Supporting Information

Ethynylphosphonamidates for the Rapid and Cysteine-Selective Generation of Efficacious Antibody–Drug Conjugates

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1.1. Figure S1

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1.2. Figure S2

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1.3. Figure S3
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1.4. Figure S4
Effect of trastuzumab-4 treatment on mitotic tubulin organization in BT474 (Her2+) cells. Shown are three representative images of mitotic BT474 cells after 4 days of treatment with 0.3 nM trastuzumab-4 (a) and untreated (b). DAPI stain visualizes DNA condensation. Anti α-tubulin immunostaining visualizes spindle organization in untreated mitotic cells. Cells treated with trastuzumab-4 show DNA condensation but a strongly altered and distorted α-tubulin pattern.
1.5. **Figure S5**

Solubility measurements of 9 (red), 10 (green) and vedotin (cyan) in PBS with 5% DMSO and Inosine as an internal standard. (See chapter 3.5 for details) a) Calibration curves of different analyte concentrations (5, 10, 50, 100 and 200 µM) were recorded by UPLC/UV by peak integration of the analyte in relation to Inosin as an internal standard. Shown are error bars from three independent measurements (black) and a linear fit. Values that were out of the concentration range and therefore not in the linear region were excluded from the fit (grey). b) Three independent measurements of saturated solutions and concentration calculation with the linear equation, estimated in a.
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1.7. Figure S7

Increased conjugation efficiency can be observed at 5 mg/ml brentuximab. (See chapter 3.8 for details) a) DAR-analysis of brentuximab, reduced with 200 eq. of DTT and alkylated with 10. Modification was carried out under the same conditions for every reaction: 5 mg/ml antibody, 50 mM tris-buffer, 1 mM EDTA pH 8.5 and 5% DMSO; reaction over-night at 14°C as described in chapter 3.7. DAR-analysis was carried as described in chapter 3.9. b) Exemplary MS-spectrum and calculated DAR of sample in lane 2 (modification carried out with 6 eq. 10). HC: heavy chain, LC: light chain.

1.8. Figure S8

Partial reduction of the interchain disulfides with small excess of TCEP yields ADCs with a high conjugation efficiency in a one-step procedure. (See chapter 3.9 for details) a) DAR-analysis of brentuximab, reduced and alkylated with 10. Modification was carried out under the same conditions for every reaction with varying equivalents of TCEP: 5 mg/ml antibody, 50 mM tris-buffer, 100 mM NaCl, 1 mM EDTA pH 8.5, 167 µM 10 (5.0 eq.) and 5% DMSO; reaction over-night at 14°C as described in chapter 3.8. DAR-analysis was carried as described in chapter 3.9. b) Exemplary MS-spectrum and calculated DAR of sample in lane 3 (modification carried out with 3 eq. TCEP). HC: heavy chain, LC: light chain.
1.9. Figure S9

DAR analysis of brentuximab-10 (a, green) and Adcetris® (b, cyan) after incubation in rat serum for 0, 3 and 7 days as described in chapter 3.12. Shown are values from three independent measurements for each time point. We measured a DAR of 3.05 (mean of three measurements) for Adcetris at Day 0, even though, Adcetris is known to be modified with an average of 4 drug molecules.\textsuperscript{[2]} We attribute this to a loss of modification during the analysis process of pulldown, deglycosylation, reduction and MS-analysis. Since this sample-preparation was conducted in the same way for day 0, 3 and 7, this should not influence the relative values, given in the main manuscript. c) MS analysis of two Adcetris® measurements (day 0 top and day 3 bottom) indicating hydrolysis of the vedotin molecules that are still attached to the antibody. From this we concluded complete hydrolysis of maleimides to the open ring form at day 3, resulting in no further retro-Michael addition and associated payload loss until day 7.
1.10. Figure S10

Size-exclusion HPLC analysis of Brentuximab-10 after storage at different temperatures in PBS. Chromatograms were recorded after 0, 7 and 14 days. Chromatograms are normalized to the maximal intensity. (See chapter 3.13 for details)
1.11. Figure S11

Changes in bodyweight (top) and tumor volumes (bottom) of SCID mice with a Karpas 299 tumor xenograft after treatment with PBS (magenta), Brentuximab-10 (blue) or PBS (green). Shown are tumor volumes of eight mice per group separately. a) 1. Study: Shown are tumor volumes of four mice per group separately treated at day 7 and 10 with 1 mg/kg ADC. b) 2. Study: Shown are tumor volumes of eight mice per group separately treated at day 8 and 11 with 0.5 mg/kg ADC. (See chapter 3.14 for details)
2. General Information

2.1. 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.

2.2. Flash- and thin layer chromatography

Flash column chromatography was performed, using NORMASIL 60® silica gel 40-63 µm (VWR international, USA). Glass TLC plates, silica gel 60 W coated with fluorescent indicator F254s were purchased from Merck (Merck Group, Germany). Spots were visualized by fluorescence depletion with a 254 nm lamp or manganese staining (10 g K₂CO₃, 1.5 g KMnO₄, 0.1 g NaOH in 200 ml H₂O), followed by heating.

2.3. 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). The following gradients were used: Method C: (A = H₂O + 0.1% TFA (trifluoroacetic acid), B = MeCN (acetonitrile) + 0.1% TFA, flow rate 30 ml/min, 5% B 0-5 min, 5-90% B 5-60 min, 90% B 60-65 min. Method D: (A = H₂O + 0.1% TFA, B = MeCN + +0.1% TFA), flow rate 30 ml/min, 5% B 0-5 min, 5-25% B 5-10 min, 25%-45% B 10-50 min, 45-90% 50-60 min, 90% B 60-65 min. Method E: 0.1% TFA, flow rate 18 ml/min, 5% B 0-5 min, 5-90% B 5-60 min, 90% B 60-65 min, using a VP 250/21 Macherey-Nagel Nucleodur C18 HTec Spum column (Macherey-Nagel GmbH & Co. Kg, Germany).

