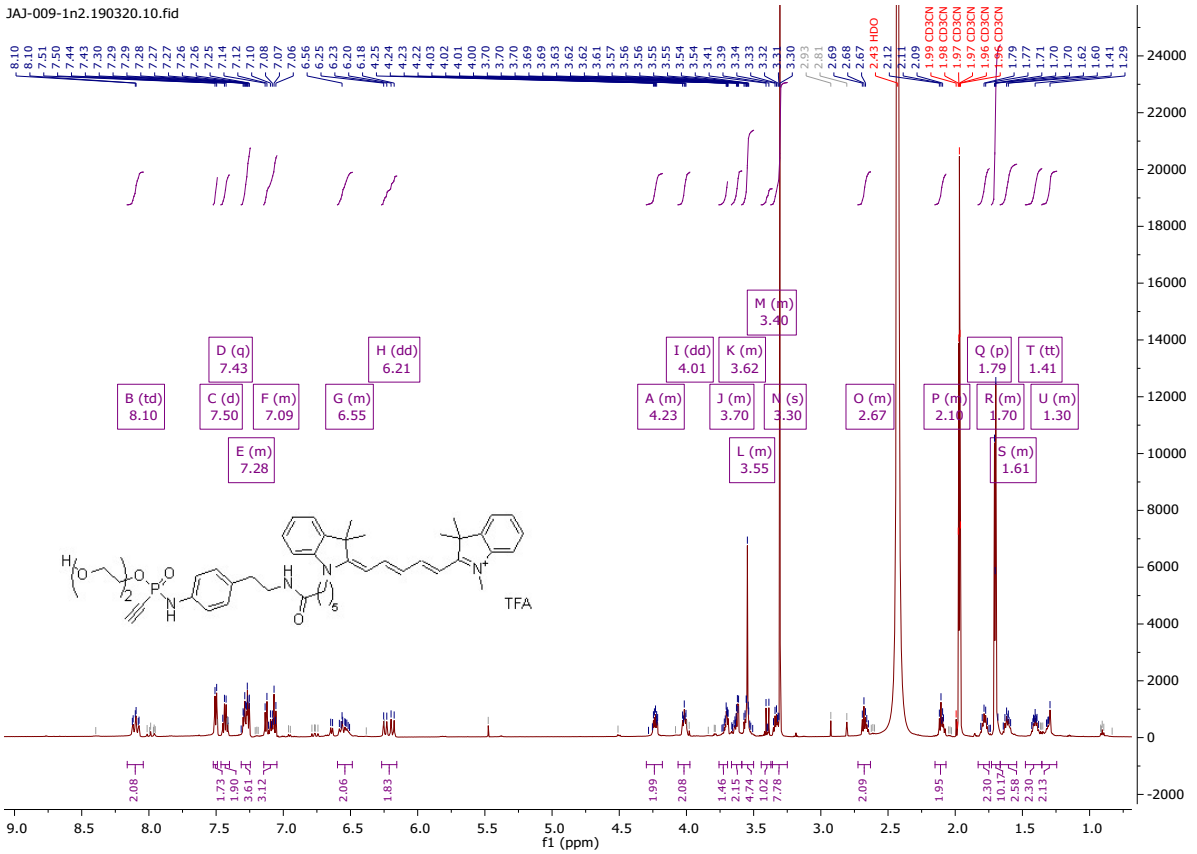
NMR-Spectra

(4-azidophenethyl)amido Cy5 (**4**)





Cy5-O-diethylene glycol-P-ethynyl-phosphonamidate (**5a**)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I (dd) 4.01
J (m) 3.70
K (m) 3.62
L (m) 3.55
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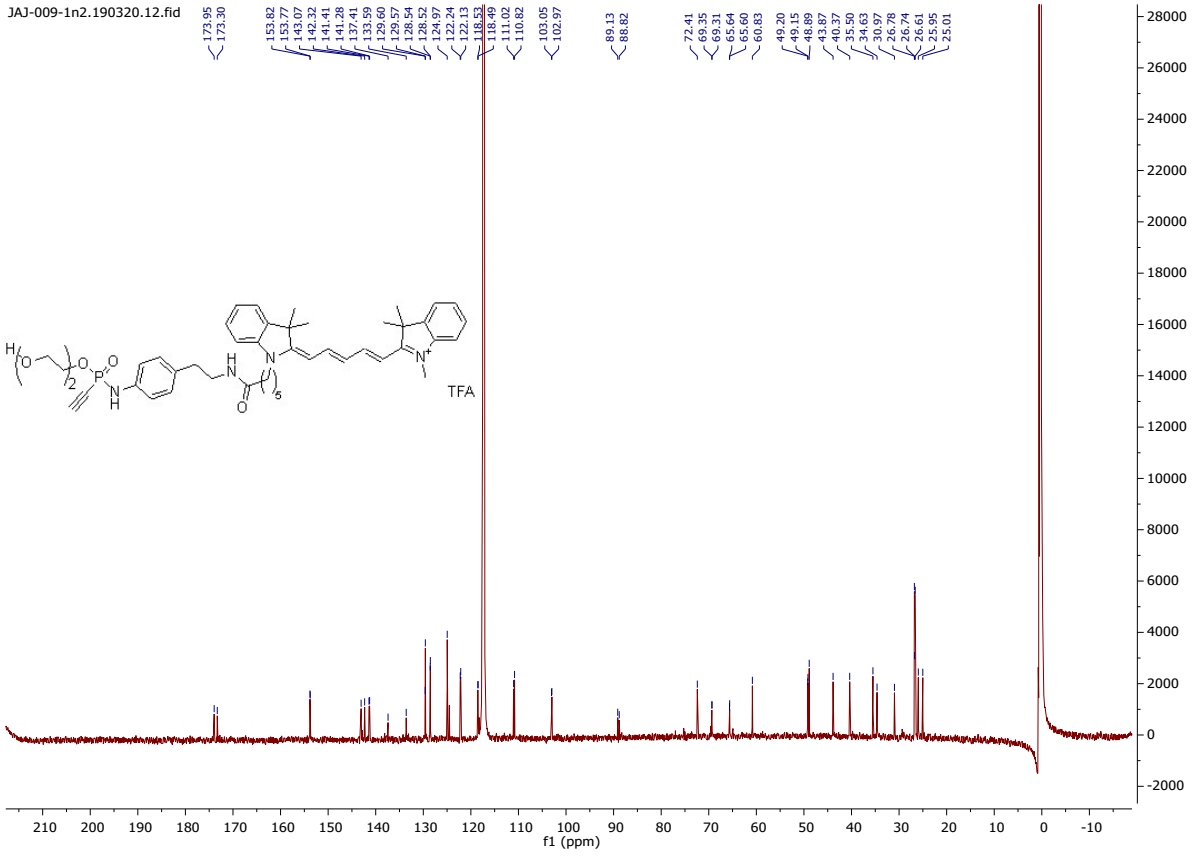
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