Expanding the payload scope in antibody-drug conjugates: Unprecedented delivery of hydroxy-containing drugs through self-immolative phosphoramidates

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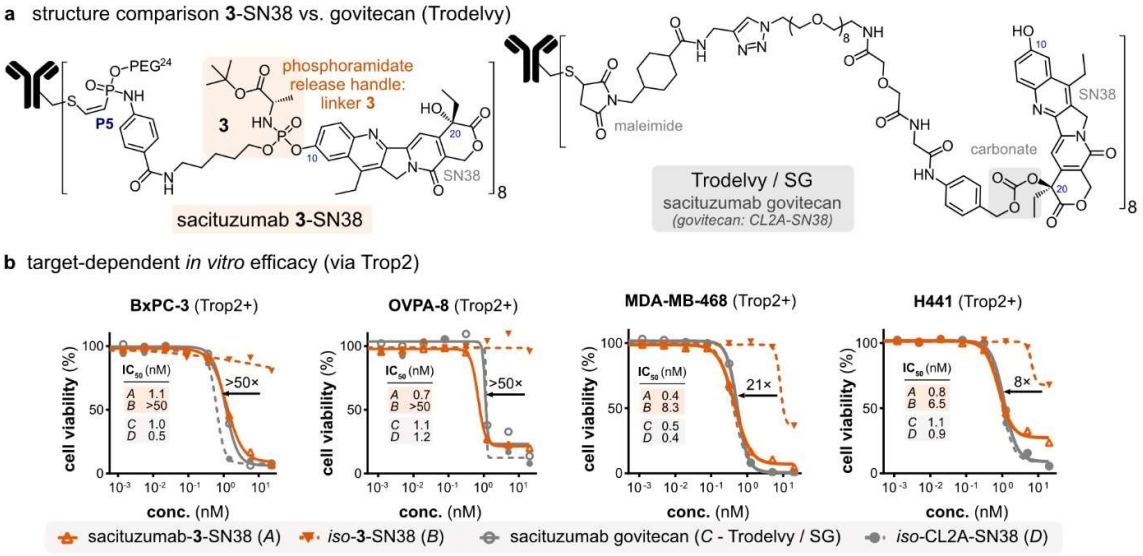
1. Supplementary Figures

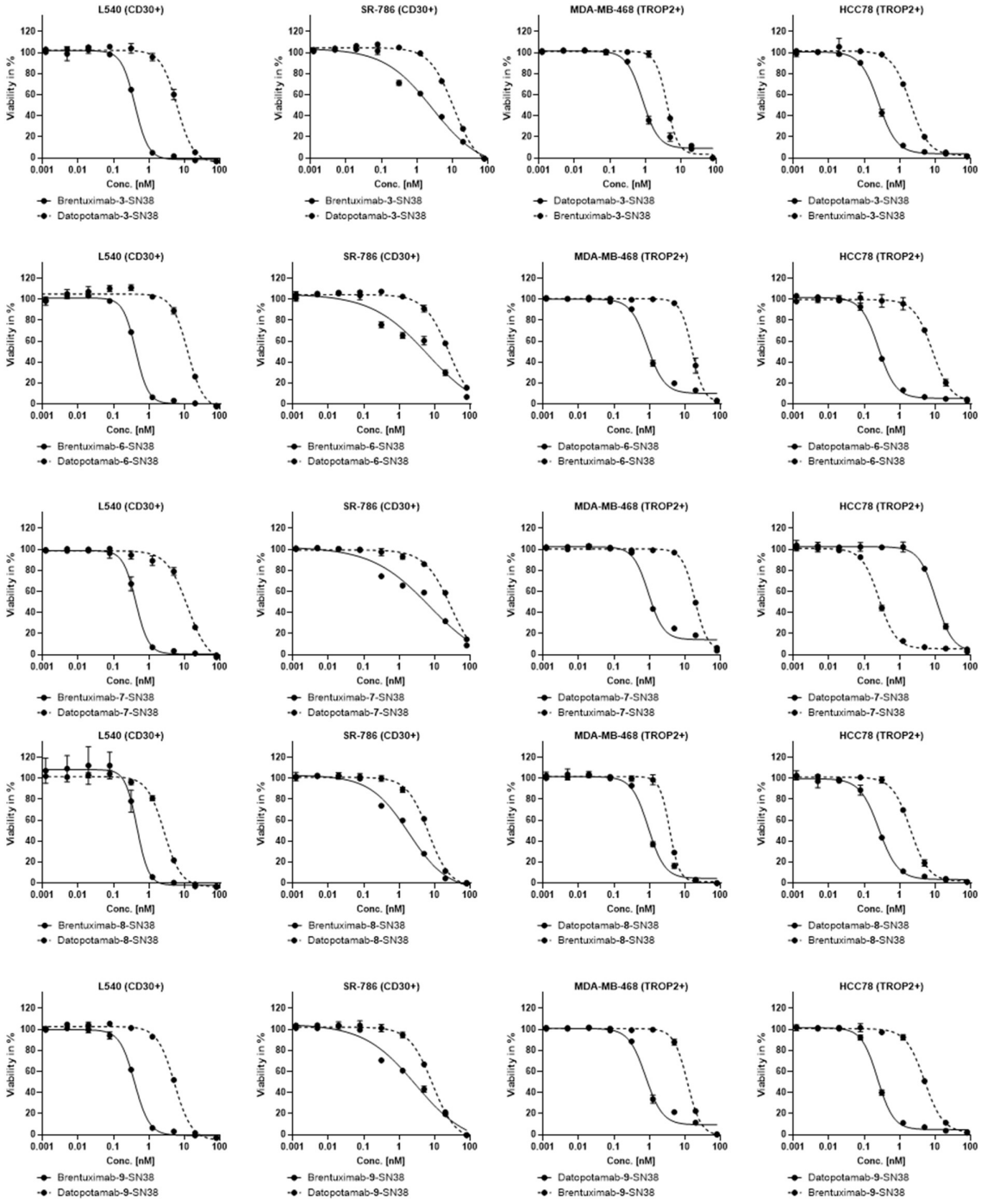
1.1. Supplementary figure 1

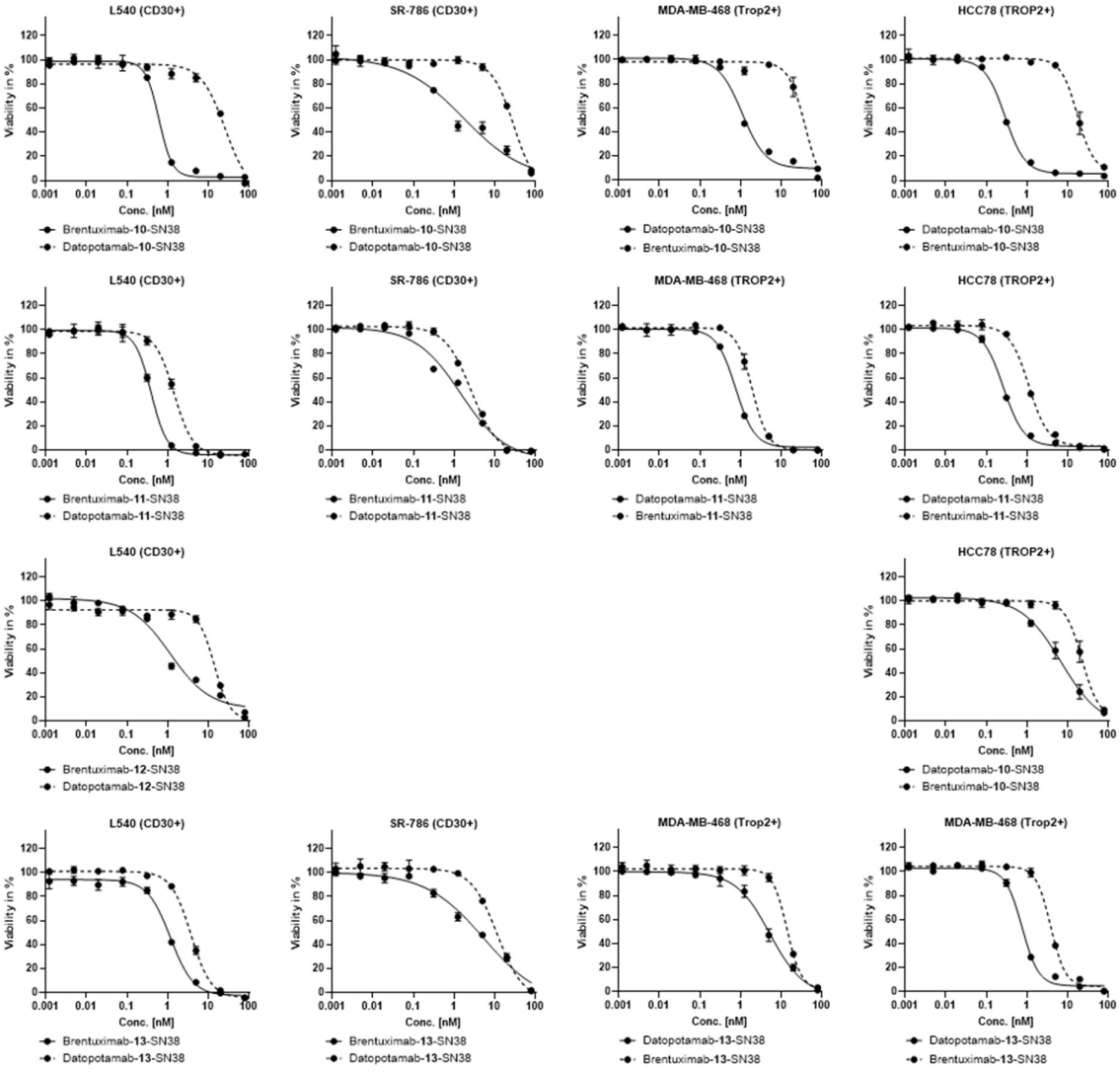


Head-to-head comparison between 3-SN38 and CL2A-SN38 *in vitro*. The sacituzumab-based ADCs are shown as solid and isotype-ADCs as dashed lines. ADCs based on **3**-SN38 are shown in orange, CL2A-SN38 in grey. (**a**) Linker structures of **3**-SN38 conjugated to sacituzumab (saci/anti-Trop2) or the isotype control (iso) and of CL2A-SN38 used in SG. (**b**) *In vitro* dose-response curves for antiproliferative activity on the Trop2+ cell lines BxPC-3, OVPA-8, MDA-MB-468 and H441. Graphs show mean, *n* = 2.

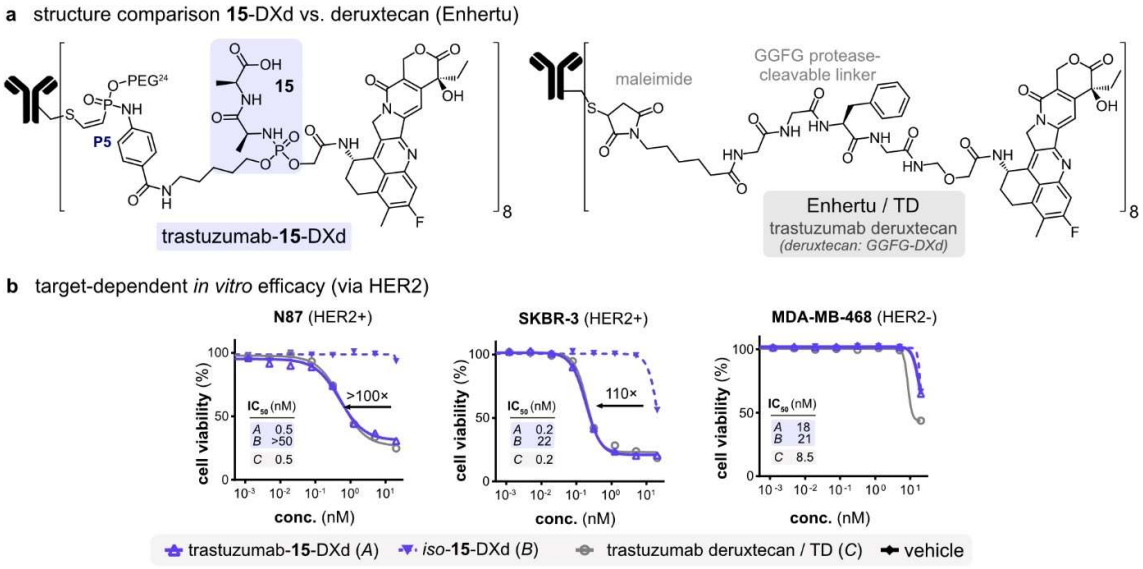
1.2. Supplementary figure 2

Antiproliferative activity dose-response curves for datopotamab- and brentuximab-based ADCs conjugated to SN38 via linkers **3**, **6**, **7**, **8**, **9**, **10**, **11**, **12** and **13** evaluated on the 4 cell lines L-540, SR-786, MDA-MB-468 and HCC78.




L540 (CD30+)
SR-786 (CD30+)
MDA-MB-468 (Trop2+)
HCC78 (TROP2+)
Viability in %
Conc. [nM]
Brentuximab-10-SN38
Datopotamab-10-SN38
Datopotamab-10-SN38
Brentuximab-10-SN38
L540 (CD30+)
SR-786 (CD30+)
MDA-MB-468 (TROP2+)
HCC78 (TROP2+)
Brentuximab-11-SN38
Datopotamab-11-SN38
Datopotamab-11-SN38
Brentuximab-11-SN38
L540 (CD30+)
HCC78 (TROP2+)
Brentuximab-12-SN38
Datopotamab-12-SN38
Datopotamab-10-SN38
Brentuximab-10-SN38
L540 (CD30+)
SR-786 (CD30+)
MDA-MB-468 (Trop2+)
MDA-MB-468 (Trop2+)
Brentuximab-13-SN38
Datopotamab-13-SN38
Datopotamab-13-SN38
Brentuximab-13-SN38

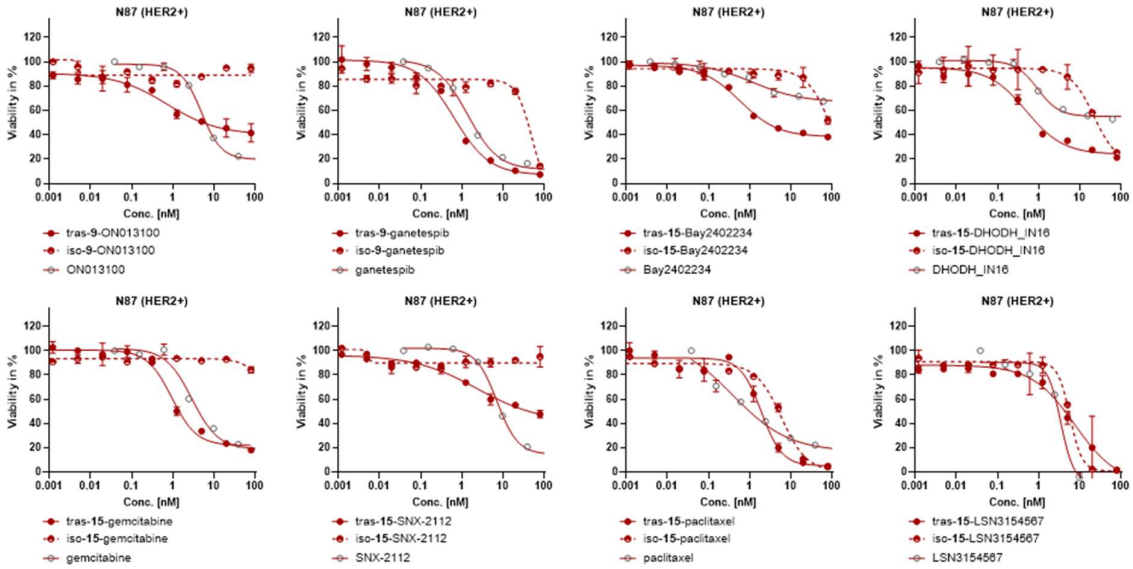
1.3. Supplementary figure 3



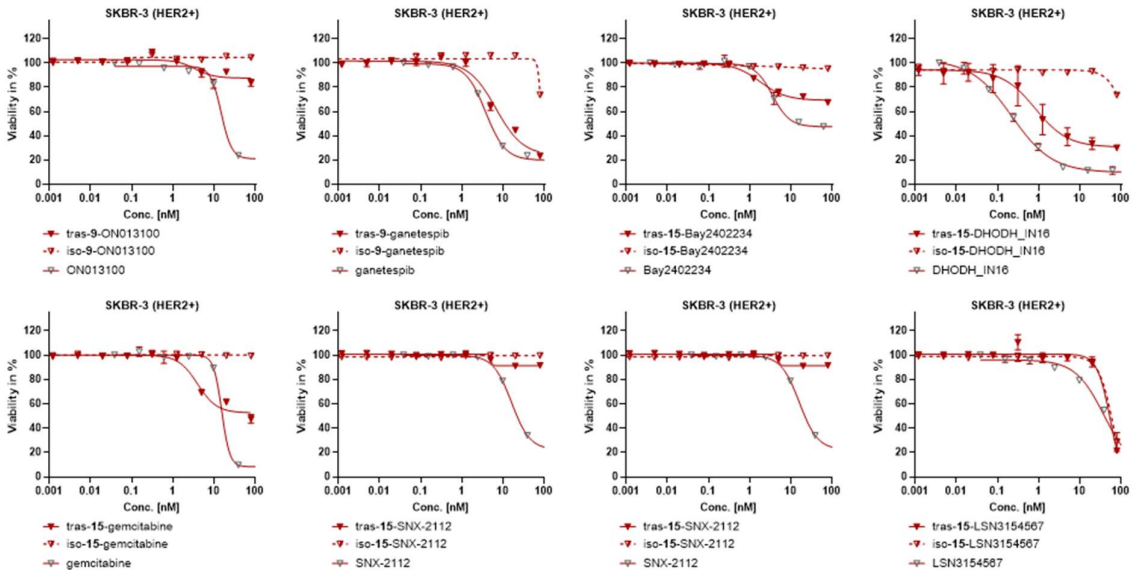
Head-to head comparison between linker 15-DXd and deruxtecan *in vitro*. Trastuzumab (anti-HER2)-based ADCs are shown as solid isotype ADCs as dashed lines. ADCs based on linker **15** are shown in lilac, deruxtecan in grey. (a) Linker structures of **15**-DXd and deruxtecan conjugated to trastuzumab (tras/anti-HER2). (b) *In vitro* dose-response curves for antiproliferative activity on the HER2-positive cell lines N87 and SKBR-3 and the HER2-negative cell line MDA-MB-468.

1.4. Supplementary figure 4

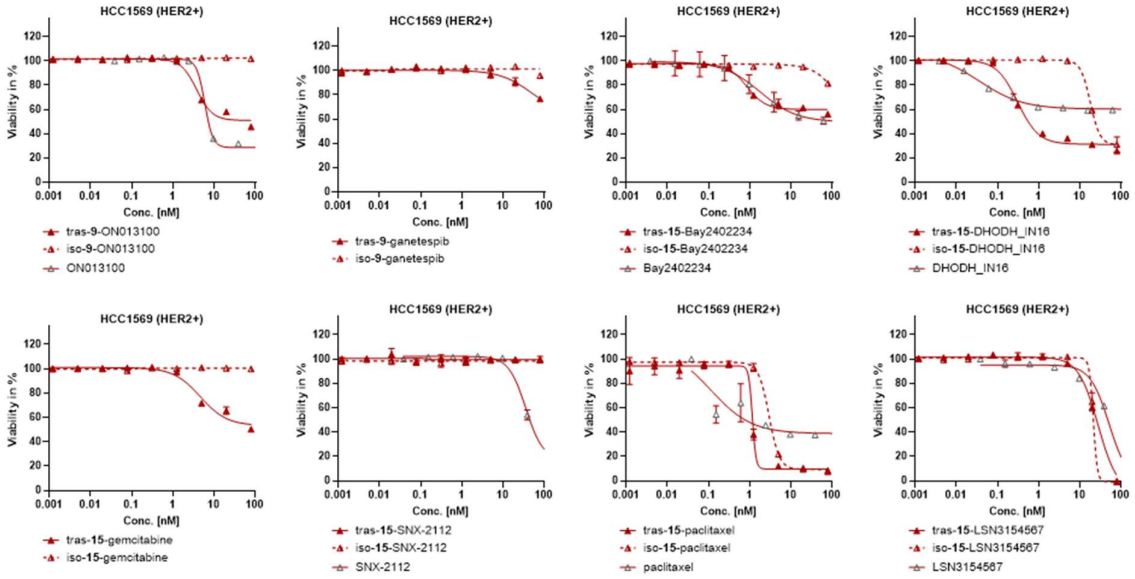
Antiproliferative activity dose-response curves for trastuzumab- and isotype-based ADCs and unconjugated drug for eight different payloads evaluated on the N87 cell line.



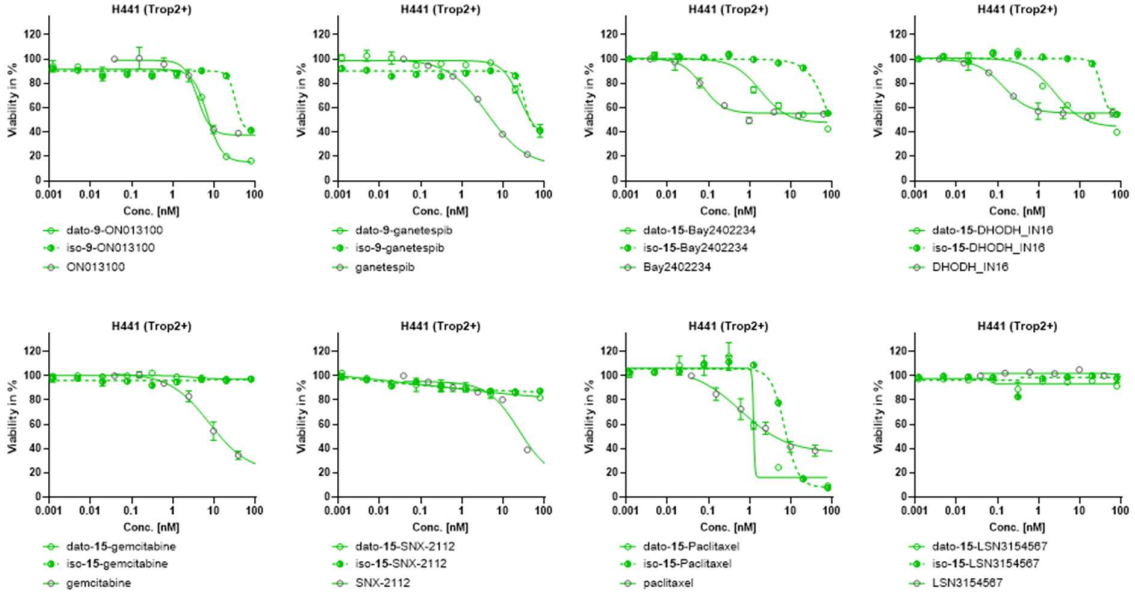
Antiproliferative activity dose-response curves for trastuzumab- and isotype-based ADCs and unconjugated drug for eight different payloads evaluated on the SKBR-3 cell line.



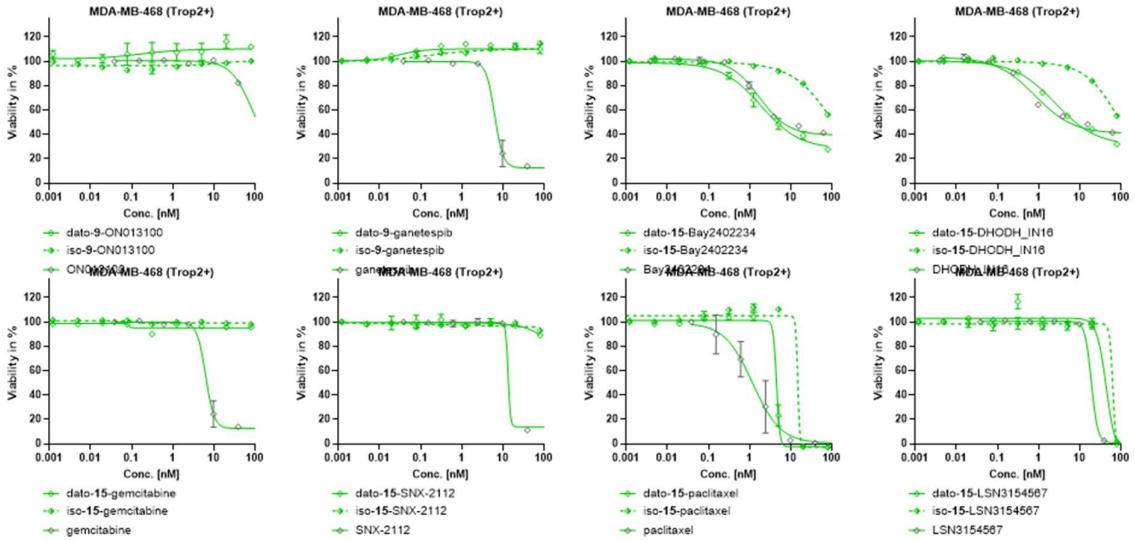
Antiproliferative activity dose-response curves for trastuzumab- and isotype-based ADCs and unconjugated drug for eight different payloads evaluated on the HCC-1569 cell line.