2.4. Semi-preparative HPLC

Semi-preparative HPLC was performed on a Shimadzu prominence HPLC system (Shimadzu Corp., Japan) with a CBM20A communication bus module, a FRC-10A fraction collector, 2 pumps LC-20AP, and a SPD-20A UV/VIS detector, using a VP250/10 Macherey-Nagel Nucleodur C18 HTec Spum column (Macherey-Nagel GmbH & Co. Kg, Germany). The following gradients were used: Method F: (A = H₂O + 0.1% TFA, B = MeCN + +0.1% TFA), flow rate 5 ml/min, 30% B 0-5 min, 30-99% B 5-65 min, 99% B 65-75 min.

2.5. 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 (CDCl₃: 7.26 [ppm]; DMSO-d₆: 2.50 [ppm] for ¹H-spectra and CDCl₃: 77.16 [ppm]; DMSO-d₆: 39.52 [ppm] for ¹³C-spectra. Coupling constants J are stated in Hz. Signal multiplicities are abbreviated as follows: s: singlet; d: doublet; t: triplet; q: quartet; m: multiplet.

2.6. 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 H₂O; 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.
2.7. UPLC-UV/MS

UPLC-UV/MS traces were recorded on a Waters H-class instrument equipped with a quaternary solvent manager, a Waters autosampler, a Waters TUV detector and a Waters Acquity QDa detector with an Acquity UPLC BEH C18 1.7 μm, 2.1 x 50 mm RP column with a flow rate of 0.6 mL/min (Waters Corp., USA). The following gradient was used for purity analyses: A: 0.1% TFA in H₂O; B: 0.1% TFA in MeCN. 5% B 0 - 1.5 min, 5-95% B 1.5-11 min, 95% B 11-13 min, 5% B 13-15 min. The following gradient was used in the solubility assay: A: 0.1% TFA in H₂O; B: 0.1% TFA in MeCN. 5% B 0 - 0.5 min, 5-95% B 0.5-3 min, 95% B 3-3.9 min, 5% B 3.9-5 min.

2.8. Intact protein MS (trastuzumab conjugates only)

Reduced antibody subunits were analyzed using a reversed-phase liquid chromatography system (Dionex Ultimate 3000 NCS-3500RS Nano, Thermo Scientific) connected to an Orbitrap Fusion mass spectrometer (Thermo Scientific). Chromatographic separation was performed with an Acquity UPLC protein BEH C4 column (300 Å, 1.7 μm, 2.1 mm x 50 mm). Component A of the mobile phase was 0.01% formic acid in water and component B consist of Acetonitrile with 0.01% formic acid. Separation was performed with a flow rate of 300 µL/min within 7.5 min linear gradient starting with 0% and ending with 40% of component B, followed by a flushing step until 80% of component B. Proteins were ionized in positive ion mode applying a spray voltage of 4.5 kV, using sheath gas (75 Arb), aux gas (12 Arb), sweep gas (1 Arb) and a vaporizer temperature of 300°C. Ionized proteins were analyzed in intact protein mode with a resolution of 15000 (FWHM), 10 microscans and a scan range of m/z 500-3000. The maximum injection time was set to 100 ms to reach an AGC-Target value of 5e5.

Raw data were analyzed with ProteinDeconvolut version 3.0 (Thermo Scientific), considering a m/z range of 800-3000 and charge states ranging from 10-100. 30000 was used as a targeted mass and the intact protein model was chosen. The output mass range was 10000-70000.

2.9. Intact protein MS (all brentuximab conjugates)

Intact proteins were analyzed using 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 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 H₂O; B: 0.01% FA in MeCN. 5-95% B 0-6 min. Mass analysis was conducted with a Waters XEVO G2-XS QTof analyzer. Proteins were ionized in positive ion mode applying a cone voltage of 40 kV. Raw data was analyzed with MaxEnt 1.

2.10. 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.

2.11. 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 have been achieved during a 30 minute isocratic gradient using a phosphate buffer at pH 7 (20 mM Na₂HPO₄/NaH₂PO₄, 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.

3. Experimental procedures
3.1. Trastuzumab production

Trastuzumab expression and purification was executed as previously published with an additional final purification by gel filtration on a Superdex 200 Increase 10/300 from GE (GE life sciences, USA) with PBS and flow rate of 0.75 ml/min.[9]

3.2. Synthesis and analysis of trastuzumab-MMAF conjugates

Trastuzumab modification was carried out by incubating freshly expressed antibody (0.5 mg/ml, 3.33 μM) with 1000 eq. of DTT in a buffer containing 50 mM sodium borate in PBS (pH 8.0) with a total volume of 80 μl at 37 °C for 40 min. Excess DTT removal and buffer exchange to a solution containing 50 mM NH₄HCO₃ and 1mM EDTA (pH 8.5) was conducted afterwards using 0.5 mL Zeba™ Spin Desalting Columns with 7K MWCO (Thermo Fisher Scientific, USA). 4 μl of O-Ethyl-P-ethynyl-phosphonamidate-VC-PAB-MMAF 4, dissolved in DMSO (5.33 mM) were added quickly to reach a final DMSO content of 5% and 80 eq. phosphonamidate with respect to the antibody. The mixture was shaken at 850 rpm and 14 °C for 16 hours. Excess reagent was again removed by buffer exchange to sterile PBS using 0.5 mL Zeba™ Spin Desalting Columns with 7K MWCO.

For SDS-page-analysis, 2 μl of the crude reaction mixture were mixed with 10 μl of ultrapure water and 4 μl Laemmli sample buffer (Bio-Rad Laboratories, USA) containing 0.4 μl 2-mercaptoethanol. Samples were heated to 95 °C for 15 minutes and completely loaded to the SDS-PAGE gel.

For MS analysis: The modified antibody was rebuffered to 100 mM NH₄HCO₃ and 500 mM NaCl using 75 μL Zeba™ Spin Desalting Columns with 7K MWCO (Thermo Fisher Scientific, USA). 8 μl of this solution were treated with 1 μl RapiGest™ (Waters Corp., USA) solution (1% in H₂O) and heated to 60 °C for 30 min. The solution was allowed to cool to room temperature, 1 μl PNGase-F solution (Pomega, Germany, Recombinant, cloned from Elizabethkingia miricola 10 u/μl) was added and the solution was incubated at 37 °C over night. Remaining disulfide bridges were reduced by addition of 1 μl DTT solution (50 mM in H₂O) and incubation at 37°C for 30 min. Samples were diluted with 1% HCl subjected to MS analysis with the Orbitrap Fusion system. (See chapter 2.8)

MS spectrum shows the ion series, which was deconvoluted to obtain the spectrum in Figure 1b.
3.3. Cell based antiproliferation assays

Antiproliferation assays were conducted as previously reported\textsuperscript{3} with the following minor changes:

- For MDAMB468 cells, a reduced amount of $2 \times 10^3$ cells were seeded in each well of a 96-well optical cell culture plate supplemented with 100 μL culture media.
- Images were acquired with an Operetta High-Content Imaging system (PerkinElmer, Waltham, MA, USA) equipped with a 20× high NA objective.
- Cell counts were calculated from duplicates.