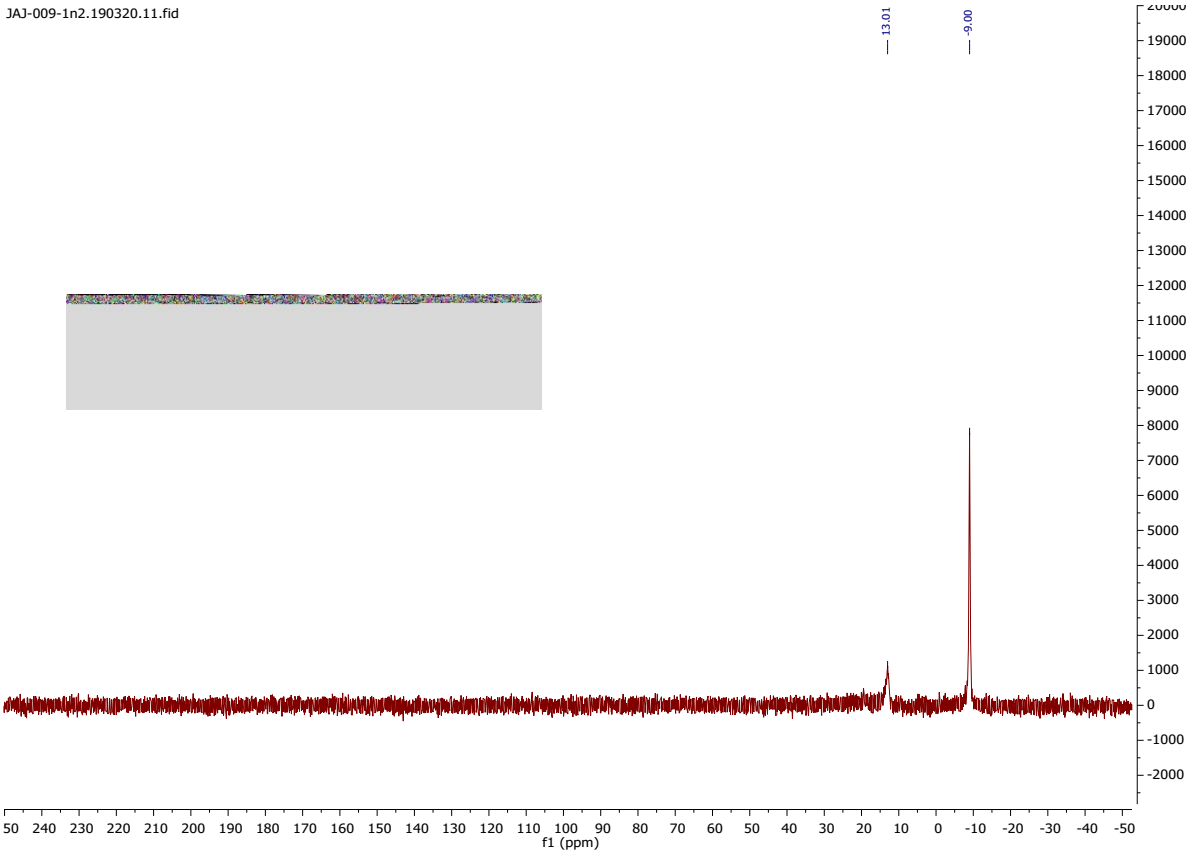
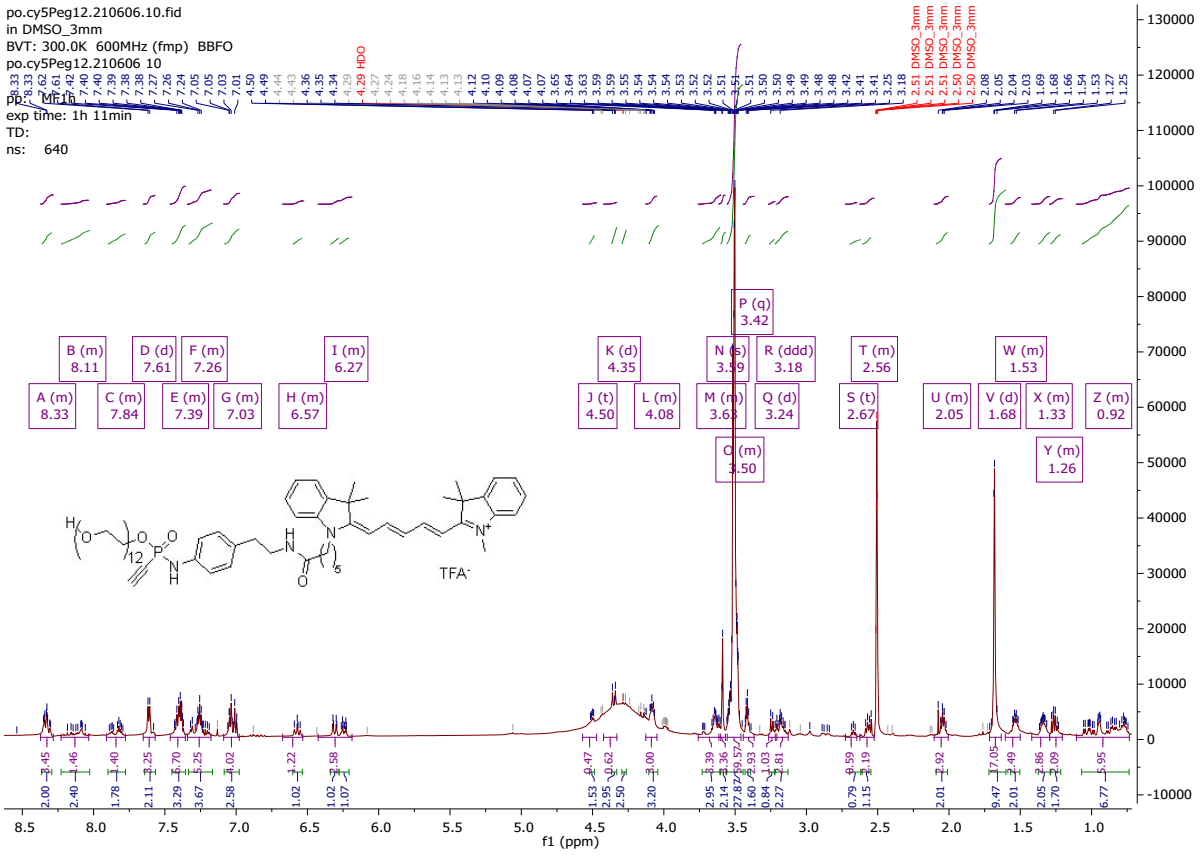
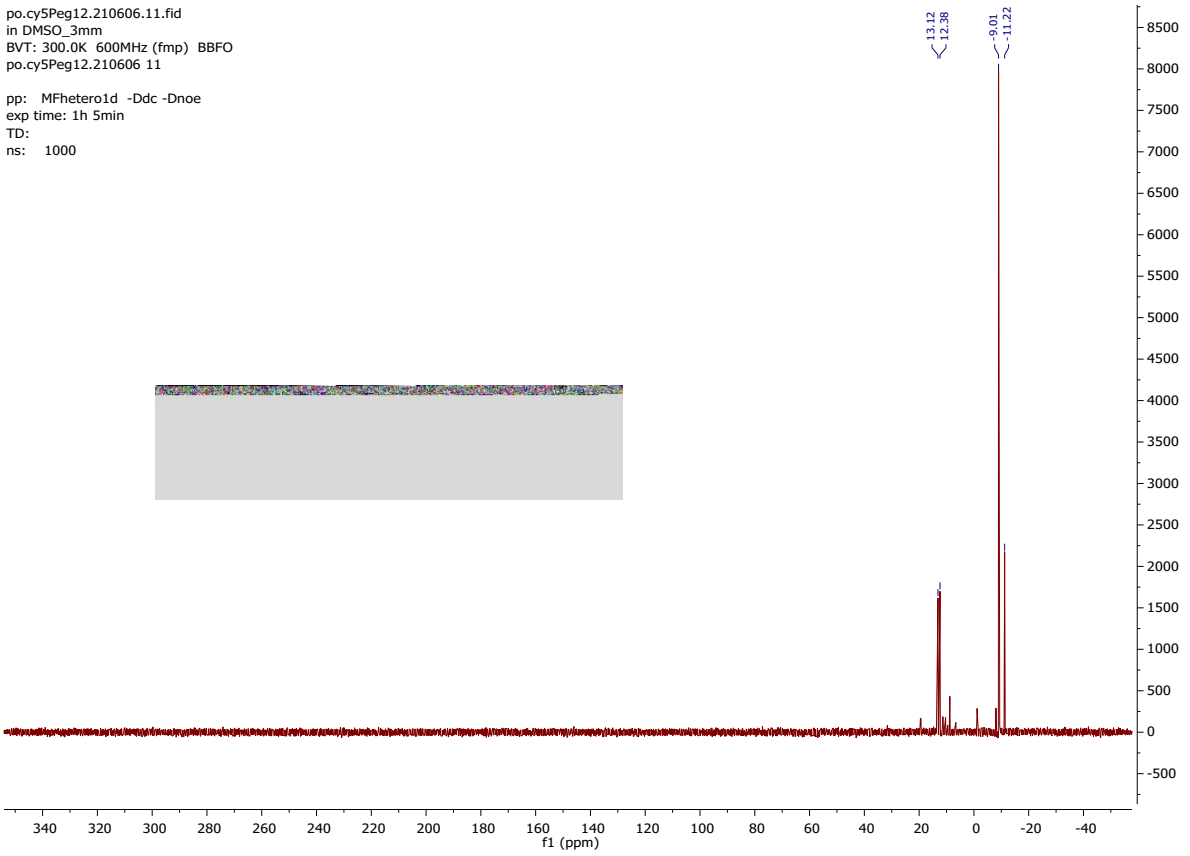
f1 (ppm)