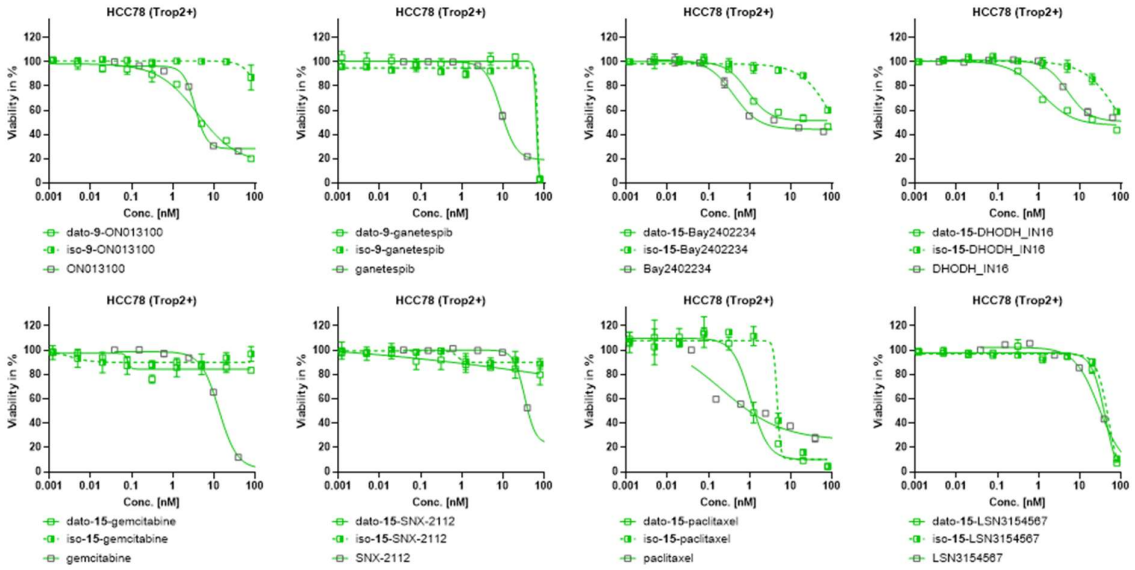
Antiproliferative activity dose-response curves for datopotamab- and isotype-based ADCs and unconjugated drug for eight different payloads evaluated on the H441 cell line.



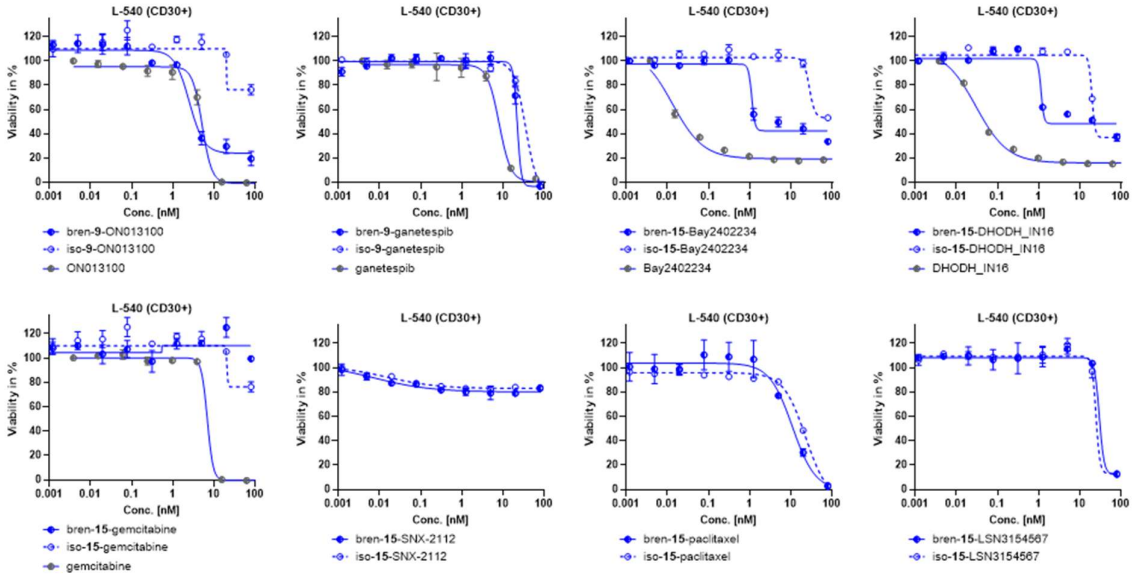
Antiproliferative activity dose-response curves for datopotamab- and isotype-based ADCs and unconjugated drug for eight different payloads evaluated on the MDA-MB-468 cell line.



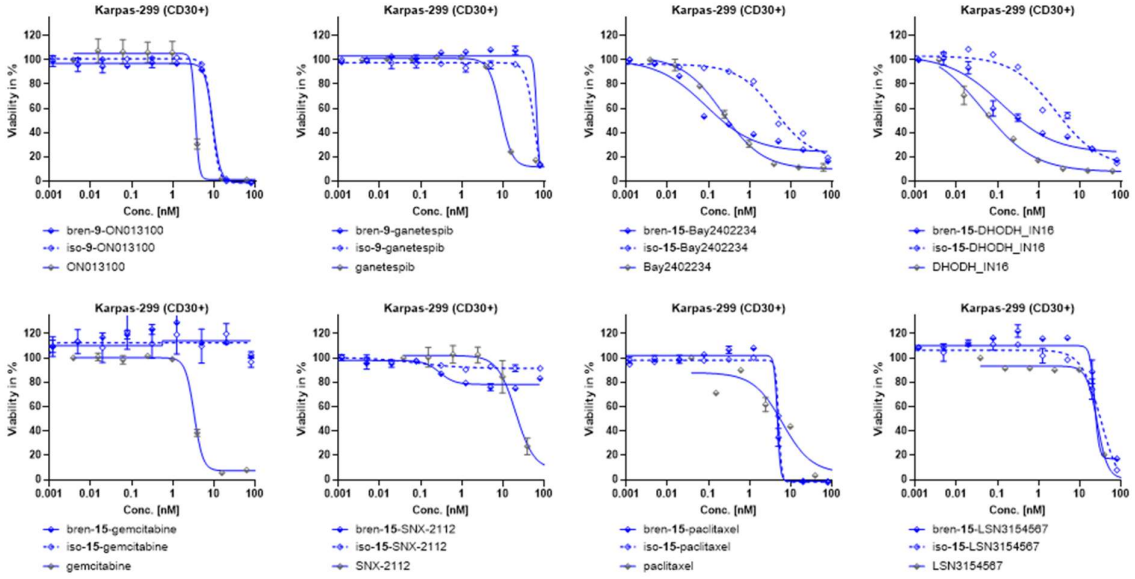
Antiproliferative activity dose-response curves for datopotamab- and isotype-based ADCs and unconjugated drug for eight different payloads evaluated on the HCC-78 cell line.



Antiproliferative activity dose-response curves for brentuximab and isotype-based ADCs and unconjugated drug for eight different payloads evaluated on the L-540 cell line.



Antiproliferative activity dose-response curves for brentuximab and isotype-based ADCs and unconjugated drug for eight different payloads evaluated on the Karpas-299 cell line.



2. General Information

2.1. Chemicals, solvents and antibodies

Chemicals and solvents were purchased from Merck (Merck group, Germany), TCI (Tokyo chemical industry CO., LTD., Japan), BLD (BLD Pharmatech Ltd., China) and Carl Roth (Carl Roth GmbH + Co. KG, Germany) and used without further purification. Dry solvents were purchased from Merck (Merck group, Germany). Trastuzumab was purchased from Roche (Hoffmann-La Roche AG, Switzerland). Enhertu (Trastuzumab Deruxtecan) was purchased from Daiichi-Sankyo (Daiichi Sankyō K.K, Japan). Trodelvy (Sacituzumab Govitecan) was purchased from Gilead (Gilead Sciences, US). CL2A-SN38, Dxd, SN38, Ganetespib, ON013100, Gemcitabine, BAY 2402234, DHODH-IN-16, SNX2112, Paclitaxel and LSN3154567 (NAMPT-IN-1) was purchased from MCE (MedChemExpress, USA).

2.2. Cell lines

Cell lines were purchased from the “German Collection of Microorganisms and Cell Cultures” (DSMZ, Leibniz Institute, Braunschweig, Germany), American Type Culture Collection (ATCC) or Merck (Merck group, Germany). Cells were cultured according to the manufacturer’s instructions in either RPMI 1640 or DMEM/F12 medium supplemented with GlutaMAX (Gibco, Thermo Fisher Scientific, USA) and 10%-20% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, USA).

2.3. Preparative HPLC

Preperative HPLC was performed on a BÜCHI Pure C-850 Flash-Prep system (BÜCHI Labortechnik AG, Switzerland) using a VP 250/10 Macherey-Nagel Nucleodur C18 HTec Spum column (Macherey-Nagel GmbH & Co. Kg, Germany) for smaller scales. The following gradients were used: Method C: (A = H_2O + 0.1% TFA (trifluoroacetic acid), B = MeCN (acetonitrile) + 0.1% TFA, flow rate 6 ml/min, 30% B 0-5 min, 30-70% B 5-35 min, 99% B 35-45 min. For bigger scales, a VP 250/21 Macherey-Nagel Nucleodur C18 HTec Spum column (Macherey-Nagel GmbH & Co. Kg, Germany) was used with the following gradients were used: Method D: (A = H_2O + 0.1% TFA (trifluoroacetic acid), B = MeCN (acetonitrile) + 0.1% TFA, flow rate 14 ml/min, 30% B 0-5 min, 30-70% B 5-35 min, 99% B 35-45 min.

2.4. LC/MS

Small molecules, linker-payloads, antibodies and ADCs 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) for antibodies and ADCs. Here, samples were eluted at a column temperature of 80°C. The following gradient was used: A: 0.1% formic acid in H2O; B: 0.1% formic acid in MeCN. 25% B 0-1 min, 0.4 mL/min, 25-95% B 1-3.5 min 0.2 mL/min, 95% B 3.5-4.5 min 0.2 mL/min, 95-25% B 4.5-5 min 0.4 mL/min, 25-95% B 5-5.5 min 0.4 mL/min, 95-25%B 5.5-7.5 min 0.4 mL/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. Small molecules and linker-payloads were analyzed with an Acquity UPLC-BEH C18 column (300 Å, 1.7 µm, 2.1 mm x 50 mm). Here, samples were eluted at a column temperature of 45°C with a flow rate of 0.4 mL/min. The following gradient was used: A: 0.1% formic acid in H2O; B: 0.1% formic acid in MeCN. 2% B 0-1 min, 2-98% B 1-5 min, 98%B 5-5.5 min, 98-2% B 5.5-6 min, 2% B 6-7min.

2.5. Preparative Size-Exclusion-Chromatography

Protein purification by size-exclusion chromatography was conducted with an ÄKTA Pure FPLC system (GE Healthcare, USA) equipped with a F9-C-fraction collector.

2.6. ADC concentration determination

The ADC concentrations were 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), in accordance with the suppliers' instructions.

2.7. Sample preparation of ADCs and antibodies for MS

0.5 µl PNGase-F solution (Pomega, Germany) and 5 µl of a 100 mM solution of DTT in water were added to 50 µl of 0.2 mg/mL antibody or ADC in PBS and the solution was incubated at 37 °C for at least 2 hours. Samples were subjected to LC/MS, injecting 2 µl for each sample.

2.8. 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 HMWS from monomers has been achieved during a 30 minute isocratic elution 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 were loaded onto the column for A-SEC analysis. UV chromatograms were recorded at 220 and 280 nm.

2.9. 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 has 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 0.7 mL/min. 15 µg sample were loaded onto the column for each analysis. UV chromatograms were recorded at 220 and 280 nm.

3. Experimental procedures

3.1. General method for the conjugation of linker payloads

50 µl of the antibody solution of 10.0 mg/ml in P5-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 linker-payload constructs 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).

3.2. Expression and purification of sacituzumab, datopotamab and brentuximab

All antibodies that were not commercially available were transiently expressed in Expi-CHO-S cells (Thermo Fisher Scientific, USA) by co-transfecting cells with pcDNA3.4 expression plasmids (Thermo Fisher Scientific, USA), coding for the heavy and light chain of the respective sequences in a 1:1 ratio, using the Expi-CHO transfection system (Thermo Fisher Scientific, USA). Cells were harvested by centrifugation at 300 g for 5 minutes at 4°C. To clear micro particles from supernatant, supernatants were centrifuged at 4000–5000 g for 30 min at 4 °C. For further clarification supernatants were passed through a 0.22 µm filter. Antibodies were purified from cleared and filtered supernatants via Protein A chromatography and analyzed by HPLC-SEC, HPLC-HIC, LC-MS. Sequences were obtained from publically available datbases.

3.3. Analysis of the *in vivo* samples by ELISA

To evaluate the PK of the ADCs *in vivo*, the total antibody concentration was measured at different time points in serum of ADC-treated mice. Total antibody was analyzed in serum over the range 2000 – 15,6 ng/ml. Nunc 96-well plates (Thermo Fisher Scientific, USA) were coated with the recombinantly expressed target of the antibody in PBS (Gibco, Thermo Fisher Scientific, USA) 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 PBST (PBS + 0.05% Tween, Merck, Germany) , blocking solution (2 % Albumin in PBST, Carl-Roth, Germany) was added and the plate was incubated at room temperature for 1 hour. The coated plates were washed 3x with PBST. Standards, quality controls (QCs) and test samples were added, the plates were sealed and incubated at room temperature for 1 hour. The plates were washed 3x with PBST. Anti-Human IgG (γ-chain specific)-peroxidase antibody (Jackson Immunoresearch, USA) was added and incubated for 1 hour at room temperature. The plates were washed 3x PBST. TMB (Thermo Fisher Scientific, USA) was added, the plates were sealed and incubated at room temperature for 15 minutes. 1 M Sulfuric Acid (Carl-Roth, Germany) was added. Using a microplate reader Infinite 200 PRO (Tecan, Switzerland), the absorbance at a wavelength of 450 nm was measured.

To evaluate the stability of the ADCs *in vivo*, the intact ADC concentration was measured at different time points in serum of ADC-treated mice. Intact ADC was analyzed in mouse serum over the range 2000 – 15,6 ng/ml. Nunc 96-well plates were coated with rabbit anti-DXd mAb 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 PBST, blocking solution (2 % Albumin in PBST) was added, the plate was sealed and incubated at room temperature for 1 hour. The coated plates were washed 3x PBST. Standards, QCs and test samples were added, the plates were sealed and incubated at room temperature for 1 hour. The plates were washed 3x PBST. HRP-labeled goat Anti-Human IgG (H+L) (Abcam, UK) was added and incubated for 1 hour at room temperature. The plates were washed 3x PBST. TMB was added, the plates were sealed and incubated at room temperature for 10 min. 1 M Sulfuric Acid was

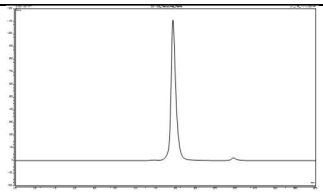
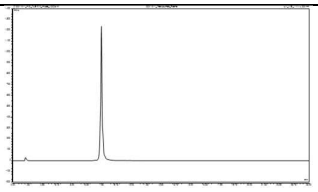
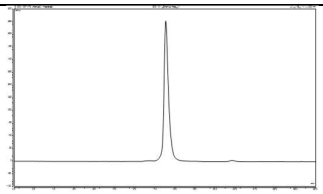
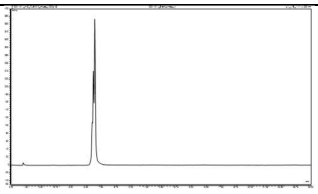
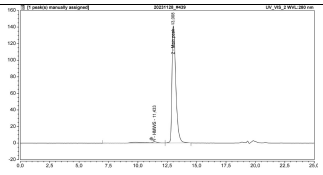
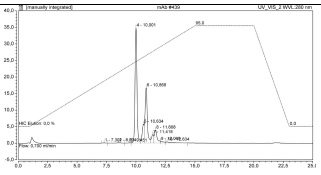
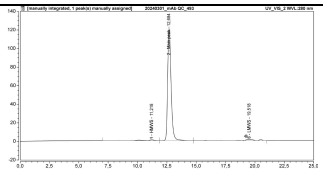
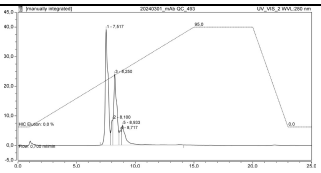
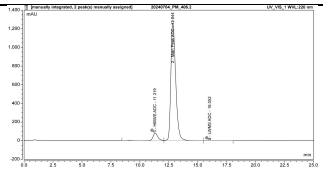
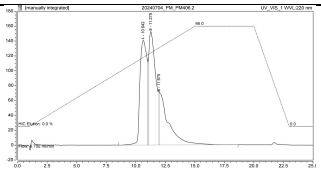
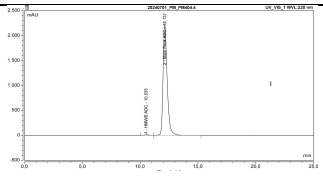
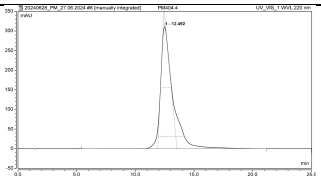
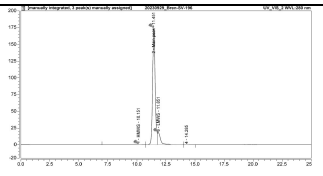
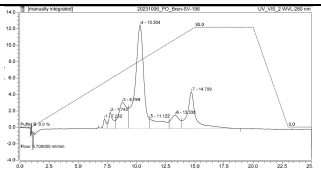
added. Using a microplate reader Infinite 200 PRO (Tecan, Switzerland), the absorbance at a wavelength of 450 nm was measured.

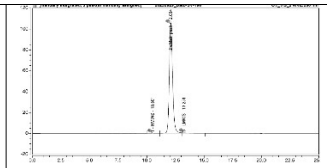
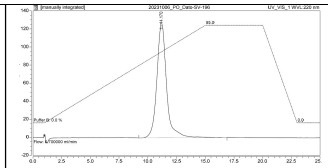
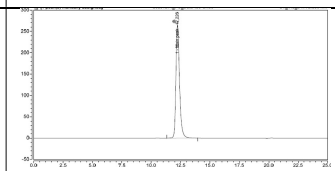
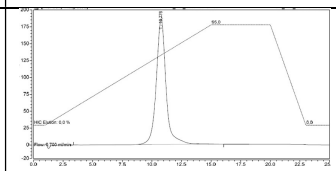
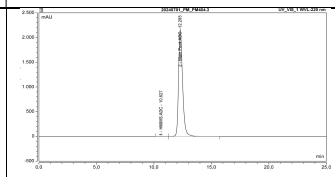
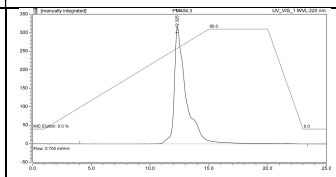
3.4. *Ex vivo* rat serum linker stability analysis (DAR determination)

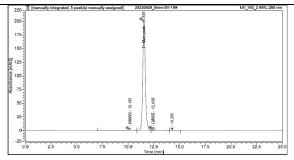
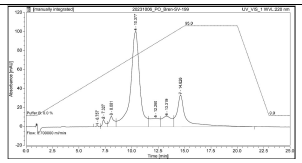
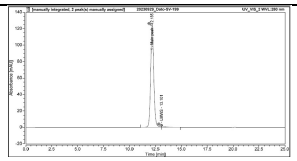
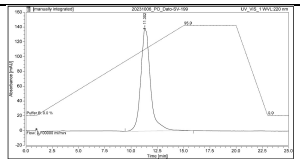
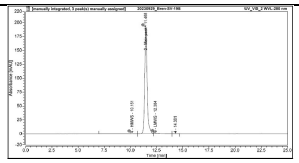
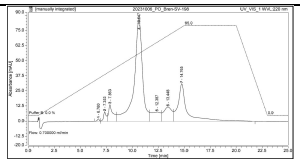
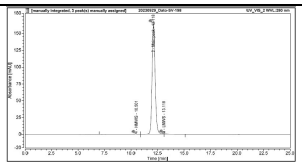
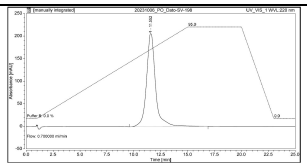
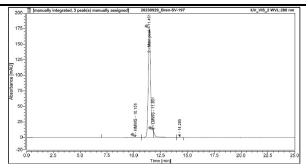
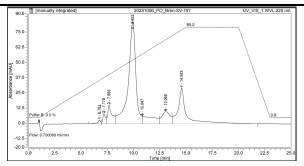
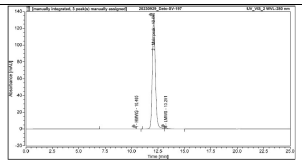
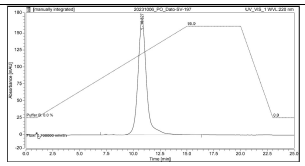
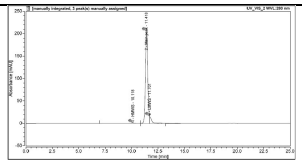
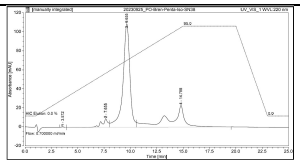
40 µl of normal rat serum (Thermo Fisher Scientific, USA), containing the corresponding ADCs in a concentration of 0.4 mg/ml in at least 80% rat serum (Thermo Fisher Scientific, USA) were sterile filtered with UFC30GV0S centrifugal filter units (Merck, Germany) and incubated at 37°C for up to 7 days. Samples for day 0 were directly processed further.