3.4. Resazurin assay

HL60 and Karpas cell lines were cultured in RPMI-1640 supplemented with 10% FCS and 0.5% Penicillin-Streptomycin. SKBR3 and MDAMB468 cell lines were cultured in DMEM/F12 supplemented with 10% FCS and 0.5% Penicillin-Streptomycin. Cells were seeded at a density of $5 \times 10^3$ cells/well (SKBR3, HL60 and Karpas) or $1 \times 10^3$ cells/well (MDAMB468) in 96-well cell culture microplate. 1:4 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 96 h 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 ($\lambda_{EX} = 560$ nm and $\lambda_{EM} = 590$ 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 R2016 software.

3.5. Solubility assay

The aqueous solubility of compounds 9, 10 and vedotin was determined using a shake flask solubility assay. Saturated solutions of the compounds in 5% DMSO/PBS at pH 7.4 were prepared in triplicates by adding 2 μL of compound (40 mM in DMSO) to 38 μL PBS, pH 7.4 containing 50 μM inosine as internal standard. The samples were incubated at 25°C for 2h and subsequently subjected to high-speed centrifugation (10 minutes, 16873 rcf). The supernatant was analyzed by UPLC/UV and the concentration was determined using a standard curve. For the standard curves, serial dilutions in 5% DMSO/PBS at pH 7.4 containing 50 μM Inosine were prepared in triplicates with final compound concentrations of 5 μM, 10 μM, 50 μM, 100 μM and 200 μM. The samples were incubated at 25°C for 2 h and subsequently subjected to high-speed centrifugation (10 minutes, 16873 rcf). The supernatant was analyzed by UPLC/UV and the peak area of compound and internal standard were integrated. The normalized peak
areas (integrated area compound divided by integrated area standard) were plotted against the concentration and a linear fit was applied within the respective solubility range.

3.6. Brentuximab production

Brentuximab expression and purification was executed in analogy to trastuzumab with final purification by gel filtration on a Superdex 200 Increase 10/300 from GE (GE life sciences, USA) with PBS and flow rate of 0.75 ml/min.

3.7. Procedure for the modification of brentuximab (1mg/ml) with different equivalents of 10

35 μl of a 70 mM solution of DTT in 50 mM sodium borate in PBS (pH 8.0) was added to 350 μl of a brentuximab solution of 1.0 mg/ml in 50 mM sodium borate in PBS (pH 8.0) and the mixture was incubated at 37 °C for 40 min. Excess DTT removal and exchange to the conjugation buffer (50 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 8.5 at 14°C) was conducted afterwards using 2 mL Zeba™ Spin Desalting Columns with 7K MWCO (Thermo Fisher Scientific, USA). 50 μl of the reduced antibody solution (0.91 mg/ml, 6.07 μM antibody) were mixed quickly afterwards with the desired amount of O-2-(2-Hydroxyethoxy)ethyl-P-ethynyl-phosphonamidate-VC-PAB-MMAE 10 dissolved in DMSO to give a final amount of 5% DMSO. The mixture was shaken at 850 rpm and 14 °C for 16 hours. For SDS-page-analysis, 2 μl of the crude reaction mixture were mixed with 10 μl of ultrapure water and 4 μl Laemmli sample buffer (Bio-Rad Laboratories, USA) containing 0.4 μl 2-mercaptoethanol. Samples were heated to 95 °C for 15 minutes and completely loaded to the SDS-PAGE gel.

5 μl of a 70 mM solution of DTT in 50 mM sodium borate in PBS (pH 8.0) was added to 50 μl of a brentuximab solution of 5.0 mg/ml in 50 mM sodium borate in PBS (pH 8.0) and the mixture was incubated at 37 °C for 40 min. Excess DTT removal and exchange to the conjugation buffer (50 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 8.5 at 14°C) was conducted afterwards using 0.5 mL Zeba™ Spin Desalting Columns with 7K MWCO (Thermo Fisher Scientific, USA). 50 μl of the reduced antibody solution (4.55 mg/ml, 30.30 μM antibody) were mixed quickly afterwards with desired amount of O-2-(2-Hydroxyethoxy)ethyl-P-ethynyl-phosphonamidate-VC-PAB-MMAE 10 dissolved in DMSO to give a final amount of 5% DMSO. The mixture was shaken at 850 rpm and 14 °C for 16 hours.

3.8. Procedure for the modification of brentuximab (5.0 mg/ml) with different equivalents of 10

50 μl of a brentuximab solution of 5.0 mg/ml in conjugation buffer (50 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 8.5 at 14°C) were mixed with 5 μl of a TCEP solution in conjugation buffer containing the appropriate amount of TCEP. Directly afterwards, 2.75 μl of a 3.03 mM solution of O-2-(2-Hydroxyethoxy)ethyl-P-ethynyl-phosphonamidate-VC-PAB-MMAE 10 (5.0 eq. with respect to the antibody) dissolved in DMSO were added to give a final amount of 5% DMSO. The mixture was shaken at 850 rpm and 14 °C for 16 hours.

3.9. Procedure for the partial reduction of brentuximabs interchain disulfide bonds with TCEP

50 μl of a brentuximab solution of 5.0 mg/ml in conjugation buffer (50 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 8.5 at 14°C) were mixed with 5 μl of a TCEP solution in conjugation buffer containing the appropriate amount of TCEP. Directly afterwards, 2.75 μl of a 3.03 mM solution of O-2-(2-Hydroxyethoxy)ethyl-P-ethynyl-phosphonamidate-VC-PAB-MMAE 10 (5.0 eq. with respect to the antibody) dissolved in DMSO were added to give a final amount of 5% DMSO. The mixture was shaken at 850 rpm and 14 °C for 16 hours.