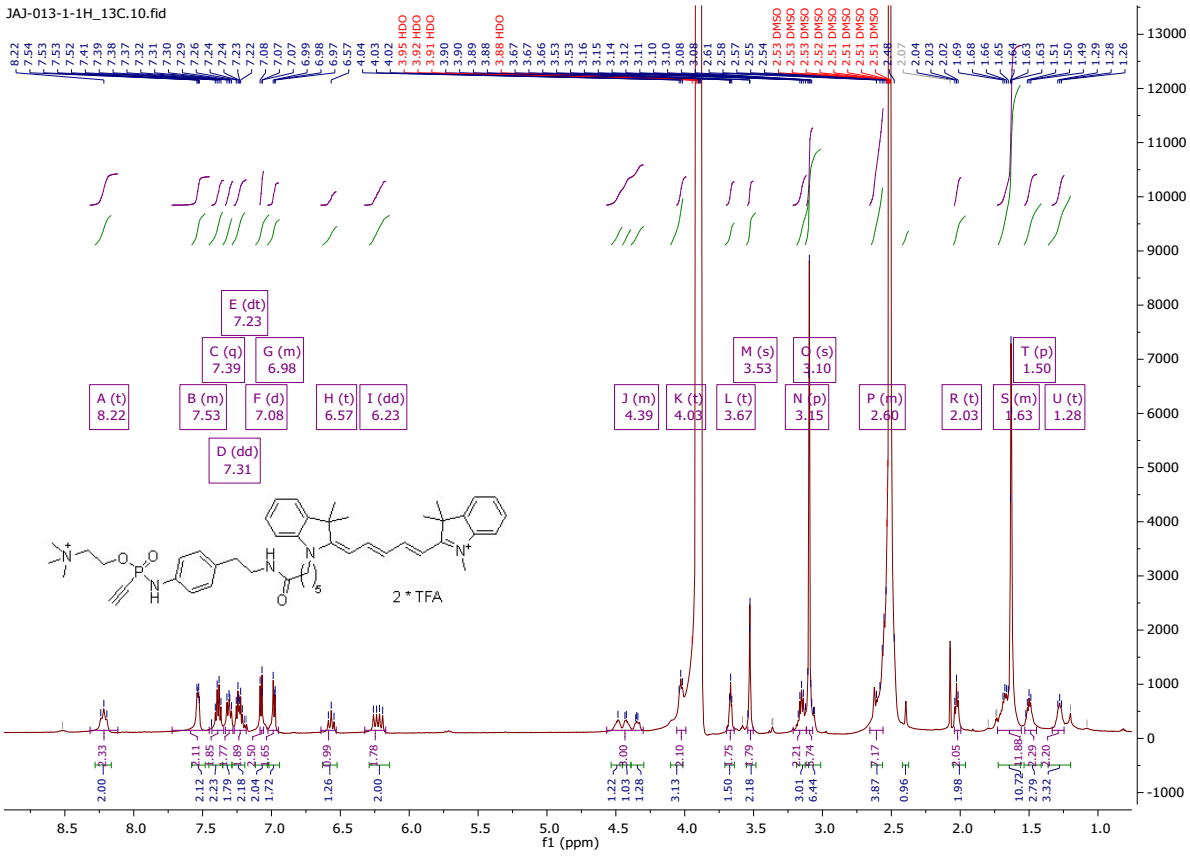


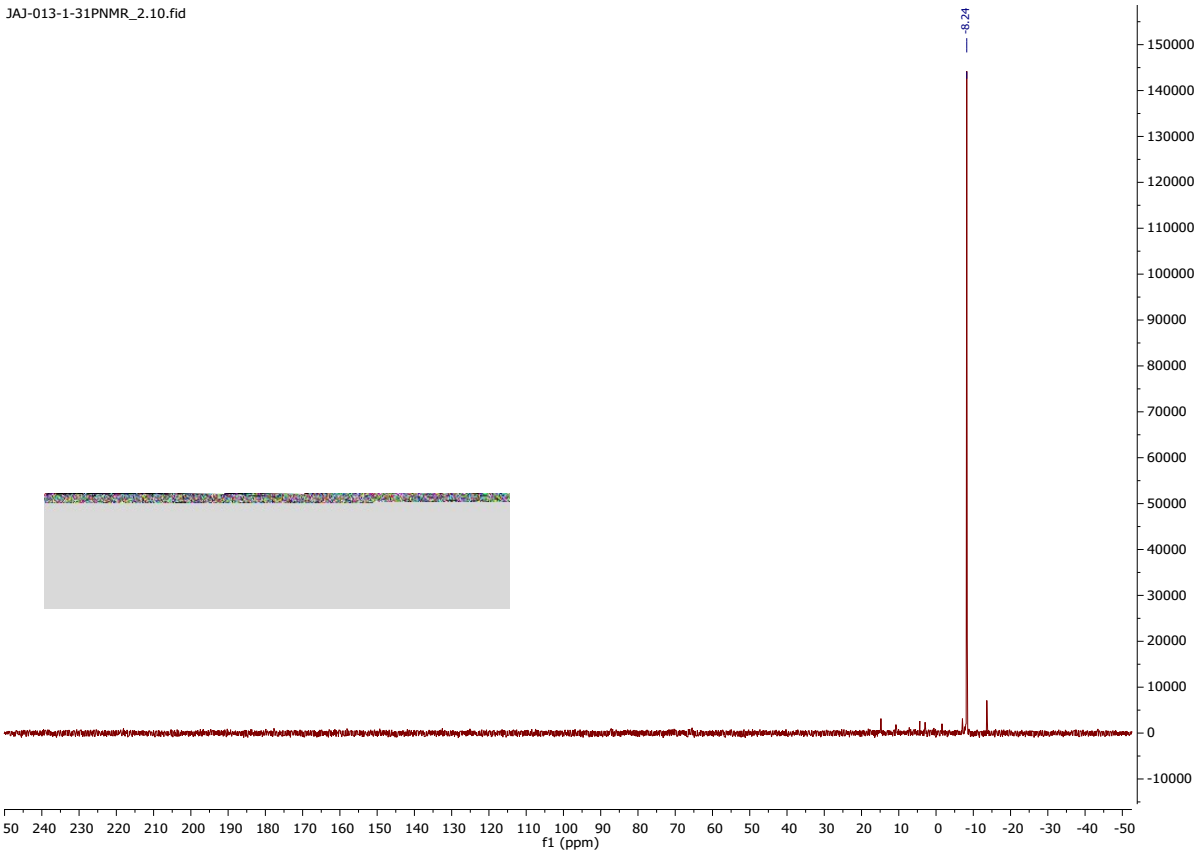
Cy5-O-dodecaethylene glycol-P-ethynyl-phosphonamidate (**5b**)