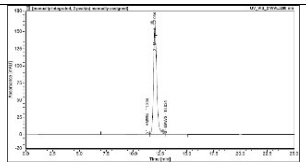
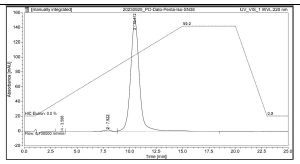
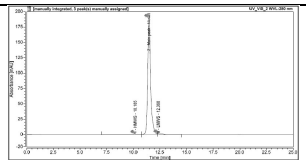
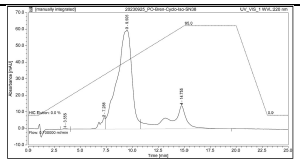
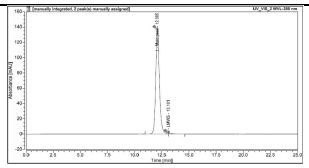
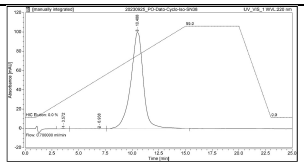
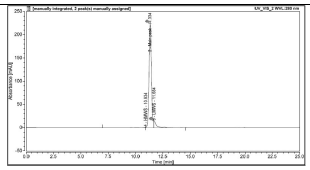
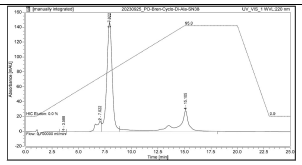
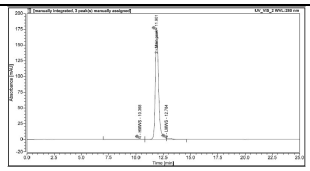
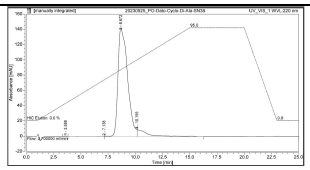
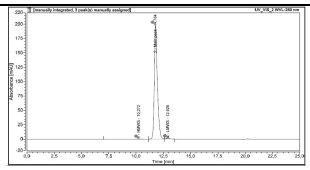
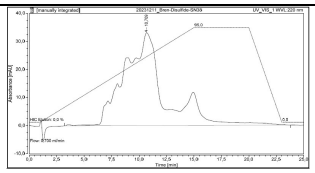
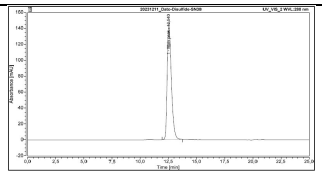
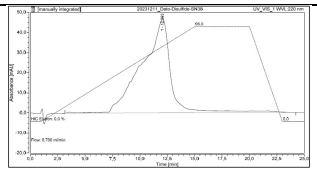
The supernatant of 50 µl anti human IgG (Fc-Specific) agarose slurry (Merck group, Germany) was removed by centrifugation and the remaining resin washed three times with 300 µl PBS. The resin was incubated with 40 µl of the serum-ADC mix for 1 hour at room temperature. Afterwards, the supernatant was removed and the resin washed 3 times with 300 µl PBS. Following by incubation for 5 minutes with 60 µl 100 mM Glycin buffer pH 2.3 at room temperature. This solution was rebuffered to PBS by using 0.5 mL Zeba™ Spin Desalting Columns with 7K MWCO (Thermo Fisher Scientific, USA). The samples were processed further for MS-measurements, as described above.

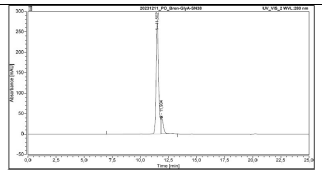
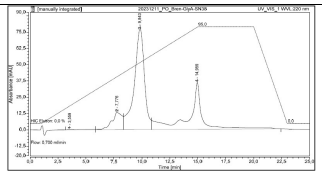
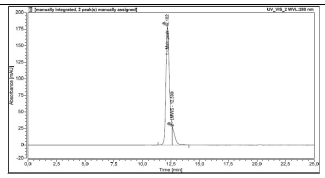
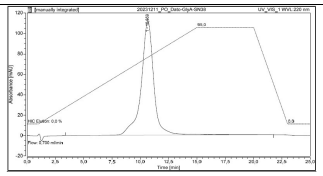
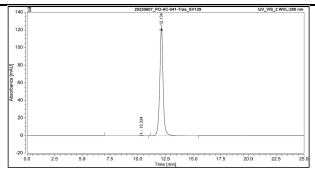
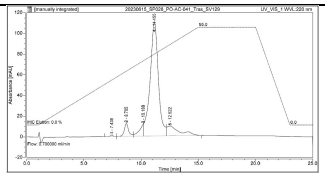
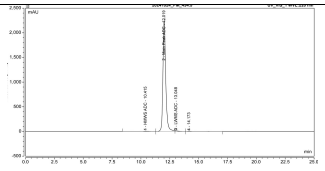
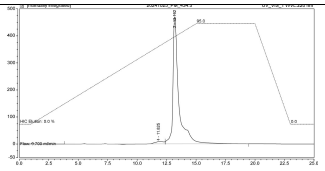
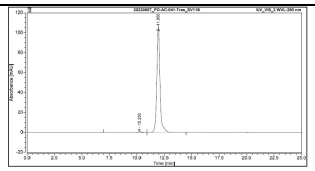
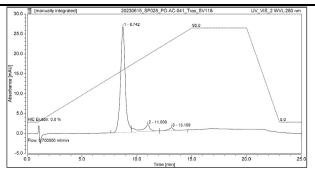
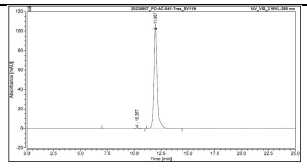
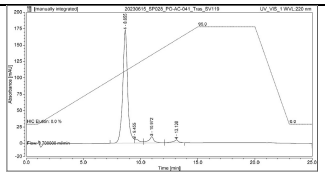
4. Analytical overview over the synthesized ADCs after purification

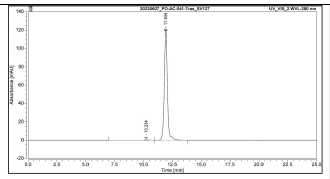
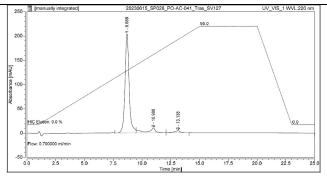
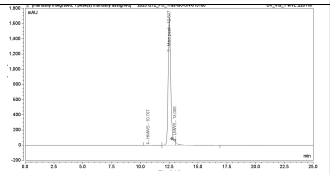
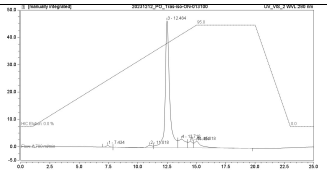
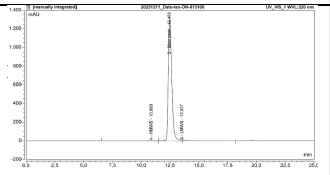
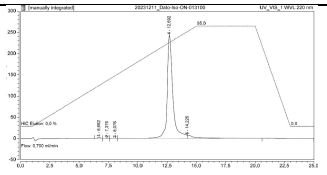
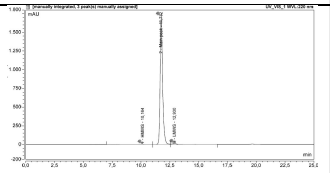
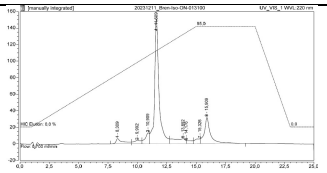
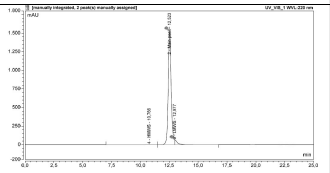
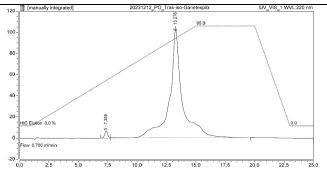
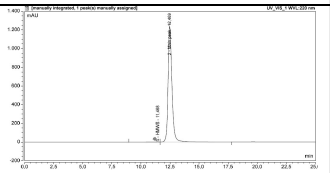
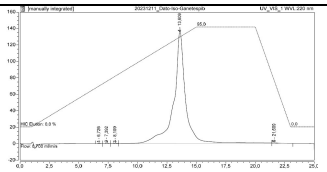
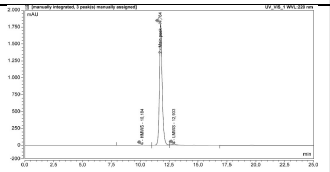
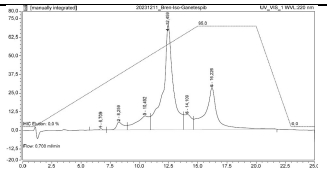
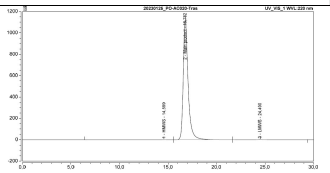
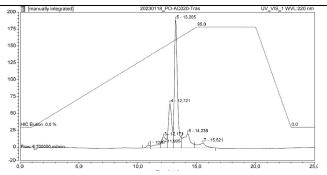
Antibody/ADC	Analytical SEC	Analytical HIC	DARav and MS results
Trastuzumab			LC: calcd.: 23439 found: 23438 HC: calcd.: 49146 found: 49149
Brentuximab			LC: calcd.: 25514 found: 25514 HC: calcd.: 48878 found: 48877
Sacituzumab			LC: calcd.: 23339 found: 23334 HC: calcd.: 49441 found: 49289
Datopotamab			LC: calcd.: 23402 found: 23397 HC: calcd.: 49116 found: 48983 (-terminal Lysine)
Sacituzumab-**1**-SN38			DARav: 8.0 LC: calcd.: 23941 found: 23941 HC: calcd.: 51110 found: 51113
Sacituzumab-**3**-SN38			DARav: 8.0 LC: calcd.: 25283 found: 25283 HC: calcd.: 55136 found: 55138
Brentuximab-**3**-SN38			DARav: 8.0 LC: calcd.: 27463 found: 27463 HC: calcd.: 54723 found: 54724

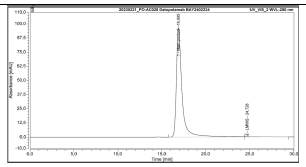
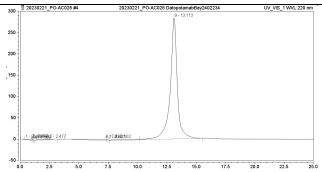
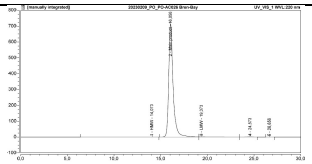
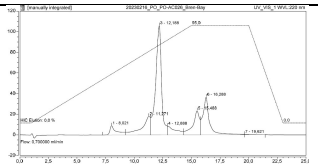
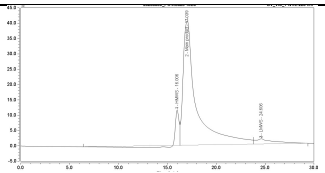
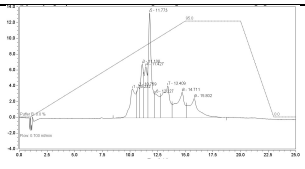
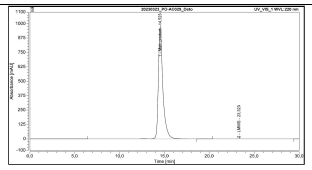
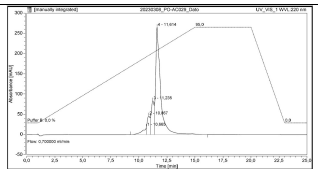
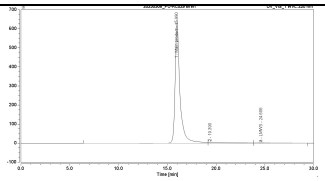
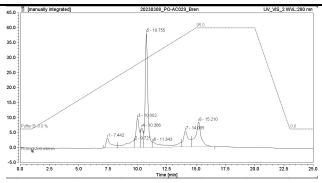
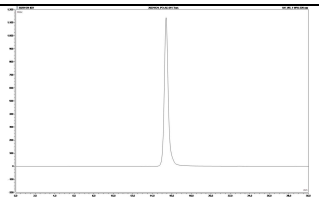
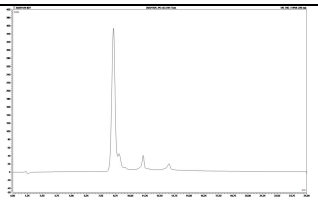
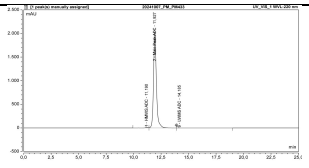
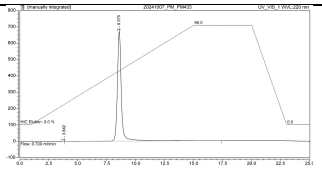
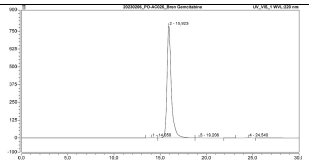
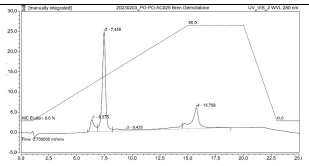
Datopotamab-**3**-SN38			DARav: 8.0 LC: calcd.: 25346 found: 25346 HC: calcd.: 54828 found: 54828
Sacituzumab-**4**-SN38			DARav: 8.0 LC: calcd.: 25269 found: 25269 HC: calcd.: 55094 found: 55096
Sacituzumab-**5**-SN38			DARav: 8.0 LC: calcd.: 25259 found: 25259 HC: calcd.: 55064 found: 55066

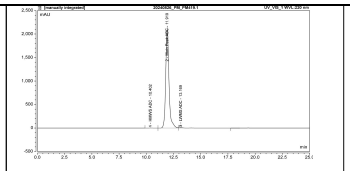
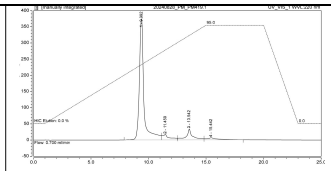
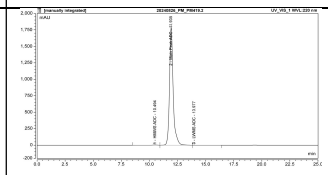
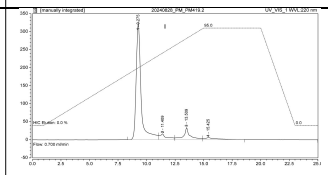
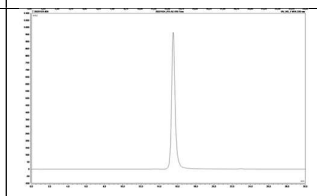
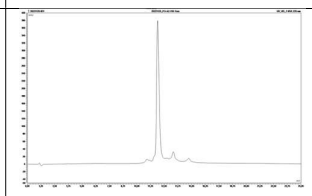
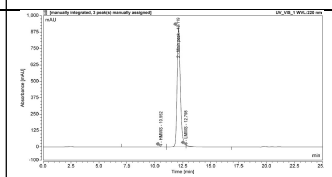
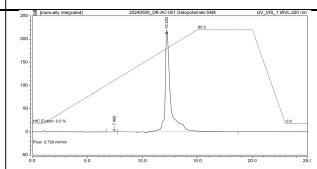
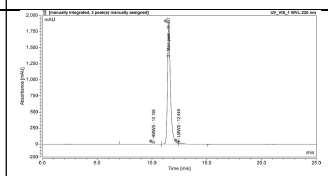
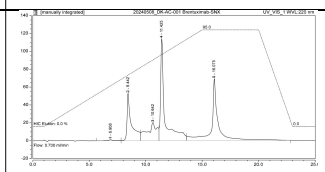
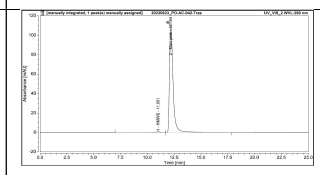
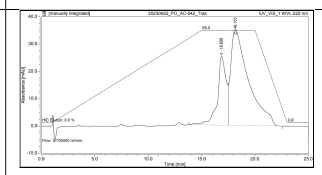
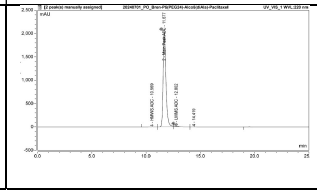
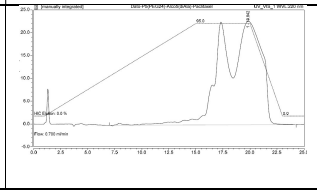
Brentuximab-**6**-SN38			DARav: 8.0 LC: calcd.: 27475 found: 27475 HC: calcd.: 54759 found: 54760
Datopotamab-**6**-SN38			DARav: 8.0 LC: calcd.: 25358 found: 25358 HC: calcd.: 54864 found: 54865
Brentuximab-**7**-SN38			DARav: 8.0 LC: calcd.: 27477 found: 27477 HC: calcd.: 54765 found: 54766
Datopotamab-**7**-SN38			DARav: 8.0 LC: calcd.: 25360 found: 25360 HC: calcd.: 54870 found: 54871
Brentuximab-**8**-SN38			DARav: 8.0 LC: calcd.: 27449 found: 27449 HC: calcd.: 54681 found: 54682
Datopotamab-**8**-SN38			DARav: 8.0 LC: calcd.: 25332 found: 25332 HC: calcd.: 54786 found: 54786
Brentuximab-**9**-SN38			DARav: 8.0 LC: calcd.: 27449 found: 27449 HC: calcd.: 54681 found: 54682

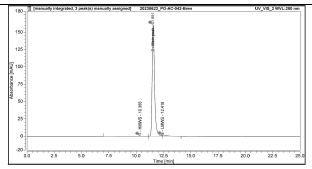
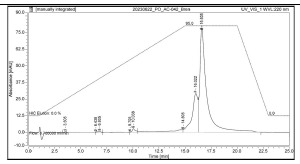
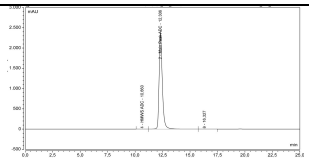
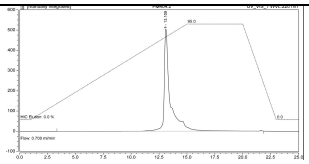
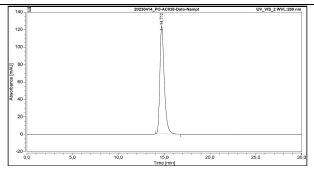
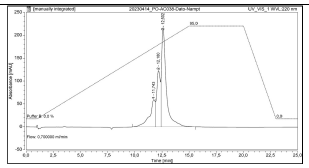
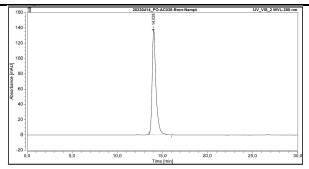
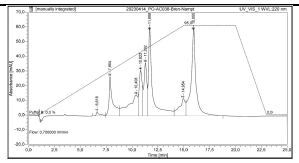
Datopotamab-**9**-SN38			DARav: 8.0 LC: calcd.: 25332 found: 25332 HC: calcd.: 54786 found: 54787
Brentuximab-**10**-SN38			DARav: 8.0 LC: calcd.: 27461 found: 27461 HC: calcd.: 54717 found: 54718
Datopotamab-**10**-SN38			DARav: 8.0 LC: calcd.: 25344 found: 25344 HC: calcd.: 54822 found: 54822
Brentuximab-**11**-SN38			DARav: 8.0 LC: calcd.: 27490 found: 27490 HC: calcd.: 54804 found: 54805
Datopotamab-**11**-SN38			DARav: 8.0 LC: calcd.: 25373 found: 25373 HC: calcd.: 54909 found: 54910
Brentuximab-**12**-SN38			DARav: 6.2 LC: calcd.: 27483 found: 27483, 25514 HC: calcd.: 56228 found: 56228, 54259, 50320
Datopotamab-**12**-SN38			DARav: 7.1 LC: calcd.: 25366 found: 25366, 23398 HC: calcd.: 56332 found: 56333, 54363, 50425

Brentuximab-**13**-SN38			DARav: 8.0 LC: calcd.: 27695 found: 27695 HC: calcd.: 55419 found: 55420
Datopotamab-**13**-SN38			DARav: 8.0 LC: calcd.: 25578 found: 25578 HC: calcd.: 5524 found: 55427
Trastuzumab-**1**-Dxd	n.a.	n.a.	DARav: 8.0 LC: calcd.: 24146 found: 24146 HC: calcd.: 51273 found: 51275
Trastuzumab-**3**-Dxd			DARav: 8 LC: calcd.: 25487 found: 25489 HC: calcd.: 55298 found: 55300
Trastuzumab-**14**-Dxd			DARav: 8.0 LC: calcd.: 25559 found: 25559 HC: calcd.: 55512 found: 55512
Trastuzumab-**15**-Dxd			DARav: 7.9 LC: calcd.: 25504 found: 25503, 23438 HC: calcd.: 55349 found: 55344
Trastuzumab-**16**-Dxd			DARav: 7.8 LC: calcd.: 25504 found: 25504, 23439 HC: calcd.: 55349 found: 55345

Trastuzumab-**17**-Dxd			DARav: 7.9 LC: calcd.: 25516 found: 25518, 23439 HC: calcd.: 55385 found: 55387
Trastuzumab-**9**-P1			DARav: 8.0 LC: calcd.: 25376 found: 25375 HC: calcd.: 56404 found: 56405
Datopotamab-**9**-P1			DARav: 8.0 LC: calcd.: 25334 found: 25334 HC: calcd.: 56236 found: 56237
Brentuximab-**9**-P1			DARav: 8.0 LC: calcd.: 27451 found: 27451 HC: calcd.: 56132 found: 56132
Trastuzumab-**9**-P2			DARav: 8.0 LC: calcd.: 25346 found: 25345 HC: calcd.: 56314 found: 56315
Datopotamab-**9**-P2			DARav: 8.0 LC: calcd.: 25304 found: 25304 HC: calcd.: 56146 found: 56147
Brentuximab-**9**-P2			DARav: 8.0 LC: calcd.: 27421 found: 27421 HC: calcd.: 56042 found: 56042
Trastuzumab-**15**-P3			DARav: 8 LC: calcd.: 25530 found: 25530 HC: calcd.: 55427 found: 55427

Datopotamab-**15**-P3			DARav: 8.0 LC: calcd.: 25489 found: 25489 HC: calcd.: 55256 found: 55258
Brentuximab-**15**-P3			DARav: 8.0 LC: calcd.: 27606 found: 27606 HC: calcd.: 55152 found: 55154
Trastuzumab-**15**-P4			DARav: 8.0 LC: calcd.: 25446 found: 25446 HC: calcd.: 55173 found: 55174
Datopotamab-**15**-P4			DARav: 8.0 LC: calcd.: 25105 found: 25403 HC: calcd.: 56449 found: 56449
Brentuximab-**15**-P4			DARav: 8.0 LC: calcd.: 27522 found: 27521 HC: calcd.: 56345 found: 56344
Trastuzumab-**15**-P5			DARav: 7.5 LC: calcd.: 25273 found: 25273, 23439 HC: calcd.: 54651 found: 54653
Datopotamab-**15**-P5			DARav: 8.0 LC: calcd.: 25230 found: 25232 HC: calcd.: 54482 found: 54487
Brentuximab-**15**-P5			DARav: 8.0 LC: calcd.: 27347 found: 27348 HC: calcd.: 54375 found: 54380

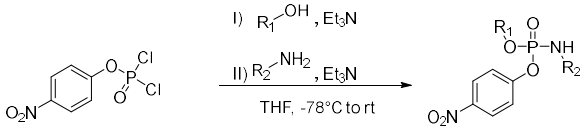
Trastuzumab-**15**-5’-O-P5			DARav: 7.5 LC: calcd.: 25273 found: 25273, 23438 HC: calcd.: 54651 found: 54656
Trastuzumab-**15**-3’-O-P5			DARav: 7.1 LC: calcd.: 25273 found: 25273, 23438 HC: calcd.: 54651 found: 54656
Trastuzumab-**15**-P6			DARav: 8 LC: calcd.: 25475 found: 25475 HC: calcd.: 55257 found: 55258
Datopotamab-**15**-P6			DARav: 8 LC: calcd.: 25433 found: 25433 HC: calcd.: 55091 found: 55090
Brentuximab-**15**-P6			DARav: 8 LC: calcd.: 27550 found: 27550 HC: calcd.: 54984 found: 54985
Trastuzumab-**14**-P7			DARav: 8.0 LC: calcd.: 25920 found: 25920
Datopotamab-**15**-P7			DARav: 8.0 LC: calcd.: 25878 found: 25878

Brentuximab-**15**-P7			DARav: 8.0 LC: calcd.: 27995 found: 27996
Trastuzumab-**15**-P8			DARav: 8.0 LC: calcd.: 25485 found: 25486 HC: calcd.: 55290 found: 55292
Datopotamab-**15**-P8			DARav: 8.0 LC: calcd.: 25444 found: 25444 HC: calcd.: 55123 found: 55125
Brentuximab-**15**-P8			DARav: 8.0 LC: calcd.: 27561 found: 27561 HC: calcd.: 55019 found: 55019

5. Organic synthesis

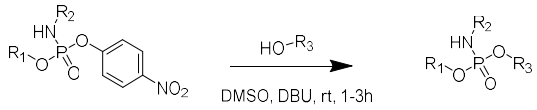
5.1. General procedures

5.1.1. General procedure 1: Synthesis of nitrophenyl phosphoramidates from 4-nitrophenyl phosphorodichloridate



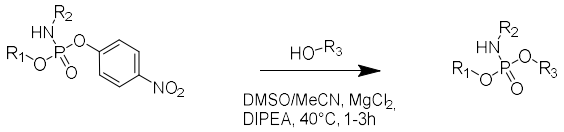
A solution of 4-nitrophenyl phosphorodichloridate (0.5 g, 1.95 mmol) in THF (6 mL) was cooled to -78°C under argon atmosphere. To this clear solution, triethylamine (1.17 mL, 8.4 mmol) was added followed by dropwise addition of a suitable alcohol (1 eq., 1.95 mmol). The suspension was stirred at room temperature for 2h. The reaction was again cooled to -78°C and to this solution, a suitable amine, amino acid or dipeptide (1 eq., 1.95 mmol) was added in one portion. The resulted solution was stirred at room temperature for 3 h. The reaction mixture was diluted with EtOAc (15 mL) and filtered through a Buchner funnel. The filtrate obtained was washed with water and brine. The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo to obtain the crude material which was purified by silica gel chromatography using a gradient elution (EtOAC: Cyclohexane; 0:100 to 25:70) to give the desired 4-nitrophenyl phophoramidate.