3.10. Procedure for DAR determination of brentuximab conjugates by intact protein MS

a) 40 μl of the crude antibody modification mixture were purified by size-exclusion chromatography with a 5 ml HiTrap® desalting column and a flow of 1.5 ml/min eluting with
100 mM NaHCO$_3$ and 500 mM NaCl over two column volumes. An exemplary chromatogram is shown below.

The antibody containing fractions were pooled and concentrated by spin-filtration to 40 µl (MWCO: 10 kDa, 0.5 ml, Sartorius, Germany).

b) 2 µl RapiGest™ solution (1% in H$_2$O) (Waters Corp., USA) were added and the solution was heated to 60 °C for 30 min. The solution was allowed to cool to room temperature, 1 µl PNGase-F solution (Pomega, Germany, Recombinant, cloned from Elizabethkingia miricola 10 u/µl) was added and the solution was incubated at 37 °C for at least 2 hours. Disulfide bridges were reduced by addition of 2 µl DTT solution (70 mM in H$_2$O) and incubation at 37°C for 30 min. Samples were diluted with 120 µl 1% HCl and subjected to intact protein MS (see chapter 2.9), injecting 5 µl for each sample.

c) For Adcetris pulldown samples from serum, 1 µl PNGase-F solution (Pomega, Germany, Recombinant, cloned from Elizabethkingia miricola 10 u/µl) was added to 20 µl of Adcetris in PBS and the solution was incubated at 37 °C for at least 2 hours. Disulfide bridges were reduced by addition of 2 µl DTT solution (70 mM in H$_2$O) and incubation at 37°C for 30 min. 10 µl of the samples were diluted with 190 µl of pure water and subjected to intact protein MS (see chapter 2.9), injecting 3 µl for each sample.

After deconvolution of the crude spectra, the DAR was determined with the following formula, where $I$ corresponds to the mass intensities of the respective species.
3.11. Synthesis and purification of an ADC from brentuximab and 10.

240 μl of a 70 mM solution of DTT in 50 mM sodium borate in PBS (pH 8.0) were added to 2.4 ml of a brentuximab solution of 1.0 mg/ml in 50 mM sodium borate in PBS (pH 8.0) and the mixture was incubated at 37 °C for 40 min. Excess DTT removal and exchange to the conjugation buffer was conducted afterwards using 10 mL Zeba™ Spin Desalting Columns with 7K MWCO (Thermo Fisher Scientific, USA). The reduced antibody solution (0.91 mg/ml, 6.07 μM) was mixed quickly afterwards with 132 μl of a 1.94 mM solution of O-2-(2-Hydroxyethoxy)ethyl-P-ethynyl-phosphonamidate-VC-PAB-MMAE 10 dissolved in DMSO to give a final amount of 5% DMSO and 16 eq. phosphonamidate. The solution was shaken at 850 rpm and 14 °C for 16 hours. Afterwards, the mixture was concentrated to 900 μl by spin-filtration (MWCO: 10 kDa, 0.5 ml, Sartorius, Germany)

Alternatively, 48 μl of a 70 mM solution of DTT in 50 mM sodium borate in PBS (pH 8.0) was added to 480 μl of a brentuximab solution of 5.0 mg/ml in 50 mM sodium borate in PBS (pH 8.0) and the mixture was incubated at 37 °C for 40 min. Excess DTT removal and exchange to the conjugation buffer was conducted afterwards using 2 mL Zeba™ Spin Desalting Columns with 7K MWCO (Thermo Fisher Scientific, USA). The reduced antibody solution (4.55 mg/ml, 30.35 μM) was mixed quickly afterwards with 27.5 μl of a 2.73 mM solution of O-2-(2-Hydroxyethoxy)ethyl-P-ethynyl-phosphonamidate-VC-PAB-MMAE 10 dissolved in DMSO to give a final amount of 5% DMSO and 4. eq. phosphonamidate. The solution was shaken at 850 rpm and 14 °C for 16 hours.

The reaction mixtures were purified in two portions by size-exclusion chromatography with a 25 ml Superose™ 6 Increase 10/300GL (GE healthcare, United States) 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 (MWCO: 10 kDa, 6 ml, Sartorius, Germany). For DAR analysis, 10 μl of this sample were mixed with 30 μl of a buffer containing 500 mM NaCl and 100 mM NaHCO₃ and the sample was processed further as described under 3.9b. The final 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.

The ADC was analyzed by analytical size exclusion chromatography (a) and intact protein MS (b) before subsequent experiments. HC: heavy chain, LC: light chain. *deconvolution artefacts (half mass of HC species and double mass of LC species). It should be noted that no species was detected in the MS that could be assigned to any form of unconjugated MMAE.
3.12. Stability studies in rat serum: ADC incubation serum and analysis of the DAR after antibody pulldown

In an Eppendorf-tube, 200 μl rat serum (Sigma Aldrich, United States) were mixed with 50 μl Brentuximab-10 (2.0 mg/ml) or Adcetris® for each sample individually to give a final solution of 0.4 mg/ml ADC in 80% rat serum. Samples were sterile filtered with UFC30GV0S centrifugal filter units (Merck, Germany) and incubated at 37°C for 3 and 7 days. Samples for day 0 were directly processed further.

The supernatant of 50 μl anti human IgG (Fc-Specific) agarose slurry (Sigma Aldrich, United States) was removed by centrifugation and the remaining resin washed three times with 200 μl PBS. The resin was incubated with 240 μl of the serum-ADC mix for 30 min at room temperature. Afterwards, the supernatant was removed and the resin washed 5 times with 200 μl PBS. Following by incubation for 5 minutes with 200 μl IgG elution buffer (Thermo-Fisher, United States) at room temperature. Now the supernatant was transferred into a Spin filter (MWCO: 10 kDa, 0.5 ml, Sartorius, Germany), rebuffered to a buffer containing 500 mM NaCl and 100 mM NaHCO₃, concentrated to 40 μl and processed further as described under 3.9b for the Brentuximab-10 samples. Since we observed decomposition of the maleimide linkage in Adcetris® under our standard deglycosylation conditions, all Adcetris® samples were rebuffered to PBS, concentrated to 100 μl and processed further as described under 3.9c. All measurements were performed in triplicates (n=3). MS Spectra that were obtained after 0, 3 and 7 days are shown below. HC: heavy chain, LC: light chain.