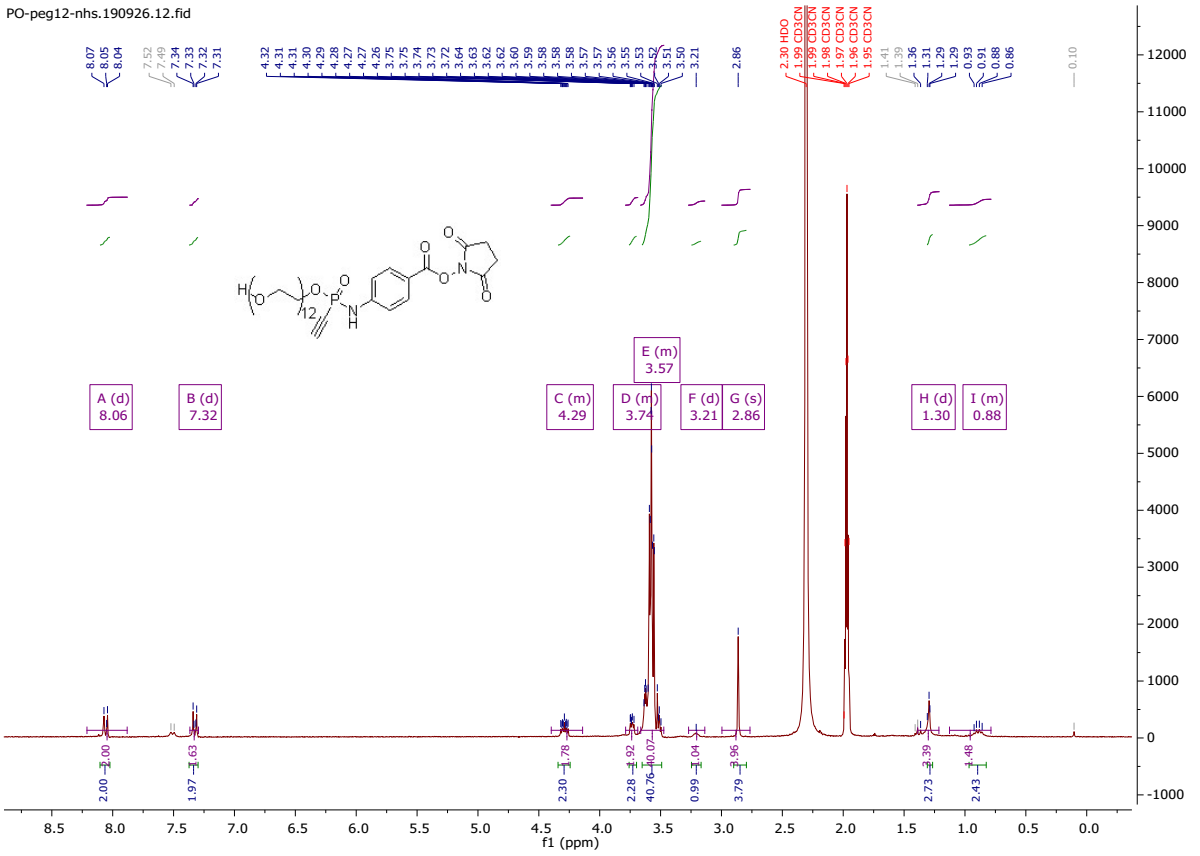


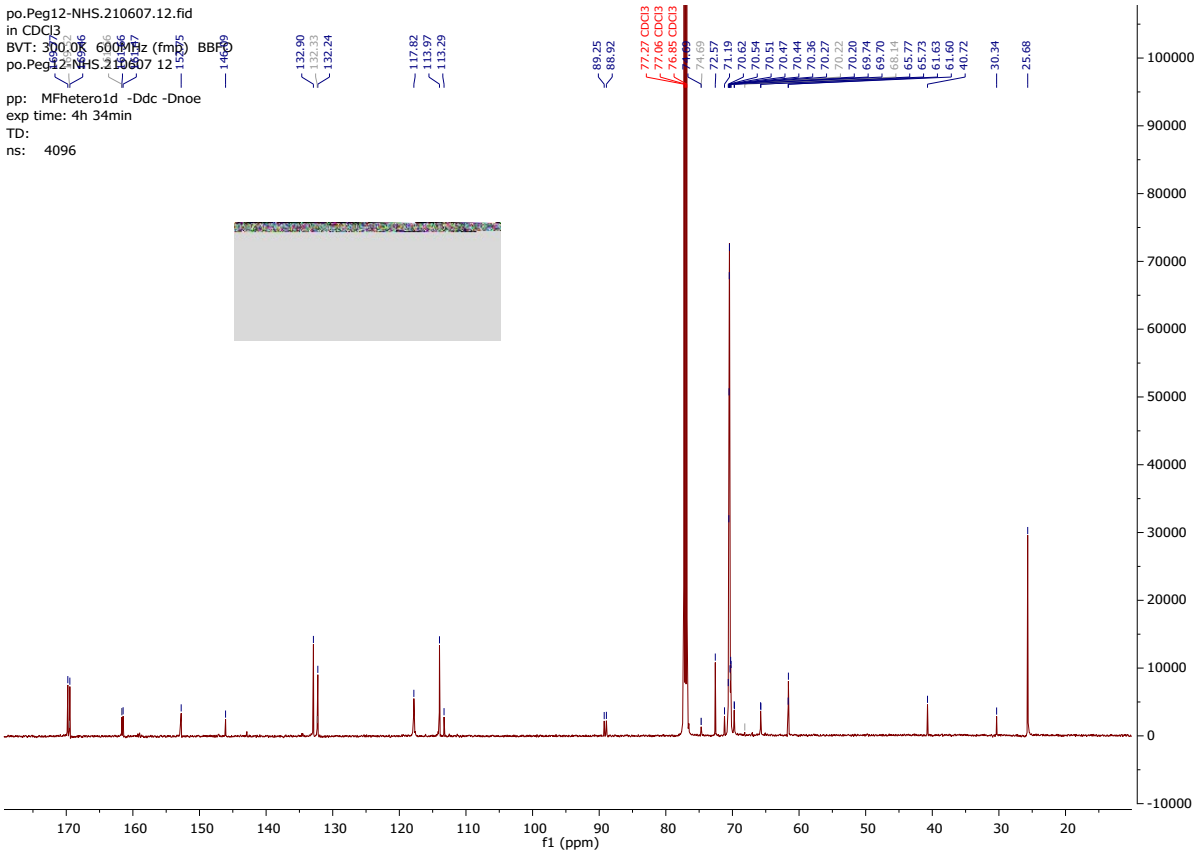
Cy5-*O*-choline-*P*-ethynyl-phosphonamidate (**5c**)