5.1.2. General procedure 2: Synthesis of drug-phosphoramidates from nitrophenyl phosphoramidates



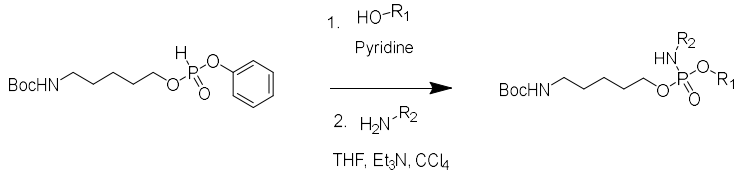
A respective alcohol was dissolved with 3 equivalents of a suitable 4-nitrophenol phosphoramidate precursor in DMSO. 6 equivalents DBU were added and the resulted reaction was stirred at room temperature for 30 minutes to 3 hours, or until the alcohol is fully consumed. After completion, the reaction was diluted with H2O/MeCN (2:1) and directly subjected to preparative HPLC purification.

5.1.3. General procedure 3: Synthesis of drug-phosphoramidates from nitrophenol phosphoramidates



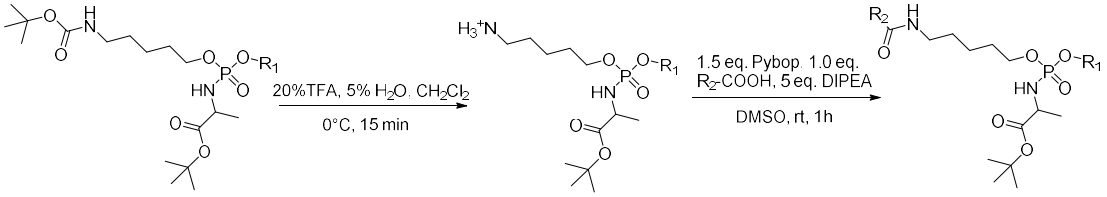
3 equivalents magnesium chloride were placed in a Schlenk flask equipped with magnetic stirrer and heat dried under vacuum. A respective alcohol is dissolved with 3 equivalents of a suitable 4-nitrophenol phosphoramidate precursor in DMSO and added to the magnesium chloride under argon. 3 equivalents DIPEA were added and the reaction was heated to 40°C and stirred for 30 minutes to 3 hours, or until the alcohol is fully consumed. After completion, the reaction is diluted with H2O/MeCN (2:1) and directly subjected to preparative HPLC purification.

5.1.4. General procedure 4: Synthesis of drug-phosphoramidates from boc-aminopentane phenyl phosphite, alcohols and amines



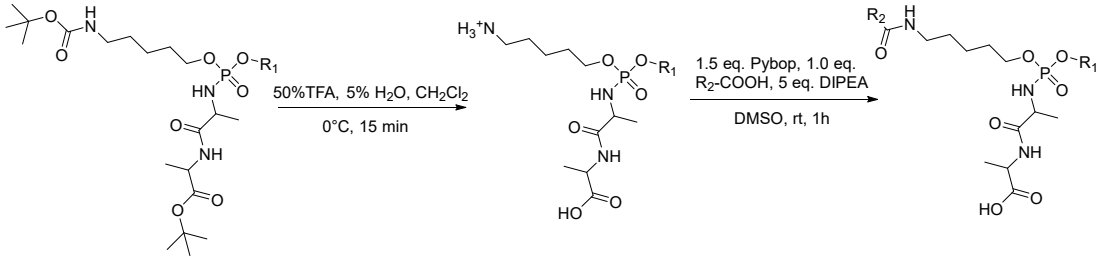
Boc-aminopentane phenyl phosphite **X6** was prepared as follows: A 25 mL Schlenk-flask, equipped with magnetic stir bar and septum, was heat dried under vacuum and flushed with argon. The Schlenk-flask was charged with 1.7 g diphenyl phosphite (7.4 mmol, 3 equivalents) and dissolved in 5 mL dry pyridine. The phosphite solution was cooled to 0°C with wet ice and 0.5 g boc-aminopentanol (2.5 mmol, 1 equivalent) dissolved in 3 mL dry pyridine were added dropwise. After complete addition, the wet ice was removed, and the reaction was stirred for 60 minutes under argon. Pyridine was evaporated under reduced pressure and the residue was suspended in cyclohexane/EtOAc (95:5) and purified by flash column chromatography (cyclohexane/EtOAc, 0-80% EtOAc, 5 CV 0% EtoAc, 10 CV to 50% EtOAc and 10 CV to 80% EtOAc). 0.53 g of boc-aminopentan phenyl phosphite (1.5 mmol, 62.4%) were isolated as clear colourless oil. HR-MS for $C_{16}H_{27}NO_5P^+$ $[M+H]^+$ calcd.: 344.1621, found: 344.16277.
10 equivalents of boc-aminopentane phenyl phosphite **X6** were dissolved in 0.5 mL dry pyridine /100 mg and transferred to a heat dried and argon flushed 25 mL Schlenk flask equipped with magnetic stir bar and septum. 1 equivalent of alcohol dissolved in 0.5 mL pyridine/10 mg was added to the phosphite solution and stirred under an argon atmosphere for 1h at room temperature or until the alcohol is fully consumed. Pyridine was evaporated and the crude residue was dissolved in dry THF. 20 equivalents of diAlaOtBu were dissolved in dry THF and transferred to a heat-dried and argon flushed 25 mL Schlenk flask equipped with magnetic stir bar and septum. The phosphite in THF was added to the stirring solution of diAlaOtBu and stirred at room temperature under argon. Subsequently, 20 equivalents of anhydrous triethylamine and 40 equivalents of carbon tetrachloride were added. The reaction was stirred at room temperature for 2h or until all phosphite was consumed. The solvents were evaporated in an argon stream and the crude residue was purified via HPLC to yield the desired phosphoramidate.

5.1.5. General procedure 5: Boc deprotection in the presence of the tert-butyl ester followed by amide coupling



The Boc protected phosphoramidate was dissolved in 0.5 mL DCM and 5 % H2O were added. After cooling the mixture to 0°C in an ice bath, 0.1 mL TFA was added, and the solution was stirred at 0°C for 30 min to 3h until deprotection was complete. The solvents were removed under N_2 stream and the product was dried in vacuo. The products were directly transferred to the next step without any further purification.
The residue was dissolved in 0.5 ml DMSO. 1.5 eq. PyBOP, dissolved in DMSO and 5.0 eq. DIPEA were added. After 5 minutes, 1.0 eq of desired carboxylic acid dissolved in DMSO was added and the mixture was stirred at RT for 1h. The mixture was diluted with H2O/MeCN (2:1) and directly subjected to preparative HPLC purification.

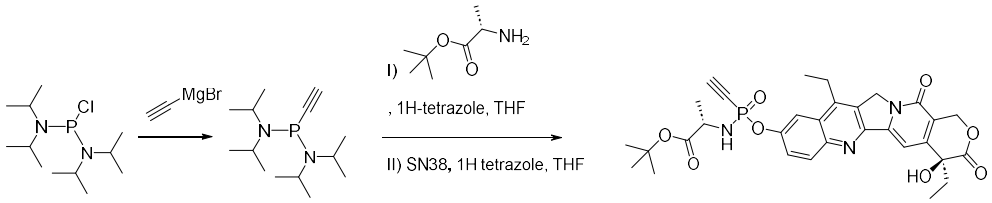
5.1.6. General procedure 6: Boc- and *tert*-butyl ester deprotection, followed by amide coupling



The Boc protected phosphoramidates were dissolved in 0.5 ml DCM and 5 % H2O were added. After cooling the mixture to 0°C in an ice bath, 0.5 ml TFA was added and the solution was stirred at 0°C for 30 min to 3h until deprotection was complete. The solvents were removed under N_2 stream and the product was dried in vacuo. The products were directly transferred to the next step without any further purification.
The residue was dissolved in 0.5 ml DMSO. 1.5 eq. PyBOP, dissolved in DMSO and 5.0 eq. DIPEA were added. After 5 minutes, 1.0 eq of desired carboxylic acid dissolved in DMSO was added and the mixture was stirred at RT for 1h. The mixture was diluted with H2O/MeCN (2:1) and directly subjected to preparative HPLC purification.

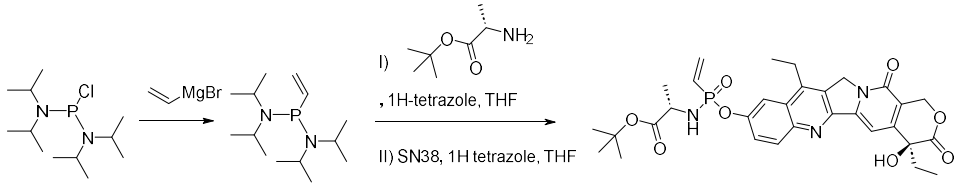
All P5(PEG)-building blocks that have been used in the procedures above have been synthesized as previously reported.[1-3]

5.2. Linker 1-SN38: Scheme and synthesis



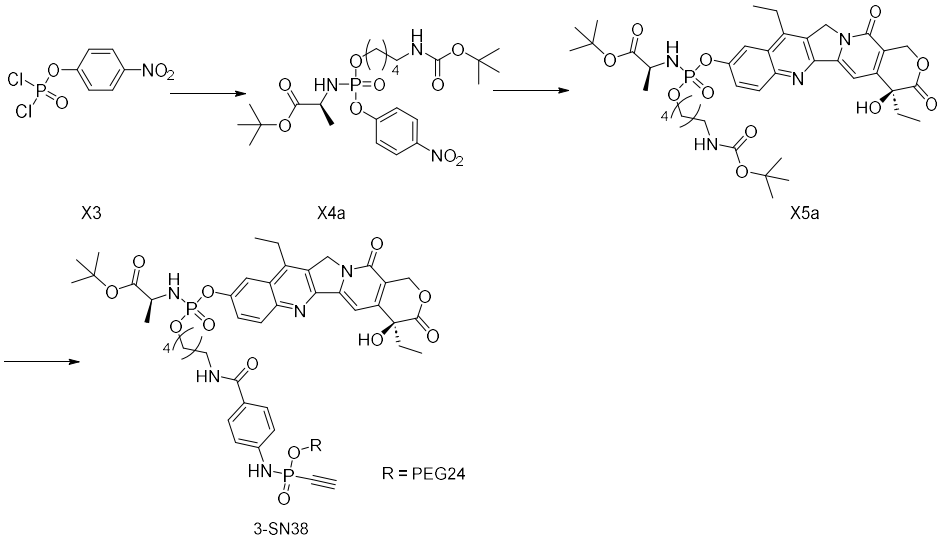
A solution of ethynylmagnesium bromide in THF (2.2 mL, 0.5 M, 1.1 eq.) and bis-(*N*,*N*-diisopropylamino)chlorophosphine (266 mg, 1 mmol) was stirred under an argon atmosphere at 0°C and allowed to attain room temperature for 30 minutes. A solution of 1H-Tetrazole in MeCN (2.2 mL, 0.45 M, 1.1 eq.) and alanine *tert*-butyl ester (145 mg, 1 mmol, 1 eq.) dissolved in 2 mL THF were added to the reaction and stirred for 3h at room temperature. A part of the reaction mix (0.165 mL, 156 mM, 25.7 µmol, 1.1 eq.) and 1H-Tetrazole in MeCN (55 µL, 0.45 M, 1.1 eq.) were added to SN38 (9.65 mg, 25 µmol, 1eq.) and stirred for 1h at room temperature. Iodine-Water-Pyridine-Tetrahydrofuran Oxidizer Solution (1.3 mL, 0.02 M, 1.1 eq.) were added and stirred for 5 minutes. All volatiles were removed in an argon stream and the residue was purified by reversed phase HPLC (30 to 80% MeCN in 50 minutes) to yield 9.9 mg of **1-SN38** (16.4 µmol, 68%). HR-MS for $C_{31}H_{35}N_3O_8P^+$ $[M+H]^+$ calcd.: 608.2156 found 608.21377.

5.3. Linker 2-SN38: Scheme and synthesis

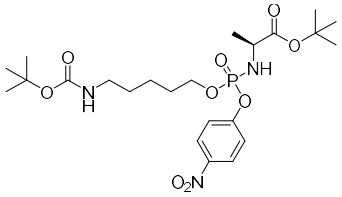


A solution of vinylmagnesium bromide in THF (1.1 mL, 1.0 M, 1.1 eq.) and bis-(*N*,*N*-diisopropylamino)chlorophosphine (266 mg, 1 mmol) in 1 mL THF was stirred under an argon atmosphere at 0°C and allowed to attain room temperature for 30 minutes. A solution of 1H-Tetrazole in MeCN (2.2 mL, 0.45 M, 1.1 eq.) and alanine *tert*-butyl ester (145 mg, 1 mmol, 1 eq.) dissolved in 2.2 mL THF were added to the reaction and stirred for 3h at room temperature. A part of the reaction mix (0.140 mL, 154 mM, 22 µmol, 1.3 eq.) and 1H-Tetrazole in MeCN (40 µL, 0.45 M, 1.1 eq.) were added to SN38 (6.7 mg, 17 µmol, 1eq.) and stirred for 1h at room temperature. Iodine-Water-Pyridine-Tetrahydrofuran Oxidizer Solution (1.1 mL, 0.02 M, 1.1 eq.) were added and stirred for 5 minutes. All volatiles were removed in an argon stream and the residue was purified by reversed phase HPLC (30 to 80% MeCN in 50 minutes) to yield 7.4 mg of **2-SN38** (12.2 µmol, 72%). HR-MS for $C_{31}H_{37}N_3O_8P^+$ $[M+H]^+$ calcd.: 610.2313, found: 610.223079.

5.4. Linker 3-SN38: Scheme

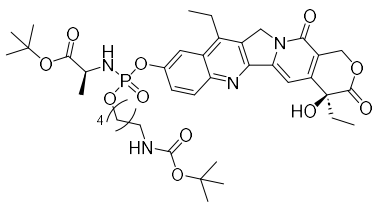


5.4.1. X4a



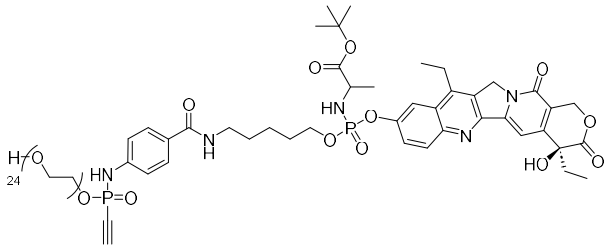
The title compound was synthesized in accordance with the general procedure 1 from 269 mg 4-nitrophenyl dichlorophosphate (1.05 mmol), 203 mg Boc-aminopentanol (1.0 mmol) and 184 mg L-alanine *tert*-butyl ester hydrochloride (1.1 mmol). The product was obtained as white solid (367 mg, 0.71 mmol, 71%). MS for $C_{22}H_{37}N_3O_9P^+$ $[M+H]^+$ calcd.: 518.2, found: 518.3.

5.4.2. X5a



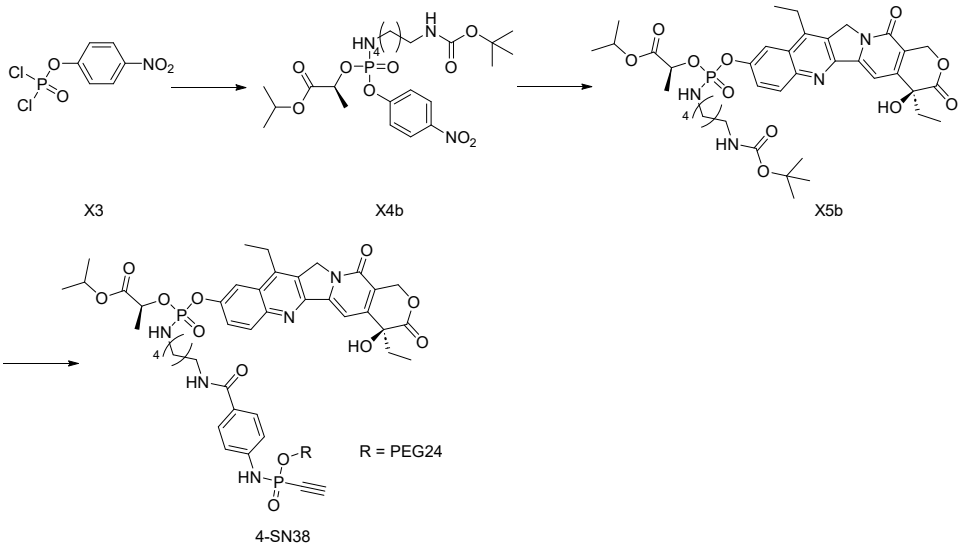
The title compound was synthesized in accordance with the general procedure 2 from 6.4 mg SN38 (0.016 mmol) and 21.7 mg **X4a** (0.040 mmol, 2.5 eq.). The product was obtained as white solid after preparative HPLC and lyophilization (9.9 mg, 0.012 mmol, 78%). HR-MS for $C_{39}H_{53}N_4NaO_{11}P^+$ $[M+H]^+$ calcd.: 785.35212, found: 785.32105.

5.4.3. 3-SN38

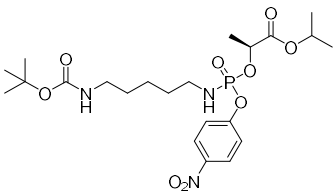


The title compound was synthesized in accordance with general procedure 5 from 10 mg **X5a** (0.013 mmol, 1 eq) and 19.6 mg P5(PEG24)-COOH (0.015 mmol, 1.2 eq.). The product was obtained as yellowish oil after preparative HPLC and lyophilization (15.4 mg, 0.008 mmol, 62%) over two steps. HR-MS for $C_{91}H_{149}N_5O_{36}P_2^{2+}$ $[M+2H]^+$ calcd.: 974.9724, found: 974.97252.

5.5. Linker 4-SN38: Scheme



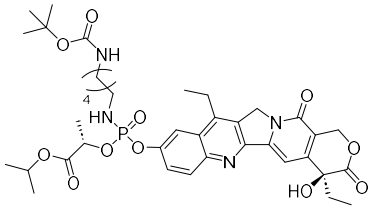
5.5.1. X4b



The title compound was synthesized in accordance with the general procedure 1 from 406 mg 4-nitrophenyl dichlorophosphate (1.6 mmol), 336 mg N-Boc-1,5-diaminopentane (1.66 mmol) and 200 mg

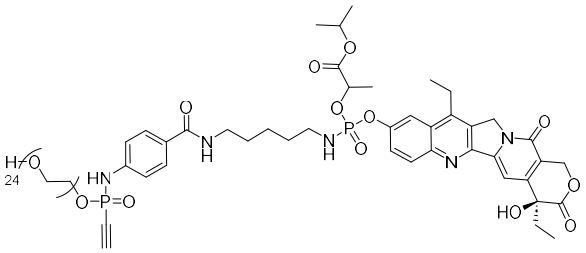
isopropyl lactate (1.5 mmol). The product was obtained as white solid (510 mg, 0.98 mmol, 65%). MS for $C_{22}H_{37}N_3O_9P^+$ $[M+H]^+$ calcd.: 518.2, found: 518.2.

5.5.2. X5b



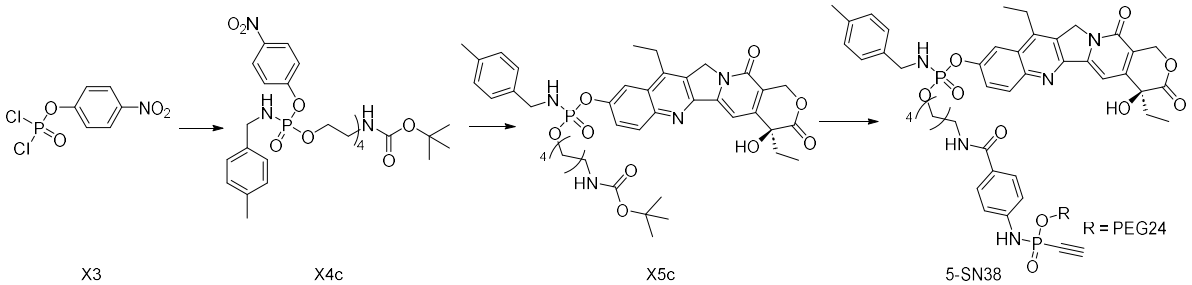
The title compound was synthesized in accordance with the general procedure 2 from 8.4 mg SN38 (0.021 mmol) and 33.2 mg **X4b** (0.064 mmol, 3.0 eq.). The product was obtained as white solid after preparative HPLC and lyophilization (11.3 mg, 0.015 mmol, 69%). HR-MS for $C_{38}H_{52}N_4O_{11}P^+$ $[M+H]^+$ calcd.: 771.3365, found: 771.33797.

5.5.3. 4-SN38

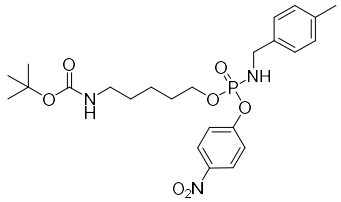


The title compound was synthesized in accordance with general procedure 5 from 11.3 mg **X5b** (0.015 mmol, 1 eq) and 33.8 mg P5(PEG24)-COOH (0.026 mmol, 1.8 eq.). The product was obtained as yellowish oil after preparative HPLC and lyophilization (24.1 mg, 0.012 mmol, 85%) over two steps. HR-MS for $C_{90}H_{147}N_5O_{36}P_2^{2+}$ $[M+2H]^+$ calcd.: 967.9645, found: 967.9618.

5.6. Linker 5-SN38: Scheme

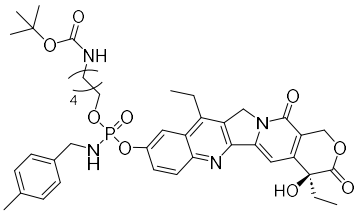


5.6.1. X4c



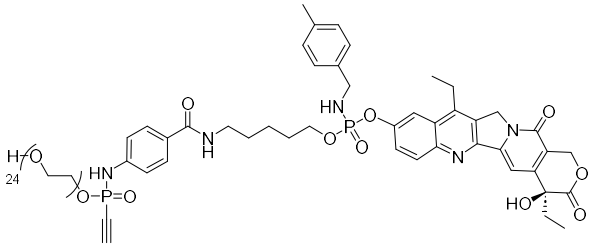
The title compound was synthesized in accordance with the general procedure 1 from 269 mg 4-nitrophenyl dichlorophosphate (1.05 mmol), 203 mg Boc-aminopentanol (1.0 mmol) and 133 mg 4-Methylbenzylamine (1.1 mmol). The product was obtained as white solid (437 mg, 0.86 mmol, 86%). MS for $C_{24}H_{35}N_3O_7P^+$ $[M+H]^+$ calcd.: 508.22, found: 508.23.