0 days of incubation of brentuximab-10 in rat serum at 37°C:
3 days of incubation of brentuximab-10 in rat serum at 37°C:
7 days of incubation of brentuximab-10 in rat serum at 37°C:

0 days of incubation of Adcetris® in rat serum at 37°C:
3.13. Stability assessment of ADCs with A-SEC

Brentuximab-10 was adjusted to a protein concentration of 1 mg/mL in PBS (Dulbecco’s Phosphate Buffered Saline, Sigma-Aldrich Merck KGaA) and filtered sterile (Ultrafree-MC Centrifugal filter units, Merck Millipore). Samples were stored at 4-8°C, 37°C and 40°C for up to 14 days. For samples stored at elevated temperatures, it was ensured that no condensate was formed. Before analysis via A-SEC the samples where centrifuged at 4°C, 4000 x g for 4 minutes.

3.14. In vivo xenograft model

The in vivo evaluations were performed at EPO GmbH. All animal experiments were conducted in accordance with German animal welfare law and approved by local authorities. In brief, 1×10^7 Karpas 299 cells were subcutaneously injected to CB17-Scid mice at day 0. Treatment was initiated when tumors reached a mean tumor volume of 0.136±0.087 cm^3 at day 7 (study
1) and day 8 (study 2). Following randomization of mice into treatment and control groups, 1 mg/kg of brentuximab-10 or Adcetris as well as vehicle (PBS) were administered as intravenous injection on days 7 and 10 for the first study and 0.5 mg/kg of brentuximab-10 or Adcetris as well as vehicle (PBS) on days 8 and 12 for the second study. Tumor volumes, body weights and general health conditions were recorded throughout the whole study.

4. Organic synthesis

4.1. \(N\)-(4-azidobenzoyl)-\(L\)-valine

A 50-ml Schlenk-flask was charged with 1.00 g of 4-azidobenzoic acid (6.13 mmol, 1.00 eq.) and suspended in 8.5 ml of dry CH\(_2\)Cl\(_2\) together with a drop of DMF under argon. 630 µl of oxalylchloride were added drop-wise at 0 °C and the reaction mixture was stirred at room temperature for 2 h until the solution became clear. All volatiles were removed under reduced pressure and the corresponding solid was redissolved in 4 ml of DMF. The corresponding solution was added drop-wise at 0 °C to a solution of 720 mg \(L\)-valin (6.13 mmol, 1.00 eq.) and 612 mg sodium hydroxide (15.33 mmol, 2.50 eq.) in 8 ml water and stirred for 2 more hours. The solution was acidified with 1 N HCl and extracted three times with diethylether. The organic fractions were pooled, dried (MgSO\(_4\)) and the solvents were removed under reduced pressure. Pure product was obtained by flash column chromatography on silicagel (30% EtOAc, 0.5% formic acid in \(n\)-hexane) as colourless fume. (954 mg, 4.96 mmol, 80.9%)

\(^1\)H NMR (600 MHz, Chloroform-\(d\)) \(\delta = 10.12\ (s, 1H), 7.79\ (d, J=8.6, 2H), 7.05\ (d, J=8.6, 2H), 6.79\ (d, J=8.5, 1H), 4.76\ (dd, J=8.5, 4.9, 1H), 2.33\ (pd, J=6.9, 4.9, 1H), 1.03\ (d, J=6.9, 3H), 1.01\ (d, J=6.9, 3H)\.

\(^{13}\)C NMR (151 MHz, CDCl\(_3\)) \(\delta = 175.82, 167.28, 144.03, 130.17, 129.13, 119.20, 77.16, 57.79, 31.40, 19.16, 17.99\.

HR-MS for C\(_{12}\)H\(_{15}\)N\(_4\)O\(_3\)\([M+H]\)^{+}\) calcd.: 263.1139, found 263.1151.

4.2. \(N\)-(4-azidobenzoyl)-\(L\)-valine-anhydride

In a 100-ml round-bottom flask, 954 mg \(N\)-(4-azidobenzoyl)-\(L\)-valine (3.64 mmol, 1.00 eq.), 750 mg dicyclohexylcarbodiimide (3.64 mmol, 1.00 eq.), 418 mg \(N\)-hydroxysuccinimide (3.64 mmol, 1.00 eq.) and 9 mg 4-(dimethylamino)-pyridine (0.07 mmol, 0.02 eq.) were dissolved in 25 ml of THF and stirred over night at room temperature. The reaction mixture was filtered, the solids were washed several times with THF, the solvent was removed under reduced pressure and the crude product was purified by flash column chromatography on silicagel (20 to 40% EtOAc in \(n\)-hexane). The compound was isolated as white powder (513 mg, 1.01 mmol, 55.7%).

\(^1\)H NMR (600 MHz, Chloroform-\(d\)) \(\delta = 8.01\ (d, J=8.7, 2H), 7.13\ (d, J=8.7, 2H), 4.29\ (d, J=4.6, 1H), 2.39\ (heptd, J=6.9, 4.6, 1H), 1.16\ (d, J=6.9, 3H), 1.03\ (d, J=6.9, 3H).\n
\(^{13}\)C NMR (151 MHz, CDCl\(_3\)) \(\delta = 177.52, 160.90, 144.51, 129.60, 122.43, 119.30, 70.68, 31.28, 18.76, 17.57.\)
4.3. \(N\)-(4-azidobenzoyl)-\textit{L}-valine-\textit{L}-citrulline

In a 50-ml round-bottom flask, 380 mg \(N\)-(4-azidobenzoyl)-\textit{L}-valine-anhydride (0.75 mmol, 1.00 eq.) were dissolved in 2 ml of 1,2-Dimethoxyethane and cooled to 0 °C. A solution of 351 mg \(\textit{L}\)-citrulline (1.50 mmol, 2.00 eq.) and 144 mg sodium hydrogencarbonate (2.25 mmol, 3.00 eq.) in 4 ml \(\text{H}_2\text{O}\) and 2 ml THF was added dropwise and stirred overnight at room temperature. All volatiles were removed under reduced pressure and the crude product was purified by flash column chromatography on silicagel (10% MeOH, 0.5% formic acid in \(\text{CH}_2\text{Cl}_2\)). The compound was isolated as colourless oil (312 mg, 0.74 mmol, 99.0%).