4-benzoic-acid-N-hydroxysuccinimideester O-dodecaethylene glycol-P-ethynyl-phosphonamidate (**S1**)

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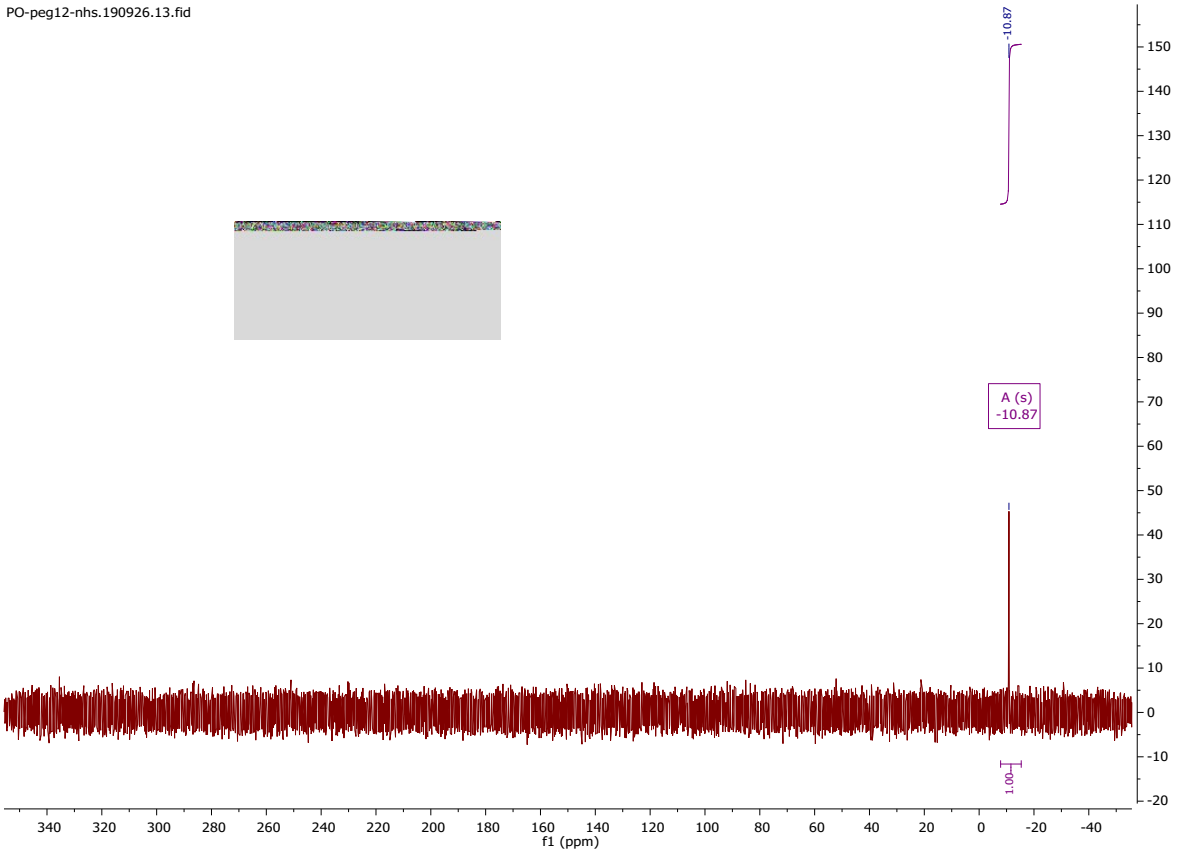
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