5.6.2. X5c



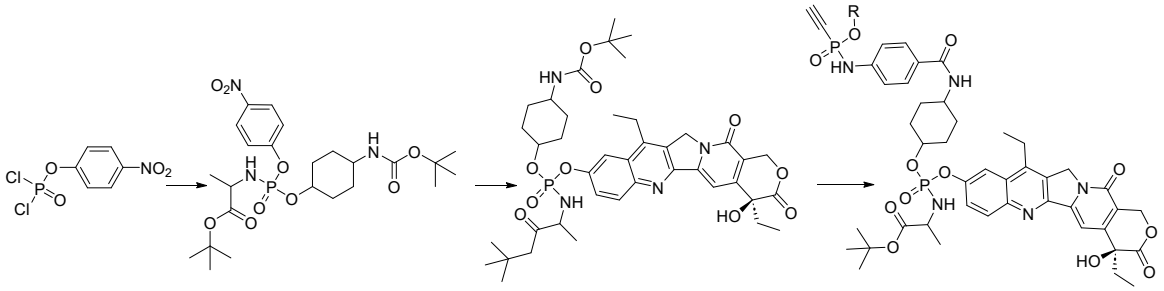
The title compound was synthesized in accordance with the general procedure 2 from 6.8 mg SN38 (0.017 mmol) and 40.5 mg **X4c** (0.080 mmol, 4.6 eq.). The product was obtained as white solid after preparative HPLC and lyophilization (10.2 mg, 0.013 mmol, 77%). HR-MS for $C_{40}H_{50}N_4O_9P^+$ $[M+H]^+$ calcd.: 761.3310, found: 761.3905.

5.6.3. 5-SN38

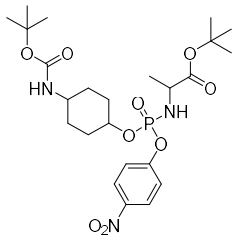


The title compound was synthesized in accordance with general procedure 5 from 10.2 mg **X5c** (0.013 mmol, 1 eq) and 20.6 mg P5(PEG24)-COOH (0.016 mmol, 1.2 eq.). The product was obtained as yellowish oil after preparative HPLC and lyophilization (18.5 mg, 0.010 mmol, 77%) over two steps. HR-MS for $C_{92}H_{145}N_5O_{34}P_2^{2+}$ $[M+2H]^+$ calcd.: 962.9618, found: 962.96109.

5.7. Linker 6-SN38: Scheme

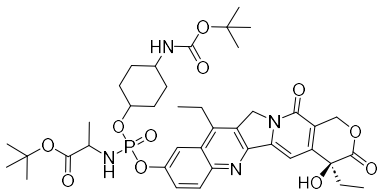


5.7.1. X4d



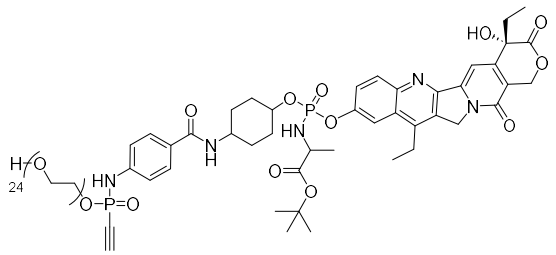
The title compound was synthesized in accordance with the general procedure 1 from 269 mg 4-nitrophenyl dichlorophosphate (1.05 mmol), 215 mg 4-(Boc-amino)cyclohexanol (1.0 mmol) and 200 mg L-alanine *tert*-butyl ester hydrochloride (1.1 mmol). The product was obtained as white solid (421.3 mg, 0.78 mmol, 77.5%). MS for $C_{24}H_{39}N_3O_9P^+$ $[M+Na]^+$ calcd.: 566.22, found: 566.25.

5.7.2. X5d



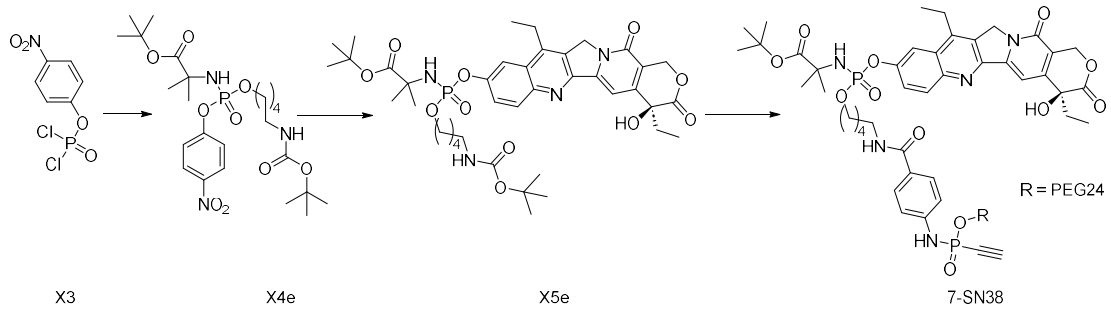
The title compound was synthesized in accordance with the general procedure 2 from 6.1 mg SN38 (0.016 mmol) and 20.6 mg **X4d** (0.039 mmol, 2.5 eq.). The product was obtained as white solid after preparative HPLC and lyophilization (10.6 mg, 0.013 mmol, 83.1%). HR-MS for $C_{40}H_{54}N_4O_{11}P^+$ $[M+H]^+$ calcd.: 797.3521, found: 797.3552.

5.7.3. 6-SN38

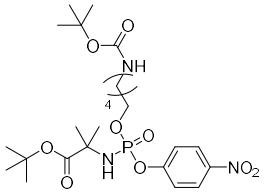


The title compound was synthesized in accordance with general procedure 5 from 10.6 mg **X5d** (0.013 mmol, 1 eq) and 20.5 mg P5(PEG24)-COOH (0.016 mmol, 1.2 eq.). The product was obtained as yellowish oil after preparative HPLC and lyophilization (16.3 mg, 0.008 mmol, 61.5%) over two steps. HR-MS for $C_{92}H_{149}N_5O_{36}P_2^{2+}$ $[M+2H]^+$ calcd.: 980.9724, found: 980.9706.

5.8. Linker 7-SN38: Scheme

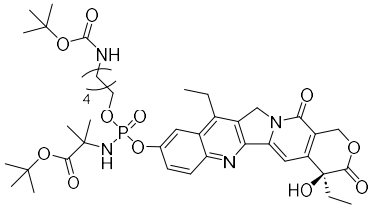


5.8.1. X4e



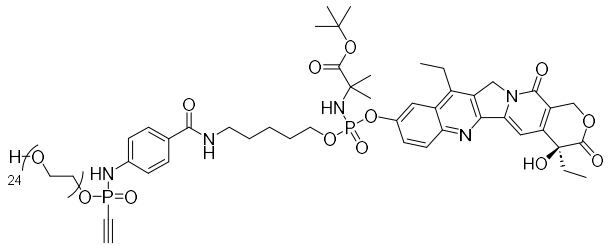
The title compound was synthesized in accordance with the general procedure 1 from 269 mg 4-nitrophenyl dichlorophosphate (1.05 mmol), 203 mg Boc-aminopentanol (1.0 mmol) and 175 mg 2,2-dimethylaminobutyric acid *tert*.-butylester (1.1 mmol). The product was obtained as white solid (327 mg, 0.6 mmol, 60%). MS for $C_{24}H_{41}N_3O_9P^+$ $[M+H]^+$ calcd.: 546.3, found: 546.4.

5.8.2. X5e



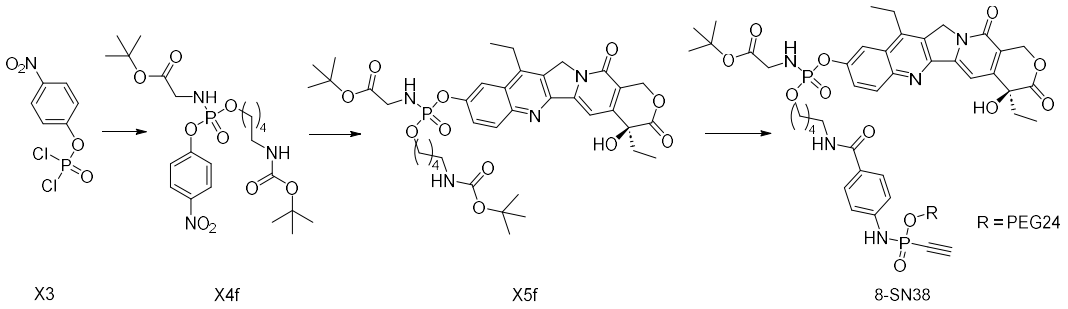
The title compound was synthesized in accordance with the general procedure 2 from 6.1 mg SN38 (0.016 mmol) and 30.6 mg **X4e** (0.05 mmol, 3.1 eq.). The product was obtained as white solid after preparative HPLC and lyophilization (9.3 mg, 0.012 mmol, 75%). HR-MS for $C_{40}H_{56}N_4O_{11}P^+$ $[M+H]^+$ calcd.: 799.3678, found: 799.3690.

5.8.3. 7-SN38

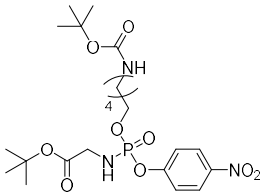


The title compound was synthesized in accordance with general procedure 5 from 9.3 mg **X5e** (0.012 mmol, 1 eq) and 33.8 mg P5(PEG24)-COOH (0.026 mmol, 2.2 eq.). The product was obtained as yellowish oil after preparative HPLC and lyophilization (16.7 mg, 0.009 mmol, 81%) over two steps. HR-MS for $C_{92}H_{151}N_5O_{36}P_2^{2+}$ $[M+2H]^{2+}$ calcd.: 981.9802, found: 981.9803.

5.9. Linker 8-SN38: Scheme

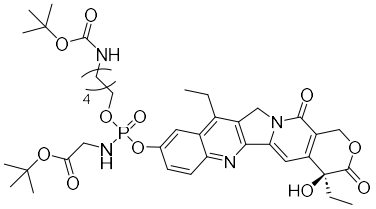


5.9.1. X4f



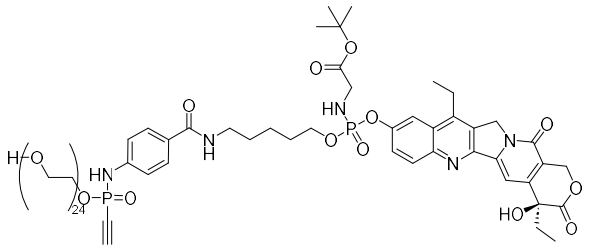
The title compound was synthesized in accordance with the general procedure 1 from 269 mg 4-nitrophenyl dichlorophosphate (1.05 mmol), 203 mg Boc-aminopentanol (1.0 mmol) and 184 mg glycine *tert*.-butyl ester hydrochloride (1.1 mmol). The product was obtained as white solid (393 mg, 0.76 mmol, 76%). MS for $C_{22}H_{37}N_3O_9P^+$ $[M+H]^+$ calcd.: 518.2, found: 518.3.

5.9.2. X5f



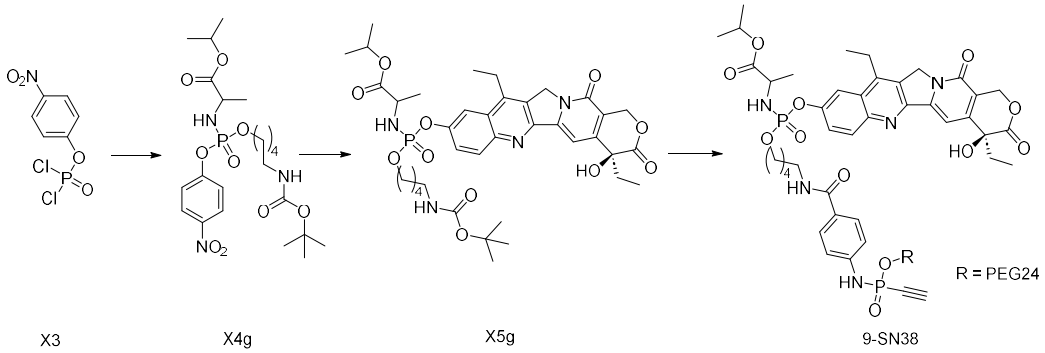
The title compound was synthesized in accordance with the general procedure 2 from 6.6 mg SN38 (0.016 mmol) and 26.0 mg **X4f** (0.05 mmol, 3.1 eq.). The product was obtained as white solid after preparative HPLC and lyophilization (11 mg, 0.014 mmol, 82%). HR-MS for $C_{38}H_{52}N_4O_{11}P^+$ $[M+H]^+$ calcd.: 771.3365, found: 771.3374.

5.9.3. 8-SN38

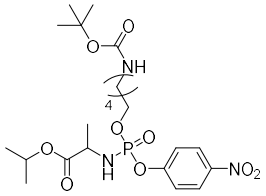


The title compound was synthesized in accordance with general procedure 5 from 11 mg **X5f** (0.014 mmol, 1 eq) and 31.0 mg P5(PEG24)-COOH (0.024 mmol, 1.7eq.). The product was obtained as yellowish oil after preparative HPLC and lyophilization (16.7 mg, 0.009 mmol, 64%) over two steps. HR-MS for $C_{90}H_{147}N_5O_{36}P_2^{2+}$ $[M+2H]^{2+}$ calcd.: 967.9645, found: 967.9616.

5.10. Linker 9-SN38: Scheme

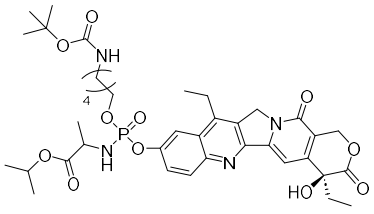


5.10.1. X4g



The title compound was synthesized in accordance with the general procedure 1 from 269 mg 4-nitrophenyl dichlorophosphate (1.05 mmol), 203 mg Boc-aminopentanol (1.0 mmol) and 184 mg L-alanine isopropyl ester hydrochloride (1.1 mmol). The product was obtained as white solid (367 mg, 0.71 mmol, 71%). MS for $C_{22}H_{37}N_3O_9P^+$ $[M+H]^+$ calcd.: 518.2, found: 518.3.

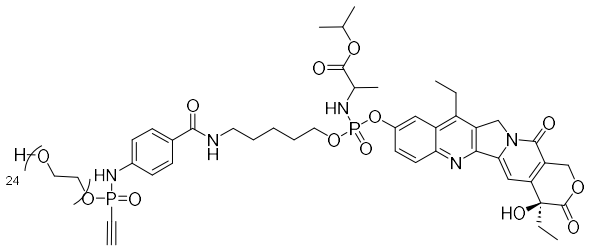
5.10.2. X5g



The title compound was synthesized in accordance with the general procedure 2 from 6.0 mg SN38 (0.016 mmol) and 16.0 mg **X4g** (0.032 mmol, 2.0 eq.). The product was obtained as white solid after

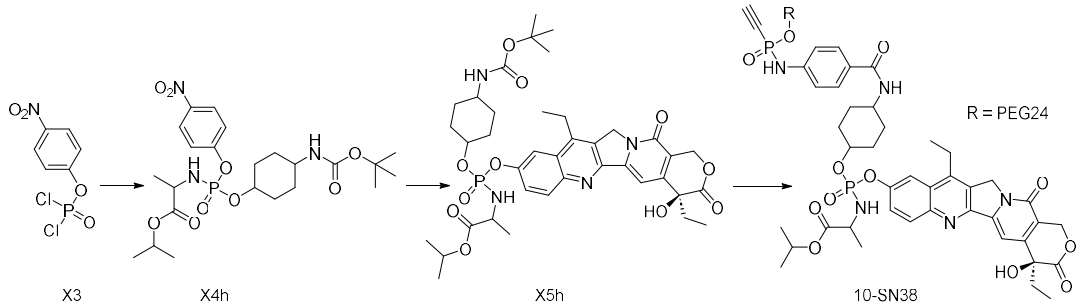
preparative HPLC and lyophilization (8.5 mg, 0.011 mmol, 69%). HR-MS for $C_{38}H_{52}N_4O_{11}P^+$ $[M+H]^+$ calcd.: 771.3365, found: 771.3374.

5.10.3. 9-SN38

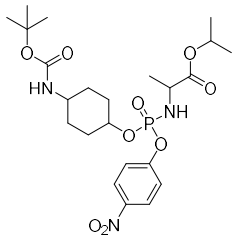


The title compound was synthesized in accordance with general procedure 5 from 8.5 mg **X5g** (0.011 mmol, 1 eq) and 30.8 mg P5(PEG24)-COOH (0.022 mmol, 2.0eq.). The product was obtained as yellowish oil after preparative HPLC and lyophilization (16.7 mg, 0.009 mmol, 81%) over two steps. HR-MS for $C_{90}H_{147}N_5O_{36}P_2^{2+}$ $[M+2H]^{2+}$ calcd.: 967.9645, found: 967.9616.

5.11. Linker 10-SN38: Scheme

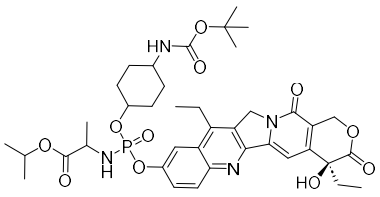


5.11.1. X4h



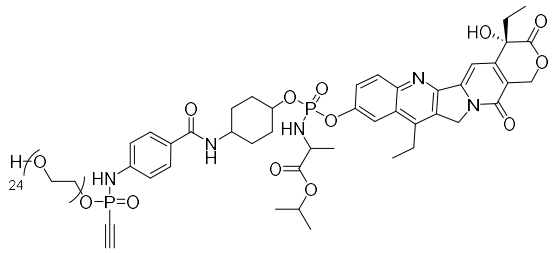
The title compound was synthesized in accordance with the general procedure 1 from 269 mg 4-nitrophenyl dichlorophosphate (1.05 mmol), 215 mg 4-(Boc-amino)cyclohexanol (1.0 mmol) and 184 mg L-alanine isopropyl ester hydrochloride (1.1 mmol). The product was obtained as white solid (402 mg, 0.67 mmol, 76%). MS for $C_{23}H_{37}N_3O_9P^+$ $[M+H]^+$ calcd.: 530.2, found: 530.3.

5.11.2. X5h



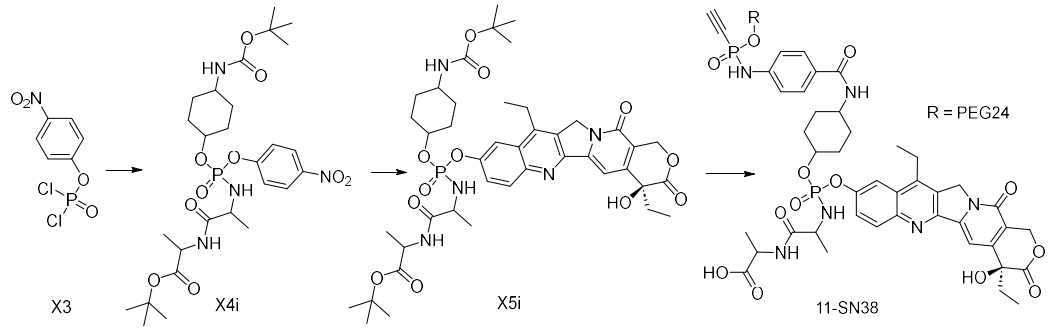
The title compound was synthesized in accordance with the general procedure 2 from 7.6 mg SN38 (0.019 mmol) and 25.2 mg **X4h** (0.048 mmol, 2.5 eq.). The product was obtained as yellow solid after preparative HPLC and lyophilization (11.0 mg, 0.014 mmol, 74%). HR-MS for $C_{39}H_{52}N_4O_{11}P^+$ $[M+H]^+$ calcd.: 783.3365, found: 783.3359.

5.11.3. 10-SN38

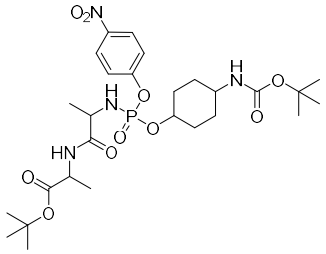


The title compound was synthesized in accordance with general procedure 5 from 11.0 mg **X5h** (0.014 mmol, 1 eq) and 21.6 mg P5(PEG24)-COOH (0.017 mmol, 1.2 eq.). The product was obtained as yellowish oil after preparative HPLC and lyophilization (22.7 mg, 0.012 mmol, 85%) over two steps. HR-MS for $C_{91}H_{147}N_5O_{36}P_2^{2+}$ $[M+2H]^{2+}$ calcd.: 973.9645, found: 973.9664.

5.12. Linker 11-SN38: Scheme

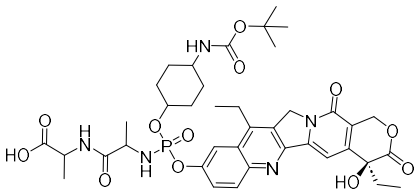


5.12.1. X4i



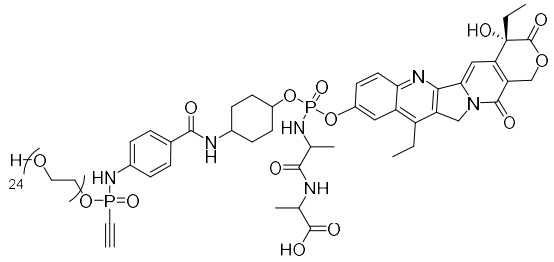
The title compound was synthesized in accordance with the general procedure 1 from 269 mg 4-nitrophenyl dichlorophosphate (1.05 mmol), 215 mg 4-(Boc-amino)cyclohexanol (1.0 mmol) and 330 mg L-alanine-L-alanine *tert*.-butyl ester trifluoroacetate (1.1 mmol). The product was obtained as white solid (412 mg, 0.67 mmol, 67%). MS for $C_{22}H_{37}N_3O_9P^+$ $[M+H]^+$ calcd.: 615.3, found: 615.3.

5.12.2. X5i



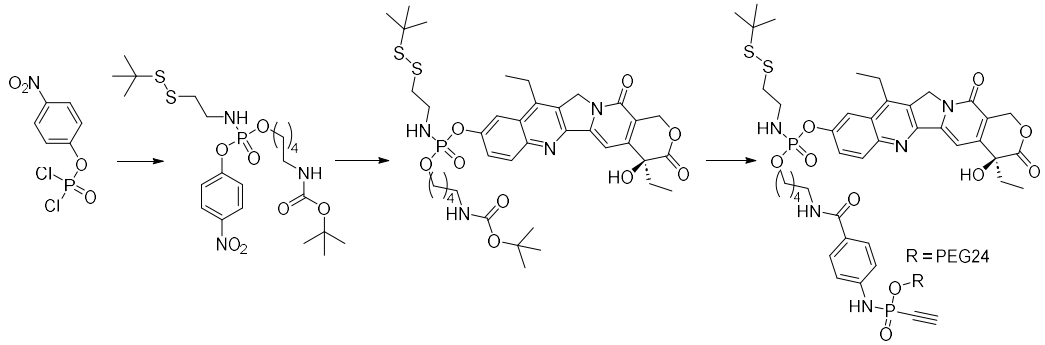
The title compound was synthesized in accordance with the general procedure 2 from 6.4 mg SN38 (0.016mmol) and 24.6 mg **X4i** (0.04 mmol, 2.5 eq.). The product was obtained as yellow solid after preparative HPLC and lyophilization (12.2 mg, 0.014 mmol, 88%). HR-MS for $C_{43}H_{59}N_5O_{12}P^+$ $[M+H]^+$ calcd.: 868.3992, found: 868.3931.

5.12.3. 11-SN38



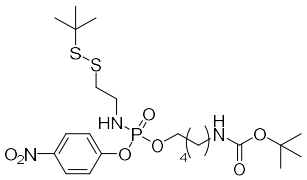
The title compound was synthesized in accordance with general procedure 6 from 6.4 mg **X5i** (0.009 mmol, 1 eq) and 17.3 mg P5(PEG24)-COOH (0.014 mmol, 1.5 eq.). The product was obtained as yellowish oil after preparative HPLC and lyophilization (8.6 mg, 0.004 mmol, 48%) over two steps. HR-MS for $C_{91}H_{146}N_6O_{37}P_2^{2+}$ $[M+2H]^{2+}$ calcd.: 988.4596, found: 988.4565.