\(^1\text{H}\) NMR (600 MHz, DMSO-\(d_6\)) \(\delta = 8.31\) (d, \(J = 8.8\), 1H), 8.27 – 8.21 (m, 1H), 7.96 (d, \(J = 8.6\), 2H), 7.20 (d, \(J = 8.6\), 2H), 6.05 (t, \(J = 5.5\), 1H), 5.47 (s, 2H), 4.37 (t, \(J = 8.1\), 5.1, 1H), 2.98 (q, \(J = 6.4\), 2H), 2.15 (dq, \(J = 13.6\), 6.8, 1H), 1.78 – 1.68 (m, 1H), 1.68 – 1.56 (m, 1H), 1.51 – 1.35 (m, 2H), 0.96 (d, \(J = 6.8\), 3H), 0.94 (d, \(J = 6.8\), 3H). \(^{13}\text{C}\) NMR (151 MHz, DMSO) \(\delta = 174.09, 171.54, 165.99, 159.40, 142.77, 131.36, 129.93, 119.23, 59.31, 52.57, 49.07, 30.77, 29.01, 27.07, 19.75, 19.28.\) HR-MS for \(\text{C}_{18}\text{H}_{26}\text{N}_7\text{O}_5\)\(^{+}\) [M+H]\(^{+}\) calcd.: 420.1990, found 420.1990.

4.4. \(N\)-(4-azidobenzoyl)-\textit{L}-valine-\textit{L}-citrulline-4-aminobenzyl alcohol (1)

In a 50-ml round-bottom flask, 330 mg \(N\)-(4-azidobenzoyl)-\textit{L}-valine-\textit{L}-citrulline (0.787 mmol, 1.0 eq.) and 107 mg 4-aminobenzyl alcohol (0.866 mmol, 1.10 eq.) were dissolved in 8 ml \(\text{CH}_2\text{Cl}_2\) and 4 ml \(\text{MeOH}\) under an argon atmosphere and cooled to 0 °C. 390 mg \(N\)-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (1.574 mmol, 2.00 eq.) were added portion-wise and the resulting solution was allowed to warm to room temperature overnight. All volatiles were removed under reduced pressure and the crude product was isolated by flash column chromatography on silicagel (10% to 15% MeOH in \(\text{CH}_2\text{Cl}_2\)) and obtained as white solid (164 mg, 0.313 mmol, 39.8%). Enantiomeric pure compound was isolated by preparative HPLC (Method D) and obtained as a white solid after lyophilisation.

\(^1\text{H}\) NMR (600 MHz, DMSO-\(d_6\)) \(\delta = 9.93\) (s, 1H), 8.32 (d, \(J = 8.4\), 1H), 8.21 (d, \(J = 7.6\), 1H), 7.96 (d, \(J = 8.6\), 2H), 7.55 (d, \(J = 8.6\), 2H), 7.24 (d, \(J = 8.6\), 2H), 7.21 (d, \(J = 8.6\), 2H), 6.12 (bs, 2H), 4.44 (s, 2H), 4.46 – 4.40 (m, 1H), 4.36 (t, \(J = 8.1\), 1H), 3.09 – 2.93 (m, 2H), 2.24 – 2.04 (m, \(J = 6.7\), 1H), 1.84 – 1.58 (m, 2H), 1.55 – 1.34 (m, 2H), 0.95 (d, \(J = 6.7\), 3H), 0.94 (d, \(J = 6.7\), 3H). \(^{13}\text{C}\) NMR (151 MHz, DMSO) \(\delta = 171.62, 170.79, 166.15, 159.46, 142.83, 137.95, 137.91, 131.29, 129.96, 127.38, 119.34, 119.26, 63.07, 59.56, 53.64, 39.20, 30.61, 29.88, 27.16, 19.79, 19.37.\) HR-MS for \(\text{C}_{26}\text{H}_{33}\text{N}_8\text{O}_5\)\(^{+}\) [M+H]\(^{+}\) calcd.: 525.2568, found 525.2563. \([\alpha]_D^{24} = -49.6\) (c = 0.81; MeOH)
4.5. \textit{N-(4-(O-Ethyl-P-ethynyl-phosphonamidato-N-benzoyl)-L-valine-L-citrulline-4-aminobenzyl-4-nitrophenyl carbonate (2)}

![Chemical Structure Image]

A 5-ml round-bottom flask was charged with 31 mg \textit{N-(4-(O-Ethyl-P-ethynyl-phosphonamidato-N-benzoyl)-L-valine-L-citrulline-4-aminobenzyl alcohol (1) (0.050 mmol, 1.00 eq.) and 31 mg Bis(4-nitrophenyl) carbonate (0.101 mmol, 2.00 eq.). The solids were dissolved in 140 µl of DMF and 17.4 µl DIPEA (0.101 mmol, 2.00 eq.) were added. The yellow solution was stirred for 1 h at room temperature and the solution was added to 30 ml of ice-cold diethyl ether. The precipitate was collected by centrifugation, redissolved in DMF and again precipitated with ether. The procedure was conducted three times in total and finally the solid was dried under high vacuum conditions. The compound was isolated in quantitative yields and sufficiently pure for the next step. Analytical pure material was purified by preparative HPLC using method C. \(^1\)H NMR (600 MHz, DMSO-\textit{d}_{6}) \(\delta = 10.10\) (s, 1H), 8.79 (d, \(J = 8.5\), 1H), 8.32 (d, \(J = 9.1\), 1H), 8.23 (d, \(J = 7.4\), 1H), 8.07 (dd, \(J = 8.5, 2.2\), 1H), 7.81 (d, \(J = 8.7\), 2H), 7.66 (d, \(J = 8.5\), 2H), 7.57 (d, \(J = 9.1\), 1H), 7.42 (d, \(J = 8.5\), 2H), 7.13 (d, \(J = 8.7\), 2H), 5.25 (s, 2H), 4.47 – 4.40 (m, 1H), 4.34 (t, \(J = 8.0\), 1H), 4.20 – 4.05 (m, 2H), 3.01 (ddt, \(J = 14.7, 13.4, 6.8\), 2H), 2.20 – 2.09 (m, \(J = 6.8\), 1H), 1.80 – 1.59 (m, 2H), 1.55 – 1.35 (m, 2H), 1.30 (t, \(J = 7.0\), 3H), 0.95 (d, \(J = 6.7\), 3H), 0.93 (d, \(J = 6.7\), 3H). \(^{13}\)C NMR (151 MHz, DMSO-\textit{d}_{6}) \(\delta = 171.79, 171.17, 166.58, 159.44, 155.75, 152.42, 145.63, 143.50, 139.83, 129.95, 129.77, 129.30, 127.59, 125.86, 123.08, 119.51, 117.24 (d, \(J = 7.8\), 91.67 (d, \(J = 45.6\), 77.26 (d, \(J = 261.0\), 70.71, 62.26 (d, \(J = 5.0\), 59.31, 53.68, 39.14, 30.71, 29.76, 27.19, 19.80, 19.30, 16.41 (d, \(J = 6.9\)). \(^{31}\)P NMR (243 MHz, DMSO) \(\delta = -10.39, -10.44\). HR-MS for C\textsubscript{36}H\textsubscript{43}N\textsubscript{7}O\textsubscript{11}P\textsuperscript{+} [M+H]\textsuperscript{+} calcd.: 780.2753, found 780.2744.