5.13. Linker 12-SN38: Scheme



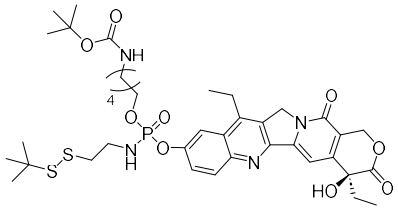
X3 X4j X6j 12-SN38

5.13.1. X4j



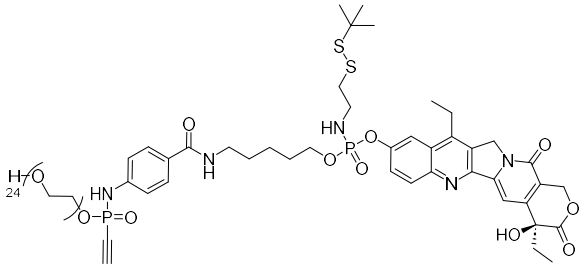
The title compound was synthesized in accordance with the general procedure 1 from 250 mg 4-nitrophenyl dichlorophosphate (0.98 mmol), 199 mg Boc-aminopentanol (0.98 mmol) and 165 mg cysteamine *tert*.-butyl disulfide (0.98 mmol). The product was obtained as white solid (215 mg, 0.4 mmol, 40%). MS for $C_{22}H_{39}N_3O_7PS_2^+$ $[M+H]^+$ calcd.: 552.2, found: 552.3.

5.13.2. X5j



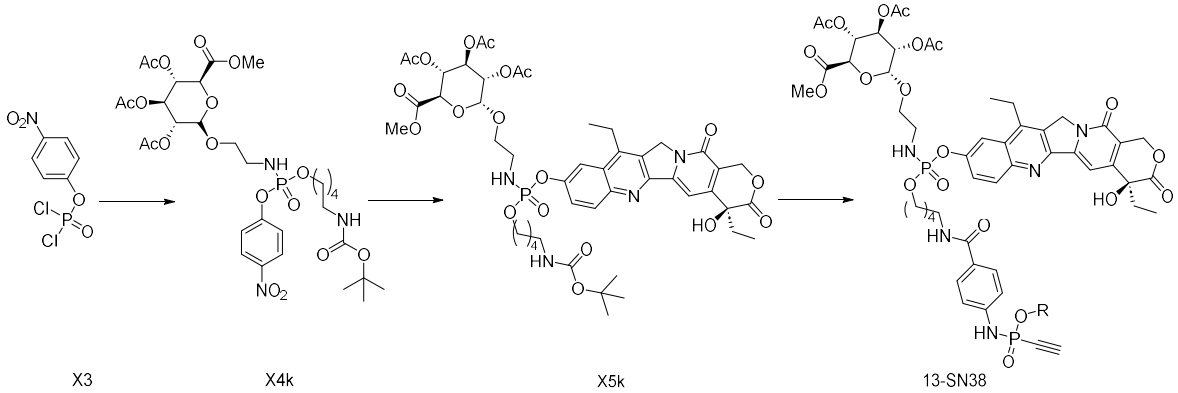
The title compound was synthesized in accordance with the general procedure 2 from 5.0 mg SN38 (0.013 mmol) and 21.0 mg **X4j** (0.040 mmol, 3.1 eq.). The product was obtained as yellow solid after preparative HPLC and lyophilization. (9.0 mg, 0.011 mmol, 84%). HR-MS for $C_{38}H_{54}N_4O_9PS_2^+$ $[M+H]^+$ calcd.: 805.3064, found: 805.3097.

5.13.3. 12-SN38

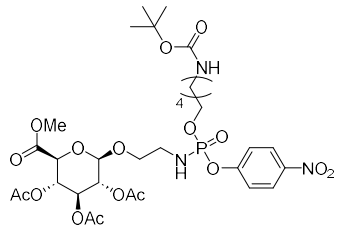


The title compound was synthesized in accordance with general procedure 5 from 8.1 mg **X5j** (0.010 mmol, 1 eq) and 19.2 mg P5(PEG24)-COOH (0.015 mmol, 1.5 eq.). The product was obtained as yellowish oil after preparative HPLC and lyophilization (13.0 mg, 0.007 mmol, 70%) over two steps. HR-MS for $C_{90}H_{149}N_5O_{34}P_2S_2^{2+}$ $[M+2H]^{2+}$ calcd.: 984.9495, found: 984.9486.

5.14. Linker 13-SN38: Scheme

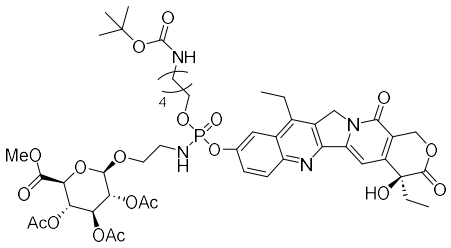


5.14.1. X4k



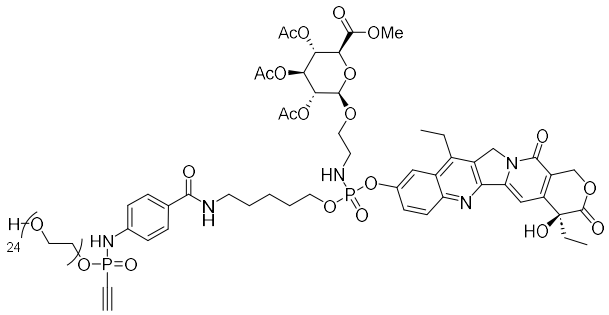
The title compound was synthesized in accordance with the general procedure 1 from 135 mg 4-nitrophenyl dichlorophosphate (0.5 mmol), 107 mg Boc-aminopentanol (0.5 mmol) and 200 mg 200 mg *O*-GlcA-aminoethanol (0.5 mmol). The product was obtained as white solid (63 mg, 0.08 mmol, 17%). MS for $C_{31}H_{47}N_3O_{17}P^+$ $[M+H]^+$ calcd.: 764.2638, found: 764.2619.

5.14.2. X5k



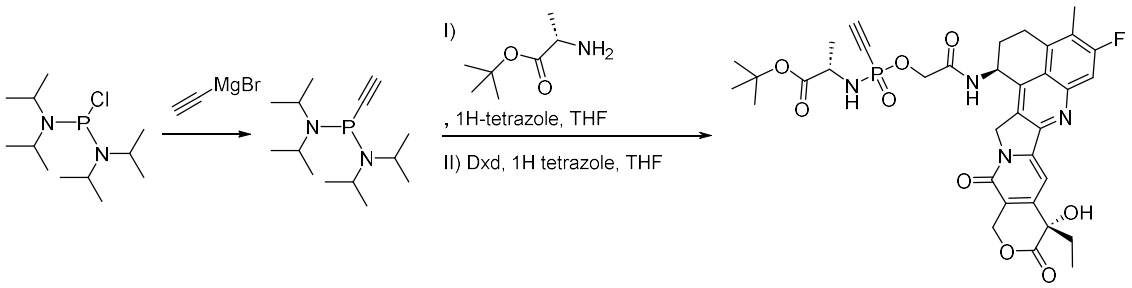
The title compound was synthesized in accordance with the general procedure 2 from 5.0 mg SN38 (0.013 mmol) and 30.0 mg **X4k** (0.038 mmol, 3.0 eq.). The product was obtained as white solid after preparative HPLC and lyophilization. (2.5 mg, 0.002 mmol, 19%). HR-MS for $C_{47}H_{62}N_4O_{19}P^+$ $[M+H]^+$ calcd.: 1017.3740, found: 1017.3703.

5.14.3. 13-SN38



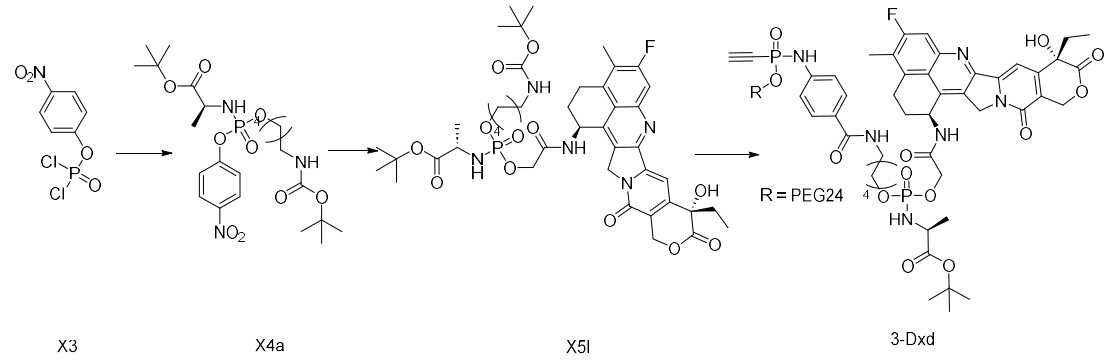
The title compound was synthesized in accordance with general procedure 5 from 2.3 mg **X5k** (0.002 mmol, 1 eq) and 3.5 mg P5(PEG24)-COOH (0.003 mmol, 1.5 eq.). The product was obtained as yellowish oil after preparative HPLC and lyophilization (1.6 mg, 0.7 µmol, 35%) over two steps. HR-MS for $C_{99}H_{157}N_5O_{44}P_2^{2+}$ $[M+2H/2]^{2+}$ calcd.: 1091.4850, found: 1091.4816.

5.15. Linker 1-Dxd: Scheme and synthesis

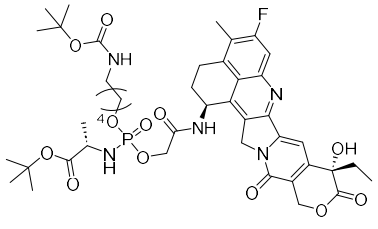


A solution of ethynylmagnesium bromide in THF (0.6 mL, 0.5 M, 1.0 eq.) and bis-(*N*,*N*-diisopropylamino)chlorophosphine (78 mg, 0.3 mmol) was stirred under an argon atmosphere at 0°C and allowed to attain room temperature for 30 minutes. A solution of 1H-Tetrazole in MeCN (0.7 mL, 0.45 M, 1.1 eq.) and alanine *tert*-butyl ester (47 mg, 0.32 mmol, 1.1 eq.) dissolved in 0.5 mL THF were added to the reaction and stirred for 1h at room temperature. A part of the reaction mix (0.180 mL, 166 mM, 30 µmol, 1.5 eq.) and 1H-Tetrazole in MeCN (45 µL, 0.45 M, 1 eq.) were added to Dxd (10 mg, 20 µmol, 1eq.) and stirred for 1h at room temperature. Iodine-Water-Pyridine-Tetrahydrofuran Oxidizer Solution (1.3 mL, 0.02 M, 1.1 eq.) were added and stirred for 5 minutes. All volatiles were removed in an argon stream and the residue was purified by reversed phase HPLC (30 to 80% MeCN in 50 minutes) to yield 8.1 mg of **1-Dxd** (11.0 µmol, 56.5%). MS for $C_{35}H_{39}FN_4O_9P^+$ $[M+H]^+$ calcd.: 709.24, found: 709.3

5.16. Linker 3-Dxd: Scheme

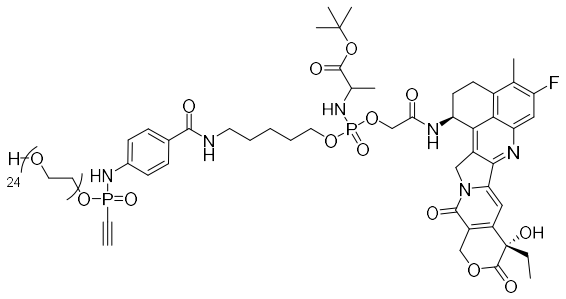


5.16.1. X5l



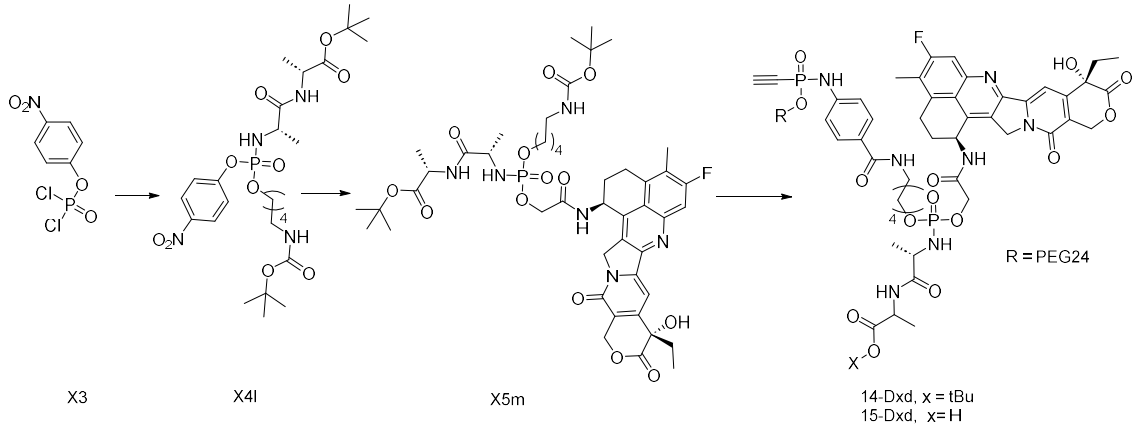
The title compound was synthesized in accordance with the general procedure 3 from 7.0 mg Dxd (0.014 mmol) and 18.6 mg **X4a** (0.035 mmol, 2.5 eq.). The product was obtained as white solid after preparative HPLC and lyophilization (1.75 mg, 0.0019 mmol, 13.9%). HR-MS for $C_{43}H_{58}FN_5O_{12}P^+$ $[M+H]^+$ calcd.: 886.3798, found: 886.3781.

5.16.2. 3-Dxd

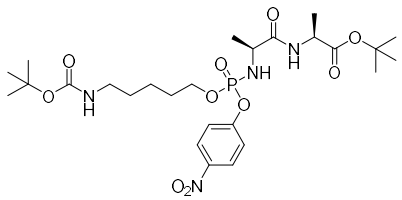


The title compound was synthesized in accordance with general procedure 5 from 8.3 mg **X5l** (0.009 mmol, 1 eq) and 13.8 mg P5(PEG24)-COOH (0.011 mmol, 1.2 eq.). The product was obtained as yellowish oil after preparative HPLC and lyophilization (13.3 mg, 0.007 mmol, 77%) over two steps. HR-MS for $C_{95}H_{153}FN_6O_{37}P_2^{2+}$ $[M+2H]^{2+}$ calcd.: 1025.4862, found: 1025.4873.

5.17. Linker 14- and 15-Dxd: Scheme

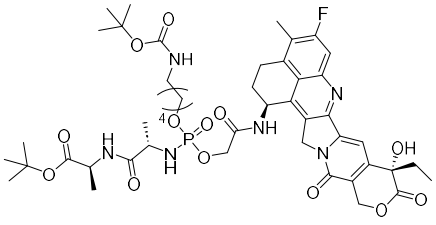


5.17.1. X4l



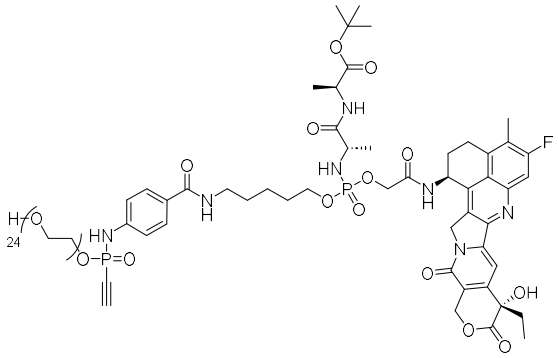
The title compound was synthesized in accordance with the general procedure 1 from 537 mg 4-nitrophenyl dichlorophosphate (2.1 mmol), 406 mg Boc-aminopentanol (2.0 mmol) and 475 mg di-L-alanine *tert*-butyl ester (2.2 mmol). The product was obtained as white solid (1.04 g, 1.7 mmol, 86%). MS for $C_{26}H_{44}N_4O_{10}P^+$ $[M+H]^+$ calcd.: 603.3, found: 603.3.

5.17.2. X5m



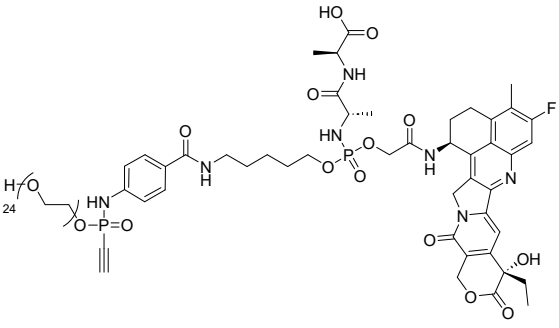
The title compound was synthesized in accordance with the general procedure 3 from 8.2 mg Dxd (0.017 mmol) and 40.1 mg **X6l** (0.066 mmol, 4 eq.). The product was obtained as white solid after preparative HPLC and lyophilization (11.4 mg, 0.012 mmol, 70.6%). HR-MS for $C_{46}H_{63}FN_6O_{13}P^+$ $[M+H]^+$ calcd.: 957.4169, found: 957.4149.

5.17.3. 14-Dxd



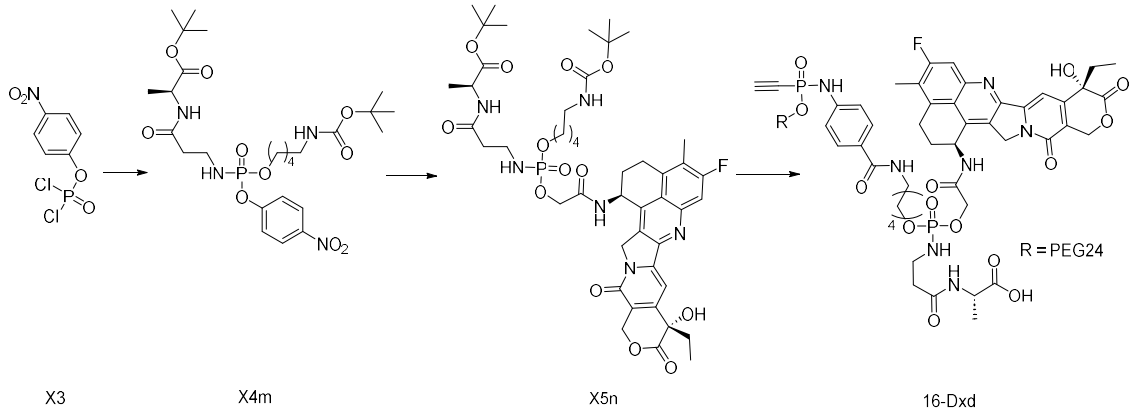
The title compound was synthesized in accordance with general procedure 5 from 11.5 mg **X5m** (0.012 mmol, 1 eq) and 15.4 mg P5(PEG24)-COOH (0.012 mmol, 1eq.). The product was obtained as yellowish oil after preparative HPLC and lyophilization (10.0 mg, 0.005 mmol, 42%) over two steps. HR-MS for $C_{98}H_{158}FN_7O_{38}P_2^{2+}$ $[M+2H]^{2+}$ calcd.: 1061.5064, found: 1061.50522.

5.17.4. 15-Dxd

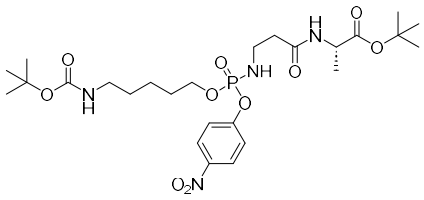


The title compound was synthesized in accordance with general procedure 6 from 4.4 mg **X5m** (0.006 mmol, 1 eq) and 10.2 mg P5(PEG24)-COOH (0.008 mmol, 1.5 eq.). The product was obtained as yellowish oil after preparative HPLC and lyophilization (7.2 mg, 3.5 µmol, 58%) over two steps. HR-MS for $C_{94}H_{150}FN_7O_{38}P_2^{2+}$ $[M+2H]^{2+}$ calcd.: 1032.9735, found: 1032.9683.

5.18. Linker 16-Dxd: Scheme

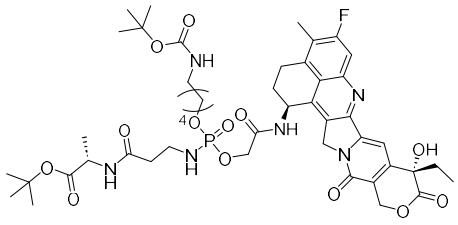


5.18.1. X4m



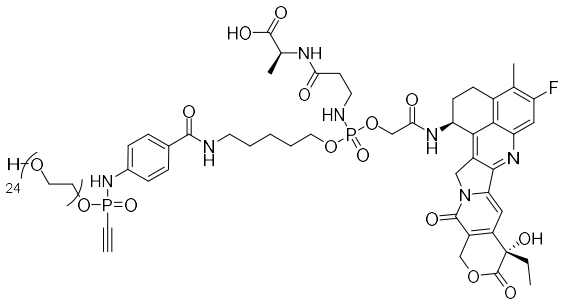
The title compound was synthesized in accordance with the general procedure 1 from 323 mg 4-nitrophenyl dichlorophosphate (1.3 mmol), 244 mg Boc-aminopentanol (1.2 mmol) and 260 mg ß-alanine-*L*-alanine *tert*-butyl ester (1.2 mmol). The product was obtained as white solid (495 mg, 0.820 mmol, 68%). MS for $C_{26}H_{44}N_4O_{10}P^+$ $[M+H]^+$ calcd.: 603.3, found: 603.3.

5.18.2. X5n



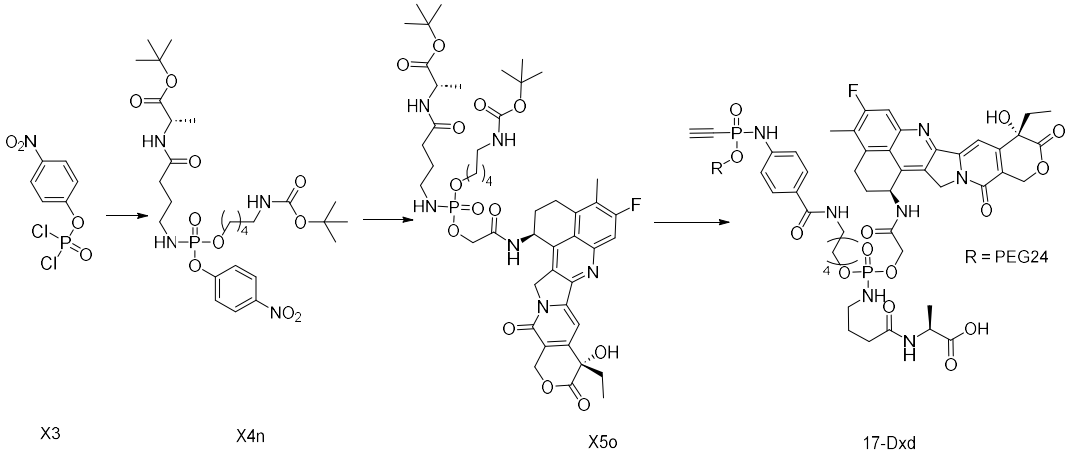
The title compound was synthesized in accordance with the general procedure 3 from 6.5 mg Dxd (0.013 mmol) and 21.4 mg **X4m** (0.036 mmol, 2.7 eq.). The product was obtained as brown solid after preparative HPLC and lyophilization (8.1 mg, 0.008 mmol, 65.2%). HR-MS for $C_{46}H_{63}FN_6O_{13}P^+$ $[M+H]^+$ calcd.: 957.4169, found: 957.4166

5.18.3. 16-Dxd

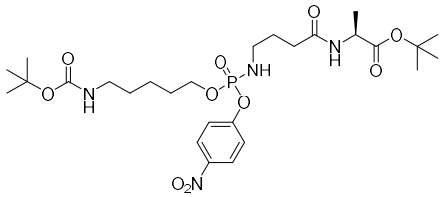


The title compound was synthesized in accordance with general procedure 6 from 1.0 mg **X5n** (0.001 mmol, 1 eq) and 2.6 mg P5(PEG24)-COOH (0.002 mmol, 2 eq.). The product was obtained as yellowish oil after preparative HPLC and lyophilization (1.2 mg, 0.58 µmol, 58%) over two steps. HR-MS for $C_{94}H_{150}FN_7O_{38}P_2^{2+}$ $[M+2H]^{2+}$ calcd.: 1032.9735, found: 1032.9786.

5.19. Linker 17-Dxd: Scheme

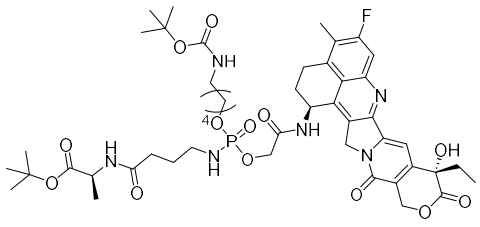


5.19.1. X4n



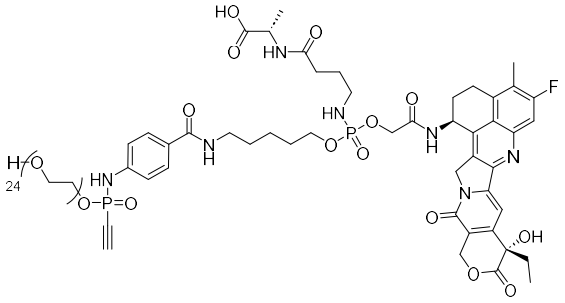
The title compound was synthesized in accordance with the general procedure 1 from 64 mg 4-nitrophenyl dichlorophosphate (0.25 mmol), 51 mg Boc-aminopentanol (0.25 mmol) and 58 mg γ-aminobutyic acid-*L*-alanine *tert*-butyl ester (0.25 mmol). The product was obtained as white solid (91.6 mg, 0.15 mmol, 60%). MS for $C_{26}H_{44}N_4O_{10}P^+$ $[M+H]^+$ calcd.: 617.3, found: 617.3.

5.19.2. X5o



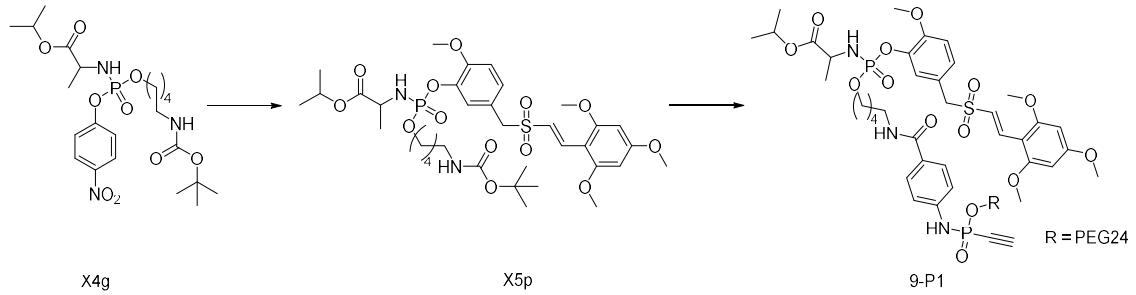
The title compound was synthesized in accordance with the general procedure 3 from 5.8 mg Dxd (0.012 mmol) and 36.2 mg **X4n** (0.059 mmol, 5.0 eq.). The product was obtained as yellow solid after preparative HPLC and lyophilization (3.3 mg, 0.0034 mmol, 28%). HR-MS for $C_{47}H_{65}FN_6O_{13}P^+$ $[M+H]^+$ calcd.: 971.4326, found: 971.4333.

5.19.3. 17-Dxd

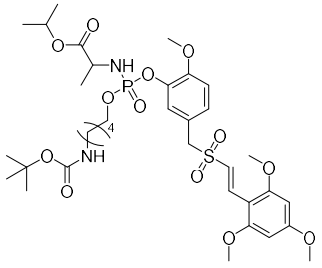


The title compound was synthesized in accordance with general procedure 6 from 3.3 mg **X5o** (0.004 mmol, 1 eq) and 7.7 mg P5(PEG24)-COOH (0.006 mmol, 1.2 eq.). The product was obtained as yellowish oil after preparative HPLC and lyophilization (5.6 mg, 0.003 mmol, 67%) over two steps. HR-MS for $C_{95}H_{152}FN_7O_{38}P_2^{2+}$ $[M+2H]^{2+}$ calcd.: 1039.9813, found: 1039.9763.

5.20. Linker 9-P1: Scheme

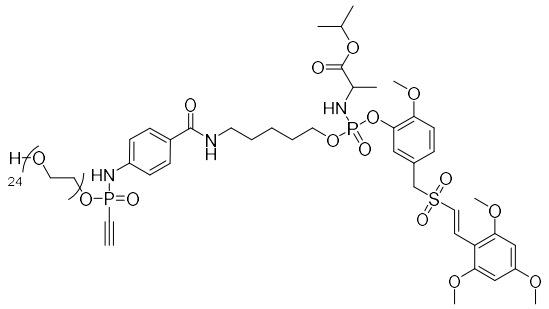


5.20.1. X5p



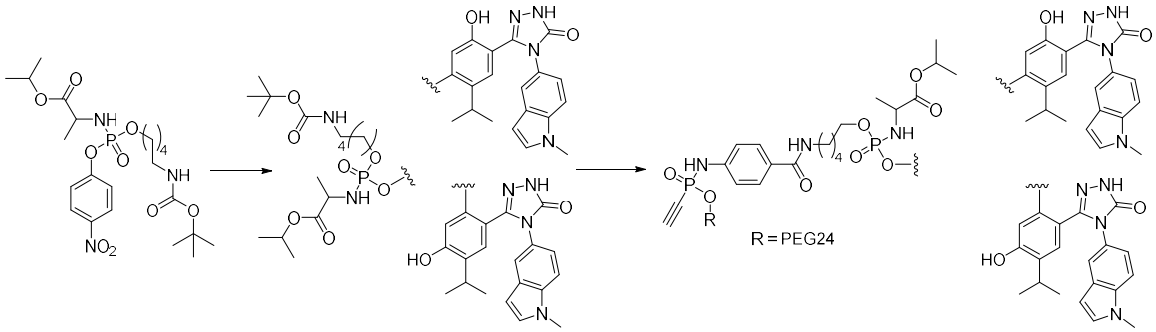
The title compound was synthesized in accordance with the general procedure 2 from 6.0 mg ON-013100 (0.015 mmol) and 24.0 mg **X4g** (0.046 mmol, 3.0 eq.). The product was obtained as yellow solid after preparative HPLC and lyophilization (8.4 mg, 0.011 mmol, 73%). HR-MS for $C_{35}H_{54}N_2O_{13}PS^+$ $[M+H]^+$ calcd.: 773.3079, found: 773.3118.

5.20.2. 9-P1

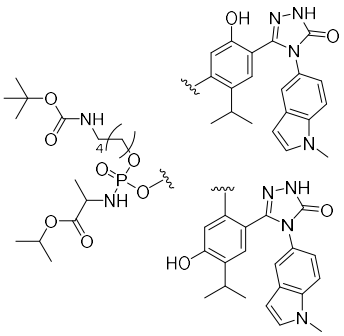


The title compound was synthesized in accordance with general procedure 6 from 8.4 mg **X5p** (0.011 mmol, 1 eq) and 30.8 mg P5(PEG24)-COOH (0.022 mmol, 2.0eq.). The product was obtained as yellowish oil after preparative HPLC and lyophilization (11.7 mg, 0.006 mmol, 55% over two steps). HR-MS for $C_{87}H_{149}N_3O_{38}P_2S^{2+}$ $[M+2H]^{2+}$ calcd.: 968.9502, found: 968.9505.

5.21. Linker 9-P2: Scheme

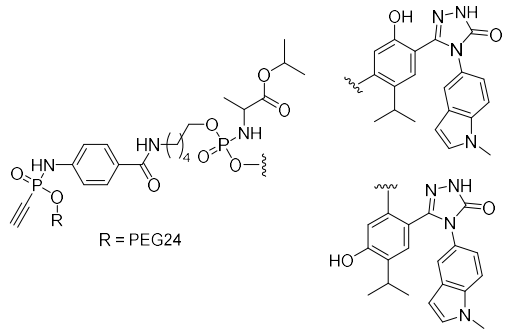


5.21.1. X5q



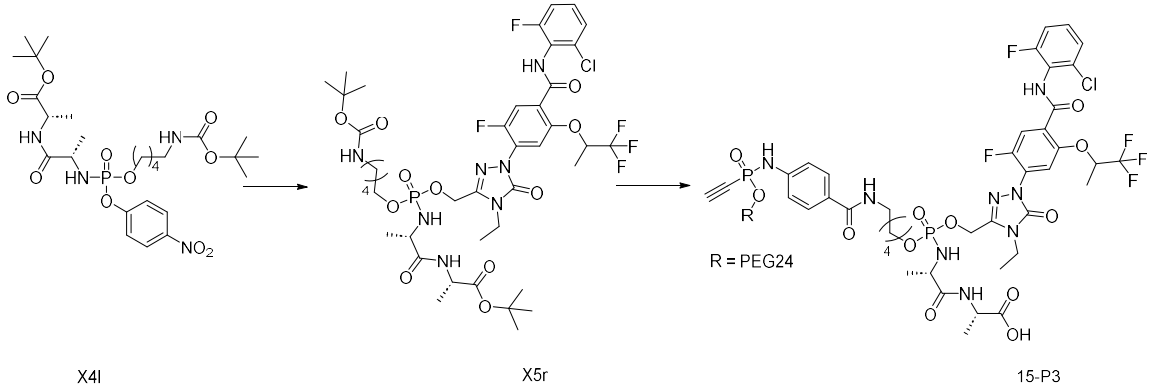
The title compound was synthesized in accordance with the general procedure 2 from 7.0 mg Ganetespib (0.019 mmol) and 10.0 mg **X4g** (0.019 mmol, 3.0 eq.). The product was obtained as white solid after preparative HPLC and lyophilization (1.6 mg, 0.002 mmol, 11%). HR-MS for $C_{36}H_{52}N_5O_9P^+$ $[M+H]^+$ calcd.: 743.3528, found: 743.3542.

5.21.2. 9-P2

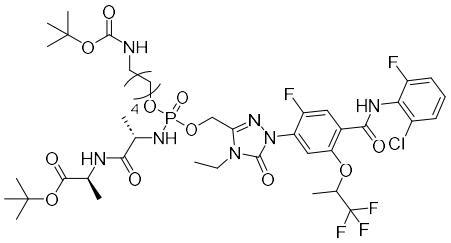


The title compound was synthesized in accordance with general procedure 6 from 1.6 mg **X5q** (0.002 mmol, 1 eq) and 5.1 mg P5(PEG24)-COOH (0.004 mmol, 2.0eq.). The product was obtained as yellowish oil after preparative HPLC and lyophilization (2.6 mg, 0.001 mmol, 65% over two steps). HR-MS for $C_{88}H_{147}N_7O_{34}P_2^{2+}$ $[M+2H]^{2+}$ calcd.: 953.9727, found: 953.9735.

5.22. Linker 15-P3: Scheme

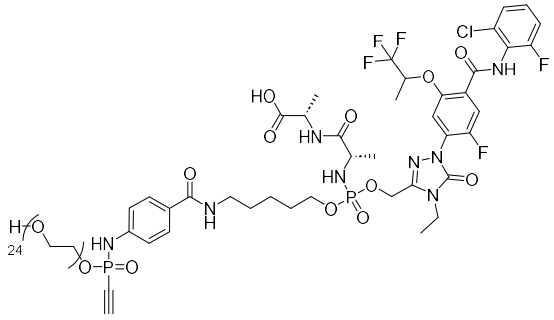


5.22.1. X5r



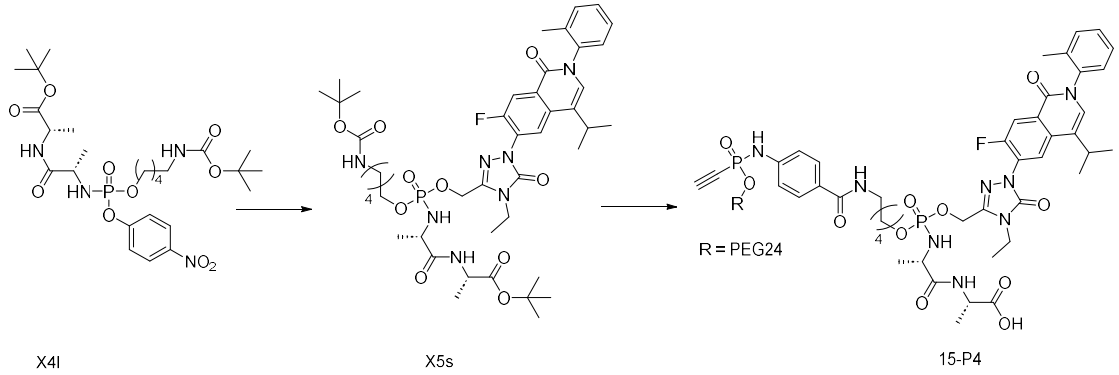
The title compound was synthesized in accordance with the general procedure 3 from 20 mg BAY-2402234 (0.038 mmol) and 80 mg **X4l** (0.133 mmol, 3.5 eq.). The product was obtained as white solid after preparative HPLC and lyophilization (26.2 mg, 0.027 mmol, 69%). HR-MS for $C_{41}H_{56}ClF_5N_7NaO_{11}P^+$ $[M+Na]^+$ calcd.: 1006.3276, found: 1006.3291.

5.22.2. 15-P3

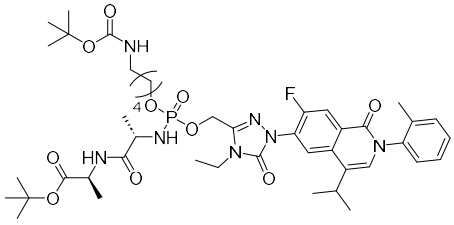


The title compound was synthesized in accordance with general procedure 6 from 14 mg **X5r** (0.014 mmol, 1 eq) and 18 mg P5(PEG24)-COOH (0.014 mmol, 1eq.). The product was obtained as yellowish oil after preparative HPLC and lyophilization (21.0 mg, 0.01 mmol, 70%) over two steps. HR-MS for $C_{89}H_{144}ClF_5N_8O_{36}P_2^{2+}$ $[M+2H]^+$ calcd.: 1046.4378, found: 1046.4386.

5.23. Linker 15-P4: Scheme

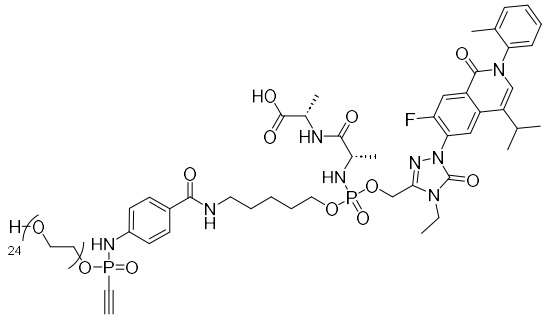


5.23.1. X5s



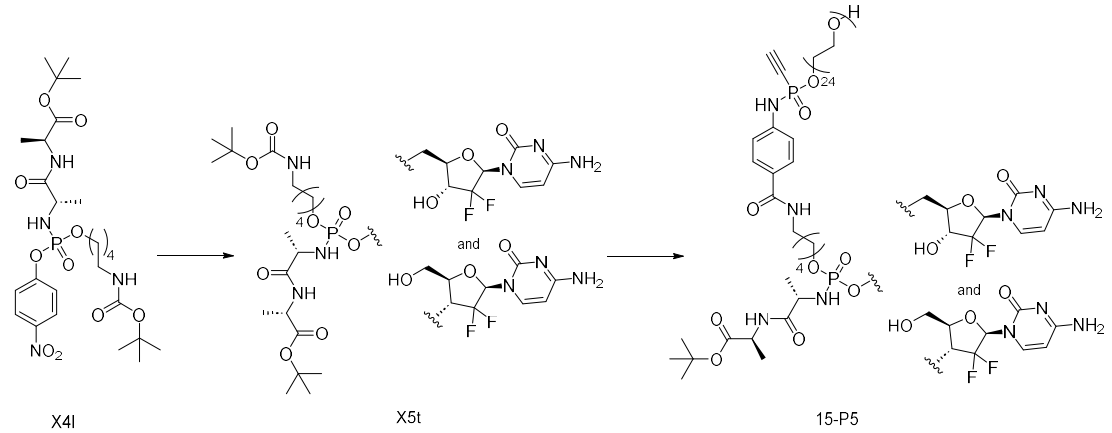
The title compound was synthesized in accordance with the general procedure 3 from 5 mg DHODH-IN-16 (0.01 mmol) and 20 mg **X4l** (0.033 mmol, 3.3 eq.). The product was obtained as white solid after preparative HPLC and lyophilization (5.3 mg, 0.0058 mmol, 51%). MS for $C_{44}H_{64}FN_7O_{10}P^+$ $[M+H]^+$ calcd.: 900.4, found: 900.5.

5.23.2. 15-P4

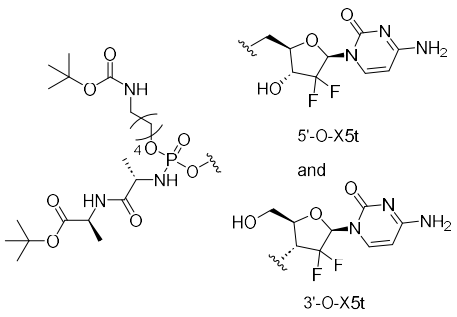


The title compound was synthesized in accordance with general procedure 6 from 5.3 mg **X5s** (0.006 mmol, 1 eq) and 7.7 mg P5(PEG24)-COOH (0.006 mmol, 1eq.). The product was obtained as yellowish oil after preparative HPLC and lyophilization (6.4 mg, 0.003 mmol, 52%) over two steps. HR-MS for $C_{92}H_{151}FN_8O_{35}P_2^{2+}$ $[M+2H]^+$ calcd.: 1004.4865, found: 1004.4886.

5.24. Linker 15-P5: Scheme



5.24.1. X5t



The title compound was synthesized in accordance with the general procedure 3 from 50 mg Gemcitabine (0.19 mmol) and 114 mg **X4l** (0.19 mmol, 1.0 eq.). The crude reaction mixture was purified by preparative HPLC to isolate 5'-O-**X5t** (16.1 mg, 0.022 mmol, 11.7%) and 3'-O-**X5t** (7.4 mg, 0.010 mmol, 5.4%) as white solid. MS for $C_{29}H_{50}F_2N_6O_{11}P^+$ $[M+H]^+$ calcd.: 727.3238, found: 727.3244.

5'-O-X6t:

^{1}H-NMR (800 MHz, DMSO-d_6) δ(ppm) = 9.03 (s, 1H, H15: -NH_2-), 8.51 (s, 1H, H15': -NH_2-), 8.10 (dd, J = 7.0, 4.1 Hz, 1H, H24: -NH-), 7.82 (dd, J = 10.3, 7.8 Hz, 1H, H11: -$C_{Ar}H$), 6.77 (q, J = 5.6 Hz, 1H, H37: -NH-), 6.50 (br-s, 1H, H9: -OH), 6.15 (q, J = 8.4 Hz, 1H, H1), 6.03 (d, J = 7.8 Hz, 1H, H12: -$C_{Ar}H$), 5.28 (dt, J = 21.8, 11.0 Hz, 1H, H20: -NH-), 4.23 – 4.16 (m, 2H, H3: -CH-; H5': -CH_2-), 4.15 – 4.04 (m, 3H, H25: -CH-; H4: -CH-; H5'': -CH_2-), 3.85 (dq, J = 16.0, 6.5 Hz, 2H, H32: -CH_2-), 3.68 (tdt, J = 16.5, 9.5, 7.1 Hz, 1H, H21: -CH-), 2.89 (p, J = 7.1 Hz, 2H, H36: -CH_2-), 1.60 – 1.51 (m, 2H, H33: -CH_2-), 1.38 (s, 9H, H29-31: -CH_3), 1.36 (s, 9H, H40-42: -CH_3), 1.35 – 1.34 (m, 2H, H35: -CH_2-), 1.29 – 1.25 (m, 2H, H34: -CH_2-), 1.24 (dd, J = 7.3, 4.2 Hz, 3H, H26: -CH_3), 1.22 (d, J = 7.0 Hz, 3H, H22: -CH_3).

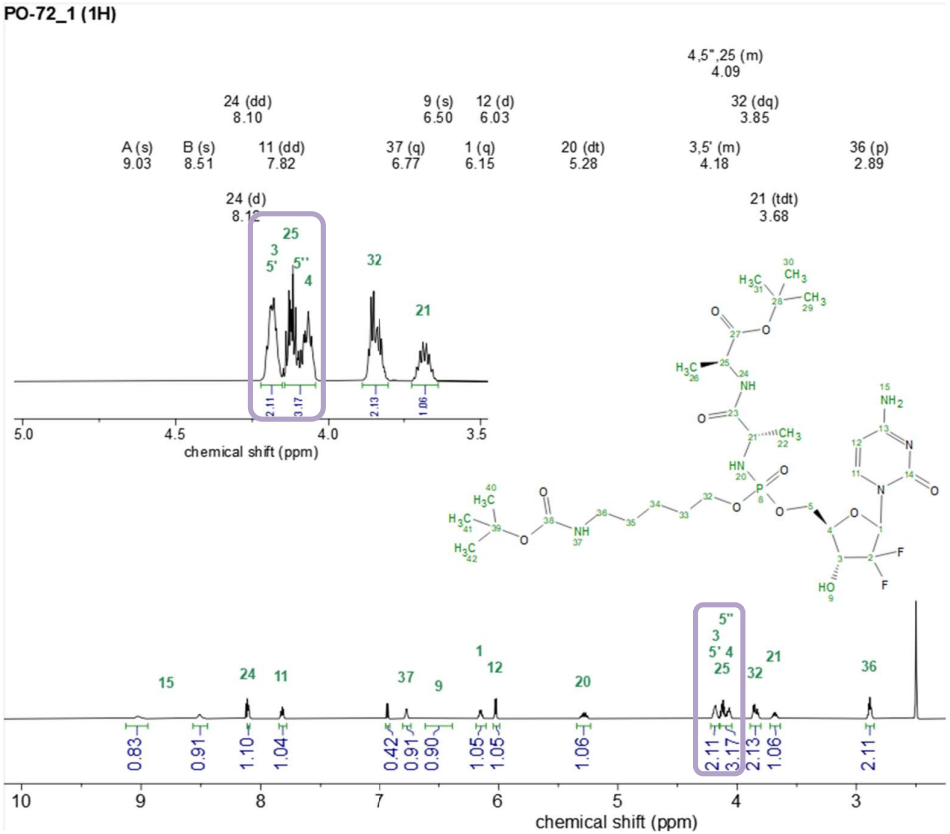
^{13}C-NMR (151 MHz, DMSO-d_6) δ(ppm) = 173.0 (2×C23), 171.7 (C27), 164.0 (4-NPh), 161.3 (C13), 158.6 (TFA), 158.4 (TFA), 155.6 (C38), 149.5 (C14), 142.9 (C11), 139.6 (4-NPh), 126.2 (4-NPh), 122.4 (t, $^{2}J_{CF}$ = 259 Hz, C2), 119.4 (TFA), 117.5, 115.8 (4-NPh), 115.5, 95.0 (2×C12), 113.5 (TFA), 83.8 (C1), 80.5 (2×C28), 79.1 (C4), 77.4 (C39), 69.3 (t, $^{3}J_{CF}$ = 22.3 Hz, C3), 65.7 (2×C32), 63.8 (C5), 49.9 (2×C21), 48.3 (2×C25), 39.5 (C36), 29.5 (C33), 29.1 (C35), 28.3 (C40-42), 27.6 (C29-31), 22.4 (C34), 21.0 (C22), 17.0 (2×C26).