4.6. \textit{N-(4-(O-Ethyl-P-ethynyl-phosphonamidato-N-benzoyl)-L-valine-L-citrulline-4-aminobenzyl-4-nitrophenyl carbonate (3)}

![Chemical Structure Image]

A 5-ml round-bottom flask was charged with 31 mg \textit{N-(4-(O-Ethyl-P-ethynyl-phosphonamidato-N-benzoyl)-L-valine-L-citrulline-4-aminobenzyl alcohol (2) (0.050 mmol, 1.00 eq.) and 31 mg Bis(4-nitrophenyl) carbonate (0.101 mmol, 2.00 eq.). The solids were dissolved in 140 µl of DMF and 17.4 µl DIPEA (0.101 mmol, 2.00 eq.) were added. The yellow solution was stirred for 1 h at room temperature and the solution was added to 30 ml of ice-cold diethyl ether. The precipitate was collected by centrifugation, redissolved in DMF and again precipitated with ether. The procedure was conducted three times in total and finally the solid was dried under high vacuum conditions. The compound was isolated in quantitative yields and sufficiently pure for the next step. Analytical pure material was purified by preparative HPLC using method C.
1H NMR (600 MHz, DMSO-\textit{d}6) \( \delta = 10.10 \text{ (s, 1H)}, 8.79 \text{ (d, } J=8.5 \text{, 1H)}, 8.32 \text{ (d, } J=9.1 \text{, 1H)}, 8.23 \text{ (d, } J=7.4 \text{, 1H)}, 8.07 \text{ (dd, } J=8.5 \text{, 2.2, 1H)}, 7.81 \text{ (d, } J=8.7 \text{, 2H)}, 7.66 \text{ (d, } J=8.5 \text{, 2H)}, 7.57 \text{ (d, } J=9.1 \text{, 1H)}, 7.42 \text{ (d, } J=8.5 \text{, 2H)}, 7.13 \text{ (d, } J=8.7 \text{, 2H)}, 5.25 \text{ (s, 2H)}, 4.47 \text{ – } 4.40 \text{ (m, 2H)}, 4.34 \text{ (t, } J=8.0 \text{, 1H)}, 4.20 \text{ – } 4.05 \text{ (m, 2H)}, 3.01 \text{ (ddt, } J=47.1 \text{, 13.4, 6.8, 2H}), 2.20 \text{ – } 2.09 \text{ (m, } J=6.8 \text{, 1H)}, 1.80 \text{ – } 1.59 \text{ (m, 2H)}, 1.55 \text{ – } 1.35 \text{ (m, 2H)}, 1.30 \text{ (t, } J=7.0 \text{, 3H)}, 0.95 \text{ (d, } J=6.7 \text{, 3H}), 0.93 \text{ (d, } J=6.7 \text{, 3H)}.

13C NMR (151 MHz, DMSO-\textit{d}6) \( \delta = 171.79, 171.17, 166.58, 159.44, 155.75, 152.42, 145.63, 143.50, 139.83, 129.95, 129.77, 127.59, 125.86, 123.08, 119.51, 117.24 \text{ (d, } J=7.8), 91.67 \text{ (d, } J=45.6), 77.26 \text{ (d, } J=261.0), 70.71, 62.26 \text{ (d, } J=5.0), 59.31, 53.68, 39.14, 30.71, 29.76, 27.19, 19.80, 19.30, 16.41 \text{ (d, } J=6.9). \)

31P NMR (243 MHz, DMSO) \( \delta = -10.39, -10.44. \)

HR-MS for C_{36}H_{43}N_{7}O_{11}P^{+} \[M+H]\^+ calcd.: 780.2753, found 780.2744.

4.7. **O-Ethyl-P-ethynyl-phosphonamidate-VC-PAB-MMAF 4**

![Chemical structure](image)

A screw-cap vial was charged with 14.35 mg \( N-\text{(4-(O-Ethyl-P-ethynyl-phosphonamidato-N-benzoyl)-L-valine-L-citrulline-4-aminobenzyl-4-nitrophenyl carbonate (3) (0.0184 mmol, 1.00 eq.), 0.50 mg 1-Hydroxybenzotriazol (0.0037 mmol, 0.20 eq.) and 13.15 mg MMAF (0.0184 mmol, 1.00 eq.). The solids were dissolved in 250 \mu l dry DMF and 25 \mu l pyridine and heated to 60 °C over-night. All volatiles were removed under reduced pressure, the crude product was purified by preparative HPLC using method E and the desired compound obtained as a white solid after lyophilization. (4.84 mg, 0.0035 mmol, 19.2 %). HR-MS for C_{69}H_{104}N_{11}O_{16}P^{2+} \[M+2H]\^{2+} calcd.: 686.8695, found 686.8694.**

4.8. **Di-(2-(2-Hydroxyethoxy)ethyl) ethynylphosphonite (5)**

![Chemical structure](image)

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. 1.06 g diethylene glycol (10.00 mmol, 1.00 eq.), dissolved in 5.56 ml 1H-tetrazole solution (0.45 M in MeCN, 2.50 mmol, 2.50 eq.) were added and the white suspension was stirred over night at room temperature. The reaction mixture was directly placed on a silica gel flash column for
purification (5% MeOH in CH₂Cl₂). The desired compound was obtained as a yellowish oil.
(112 mg, 0.421 mmol, 42.1%).

$^1$H NMR (300 MHz, Chloroform-d) $\delta = 4.14 - 3.98$ (m, 4H), 3.65 - 3.59 (m, 4H), 3.58 - 3.49 (m, 8H), 3.15 (d, $J=2.4$, 1H) $^{13}$C NMR (75 MHz, Chloroform-d) $\delta = 92.51$ (d, $J=1.4$), 84.30 (d, $J=46.8$), 72.60, 70.72 (d, $J=4.0$), 67.20 (d, $J=6.0$), 61.44. $^{31}$P NMR (122 MHz, CDCl₃) $\delta = 131.97$.

4.9. 2-(2-Hydroxyethoxy)ethyl-N-(4-benzoic-acid-N-hydroxysuccinimideester)-P-ethynyl phosphonamidate (8)

In a 5 ml round-bottom-flask, 93 mg Di-(2-(2-Hydroxyethoxy)ethyl ethynylphosphonite (5) (0.192 mmol, 1.00 eq.) and 91 mg 4-azidobenzoic-acid-N-hydroxysuccinimide ester (6) (0.192 mmol, 1.00 eq.) were dissolved in 1 ml of DMF and the solution was stirred overnight. All volatiles were removed under reduced pressure and the residue purified by column chromatographic on silicagel (100% EtOAc). The compound was obtained as colourless oil. (45 mg, 0.109 mmol, 31.4%).

$^1$H NMR (300 MHz, Chloroform-d) $\delta = 8.02$ (d, $J = 8.7$ Hz, 2H), 7.79 (d, $J = 7.6$ Hz, 1H), 7.21 (d, $J = 8.8$ Hz, 2H), 4.30 (dp, $J = 13.6$, 4.5 Hz, 2H), 3.89 - 3.67 (m, 6H), 3.09 (d, $J = 13.3$ Hz, 1H), 2.89 (s, 4H). $^{13}$C NMR (75 MHz, Chloroform-d) $\delta = 169.67, 161.45, 145.78$ (d, $J = 1.6$ Hz), 132.32, 118.04, 117.66 (d, $J = 5.1$ Hz), 89.29 (d, $J = 50.1$ Hz), 75.34 (d, $J = 294.4$ Hz), 72.59, 69.44 (d, $J = 5.1$ Hz), 66.19 (d, $J = 5.9$ Hz), 61.35, 25.68. $^{31}$P NMR (122 MHz, CDCl₃) $\delta = -9.66$. HRMS for C₁₇H₂₀N₄O₈P $[M+H]^+$ calcd.: 411.0952, found: 411.0951.

4.10. O-ethyl-P-ethynyl-phosphonamide-VC-PAB-MMAE 9

In a screw-cap-vial equipped with a stirring bar, 5 mg of H₂N-Val-Cit-PAB-MMAE 11 (4.452 μmol, 1.00 eq.) and 3.12 mg 2-ethyl-N-(4-benzoic-acid-N-hydroxysuccinimideester)-P-ethynyl phosphonamidate (7) (8.904 μmol, 2.00 eq.) were dissolved in 50 μl DMF. 3.1 μl DIPEA (17.808 μmol, 4.00 eq.) were added and the solution was stirred overnight at room temperature. The solution was diluted with 4 ml 30% MeCN in H₂O and subjected to semi-preparative HPLC purification using method F and the desired compound obtained as a white solid after lyophilization. (5.00 mg, 3.681 μmol, 82.7%). HR-MS for C₆₉H₁₀₅N₁₁O₁₅P $[M+H]^+$ calcd.: 1358.7524, found 1358.7518.
4.11. **O-2-(2-Hydroxyethoxy)ethyl-P-ethynyl-phosphonamidate-VC-PAB-MMAE**

In a screw-cap-vial equipped with a stirring bar, 5 mg of H$_2$N-Val-Cit-PAB-MMAE 11 (4.452 μmol, 1.00 eq.) and 3.65 mg 2-(2-Hydroxyethoxy)ethyl-N-(4-benzoic-acid-N-hydroxysuccinimideester)-P-ethynyl phosphonamidate (8) (8.904 μmol, 2.00 eq.) were dissolved in 50 μl DMF. 3.1 μl DIPEA (17.808 μmol, 4.00 eq.) were added and the solution was stirred overnight at room temperature. The solution was diluted with 4 ml 30% MeCN in H$_2$O and subjected to semi-preparative HPLC purification using method F and the desired compound obtained as a white solid after lyophilization. (2.73 mg, 1.589 μmol, 35.7%). HR-MS for C$_{71}$H$_{109}$N$_{11}$O$_{17}$P$^+$ [M+H]$^+$ calcd.: 1418.7735, found 1418.7729.
5. NMR spectra

\[ N-(4\text{-azidobenzoyl})-L\text{-valine} \]
N-(4-azidobenzoyl)-L-valine-anhydride
N-(4-azidobenzoyl)-L-valine-L-citrulline
$N$-(4-azidobenzoyl)-L-valine-L-citrulline-4-aminobenzyl alcohol (2)
N-(4-(O-Ethyl-P-ethynyl-phosphonamidato-N-benzoyl)-L-valine-L-citrulline-4-aminobenzyl alcohol (2)
N-(4-(O-Ethyl-P-ethylphosphonomidato-N-benzoyl)-L-valine-L-citrulline-4-aminobenzyl-4-nitrophenyl carbonate (3)
Di-(2-(2-Hydroxyethoxy)ethyl) ethynylphosphonite (5)
Di-(2-(2-Hydroxyethoxy)ethyl)-N-(4-benzoic-acid-N-hydroxysuccinimideester)-P-ethynyl phosphonamidate (8)
6. References

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