3'-O-X6t:

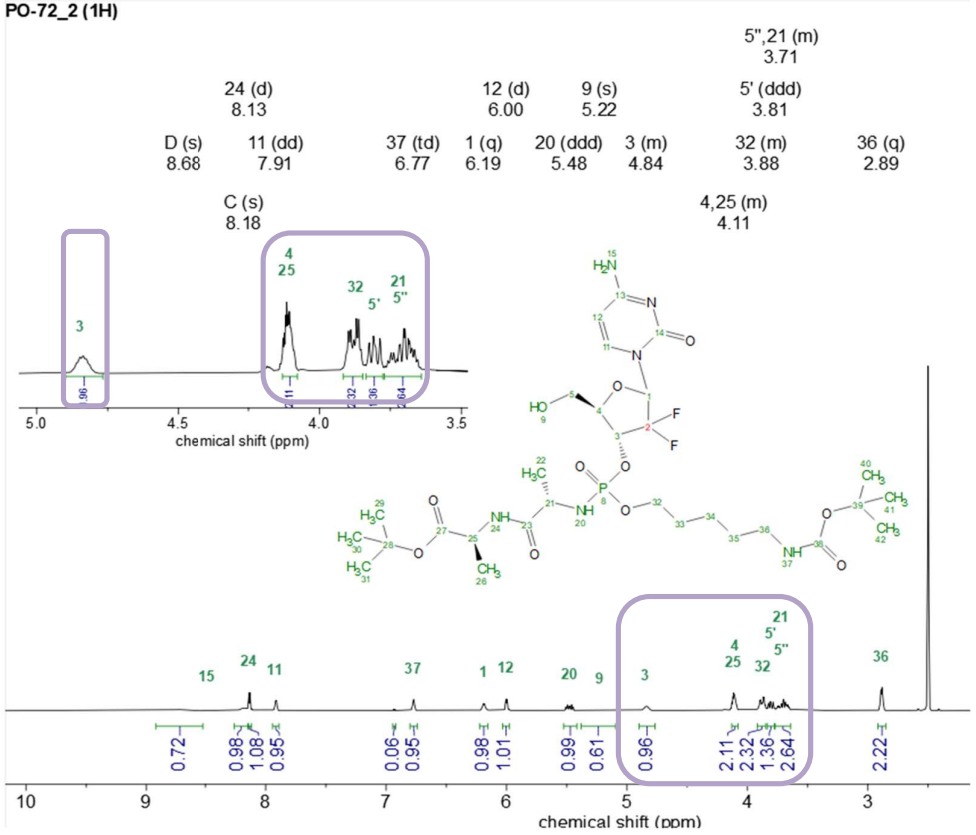
^{1}H-NMR (800 MHz, DMSO-d_6) δ(ppm) = 8.68 (s, 1H, H15: -NH_2-), 8.18 (s, 1H, H15': -NH_2-), 8.13 (d, J = 7.1 Hz, 1H, H24: -NH-), 7.91 (dd, J = 7.8, 2.9 Hz, 1H, H11: -$C_{Ar}H$), 6.77 (td, J = 5.8, 2.7 Hz, 1H, H37: -NH-), 6.19 (q, J = 8.6 Hz, 1H , H1), 6.00 (d, J = 7.7 Hz, 1H , H12: -$C_{Ar}H$), 5.48 (ddd, J = 25.4, 12.5, 10.1 Hz, 1H, H20: -NH-), 5.22 (br-s, 1H, H9: -OH), 4.87 – 4.78 (m, 1H, H3: -CH-), 4.14 – 4.07 (m, 2H, H4: -CH-; H25: -CH-), 3.93 – 3.85 (m, 2H, H32: -CH_2-), 3.81 (ddd, J = 18.3, 12.7, 2.3 Hz, 1H, H5': -CH_2-), 3.77 – 3.63 (m, 2H, H5'': -CH-; H21: -CH-), 2.89 (q, J = 6.6 Hz, 2H, H36: -CH_2-), 1.59 – 1.52 (m, 2H, H33: -CH_2-), 1.38 (d, J = 2.2 Hz, 9H, H29-31: -CH_3), 1.36 (d, J = 3.4 Hz, 9H, H40-42: -CH_3), 1.37 – 1.33 (m, 2H, H35: -CH_2-), 1.31 – 1.26 (m, 2H, H34: -CH_2-), 1.24 (dd, J = 9.3, 7.3 Hz, 3H, H26: -CH_3), 1.22 (dd, J = 16.6, 6.9 Hz, 3H, H22: -CH_3).

^{13}C-NMR (151 MHz, DMSO-d_6) δ(ppm) = 172.7 (2×C23), 171.6 (C27), 162.1 (C13), 155.6 (C38), 150.4 (C14), 142.7 (C11), 94.9 (C12), 80.4 (C28), 80.2 (C4), 77.4 (C39), 71.1 (C3), 66.2 (2×C32), 58.9 (C5), 49.9 (C21), 48.3 (C25), 39.7 (C36), 29.3 (C33), 29.0 (C35), 28.3 (C29-31), 27.6 (C40-42), 22.3 (C34), 20.8 (2×C22), 17.0 (C26).

PO-72_1 (1H)

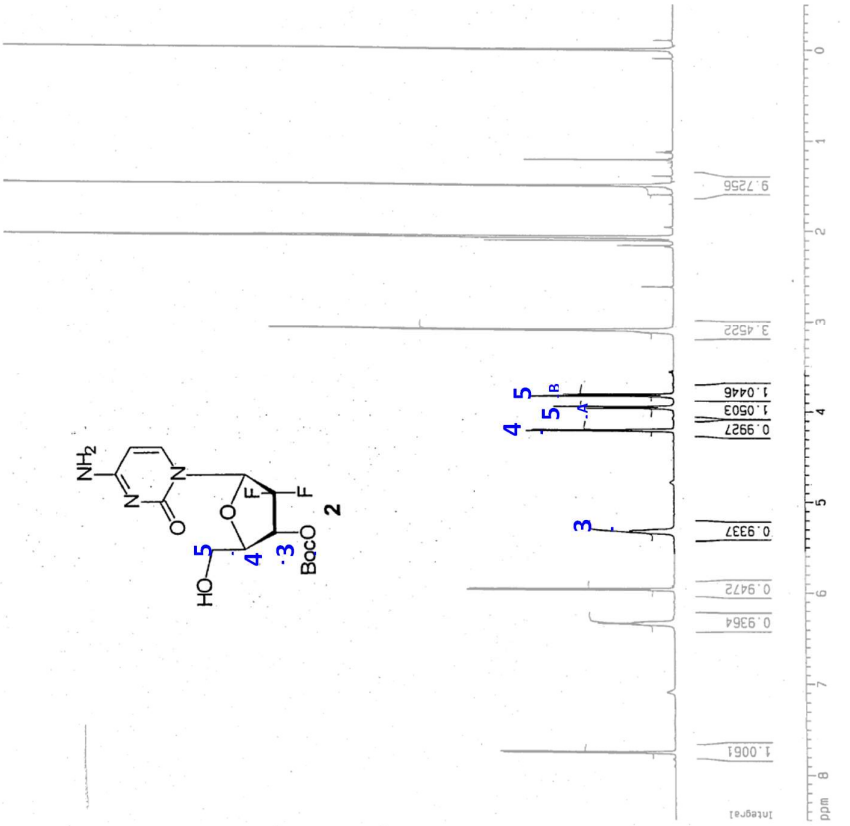


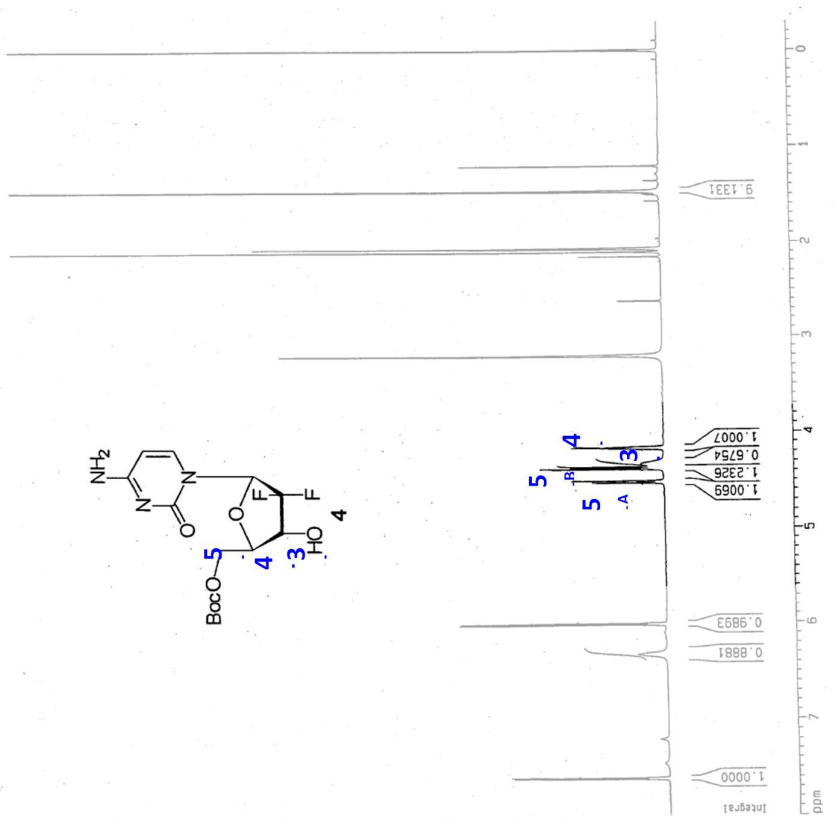
PO-72_2 (1H)



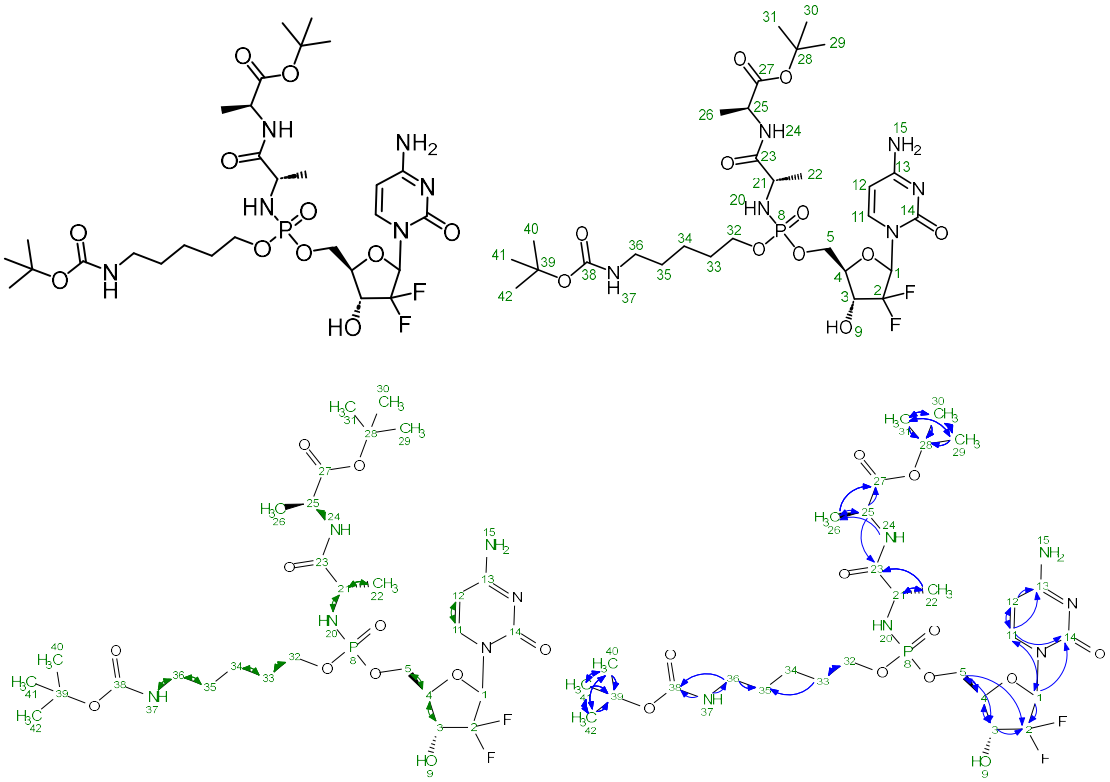
Reference: *J. Org. Chem.* **1999,** *64,* 8319-8322

Zhi-wei Guo and James M. Gallo*: Selective Protection of 2',2'-Difluorodeoxycytidine (Gemcitabine)





PO-72_1: BocNH-pentyl-Alco5(AlaAlaOtBu)-Gemcitabine *(5'-modified Gemcitabine suggested / free secondary amine)*



General:

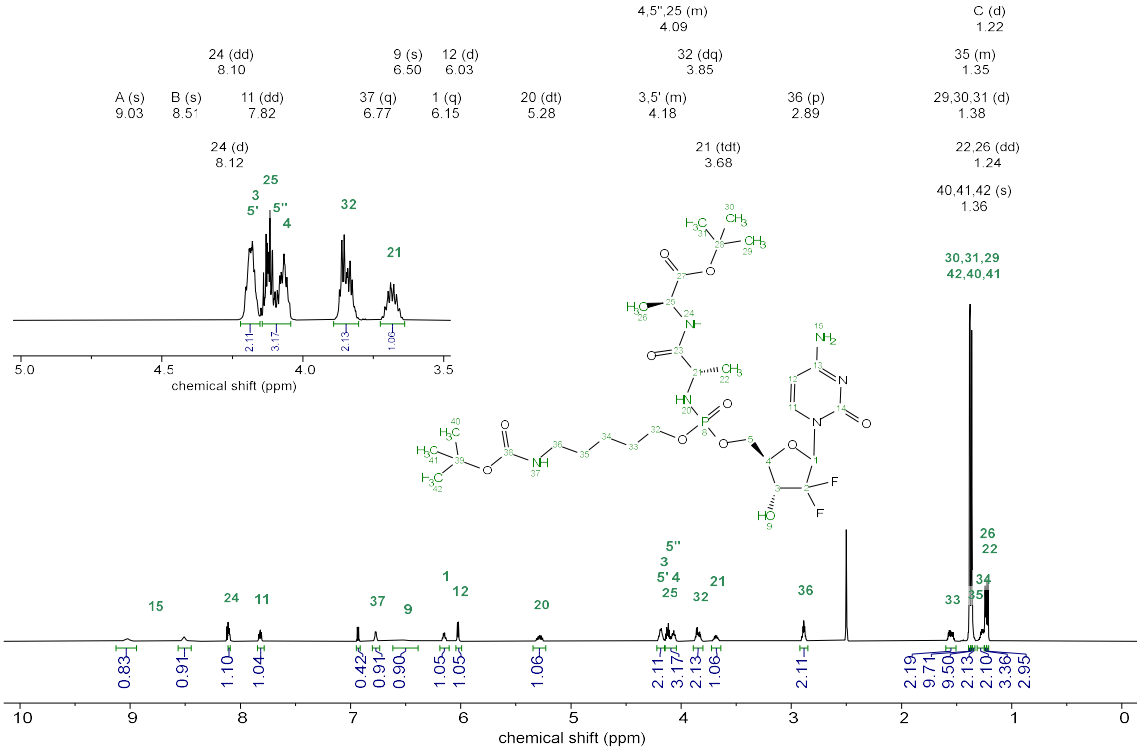
- The ^{31}P-spectrum suggests two diastereomers with a ratio of 53:47.
- The two ^{19}F lead to triplet at C43: 122.4 (t, $^{2}J_{CF}$ = 259 Hz); and a triplet at C44: 69.3 (t, $^{3}J_{CF}$ = 22.3 Hz).

- The sample contains residues of ca. 20 mol% 4-nitrophenol: **^{1}H-NMR** δ(ppm) = 11.09 (s, 1H), 8.12 (d, J = 9.2 Hz, 2H), 6.93 (d, J = 9.1 Hz, 2H). ^{13}C-NMR δ(ppm) = 164.0, 139.6, 126.2, 115.8.
- The sample also contains traces of TFA visible as d (J = 33 Hz) and q (J = 295 Hz).

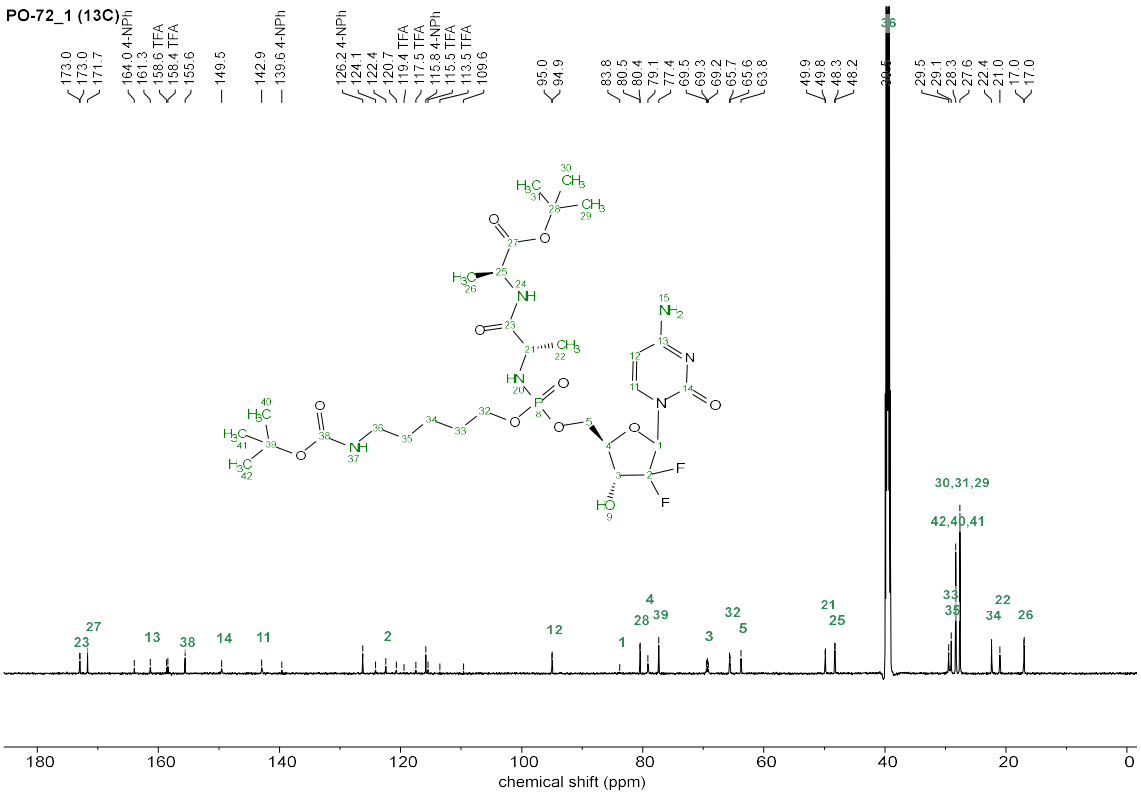
Assignment in the Gemcitabine part:

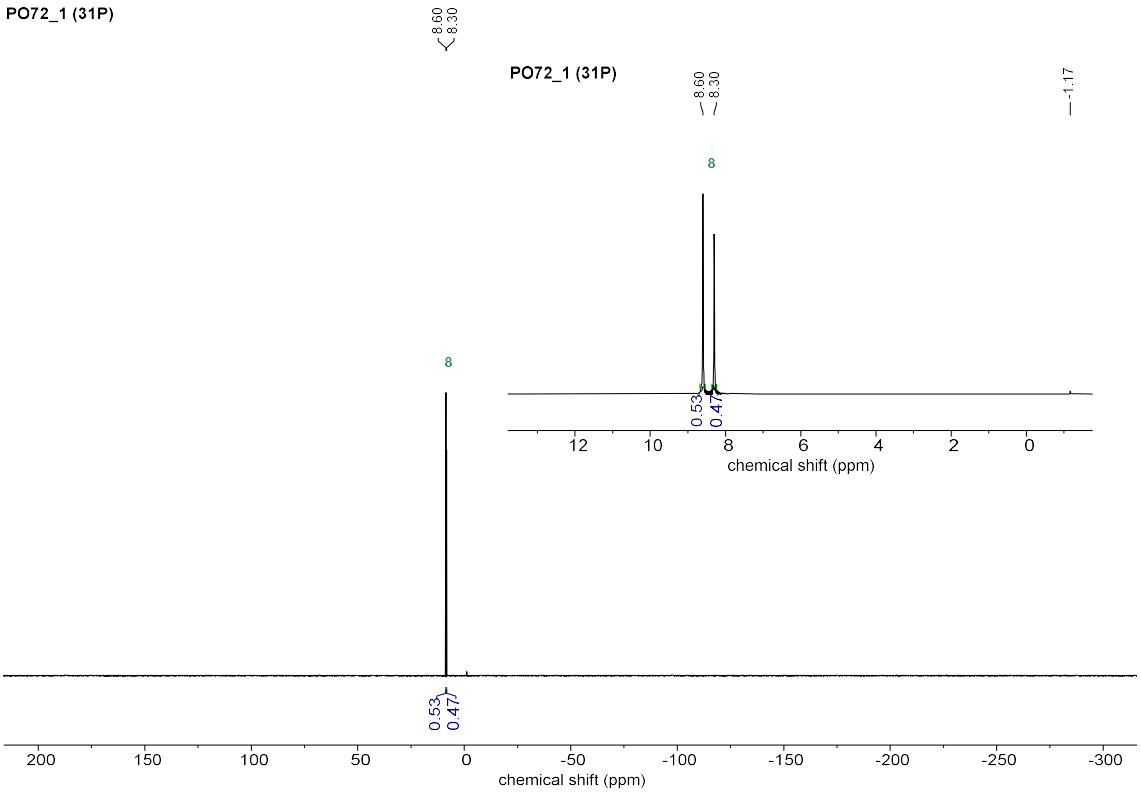
- H3-H5 overlap with each other and with H25, making COSY correlations complicated.
- The free -OH H28 (presumably at 6.50 ppm) does not have COSY correlations.

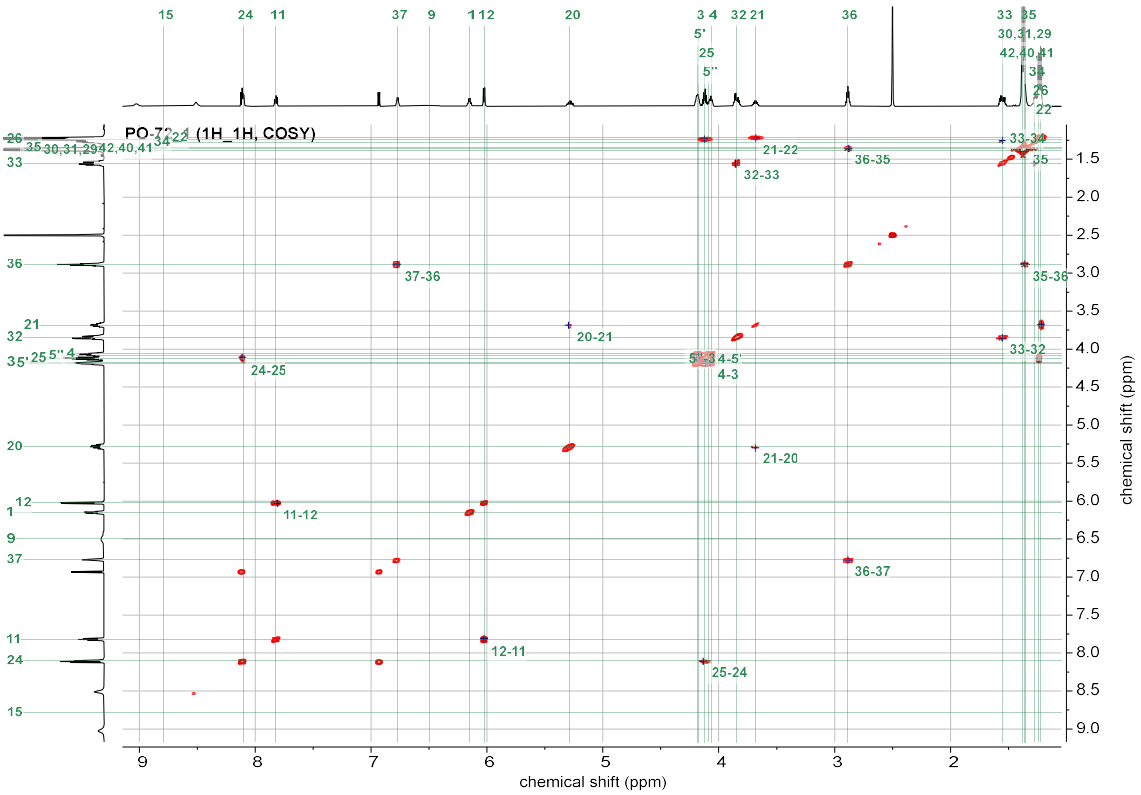
PO-72_1 (1H)

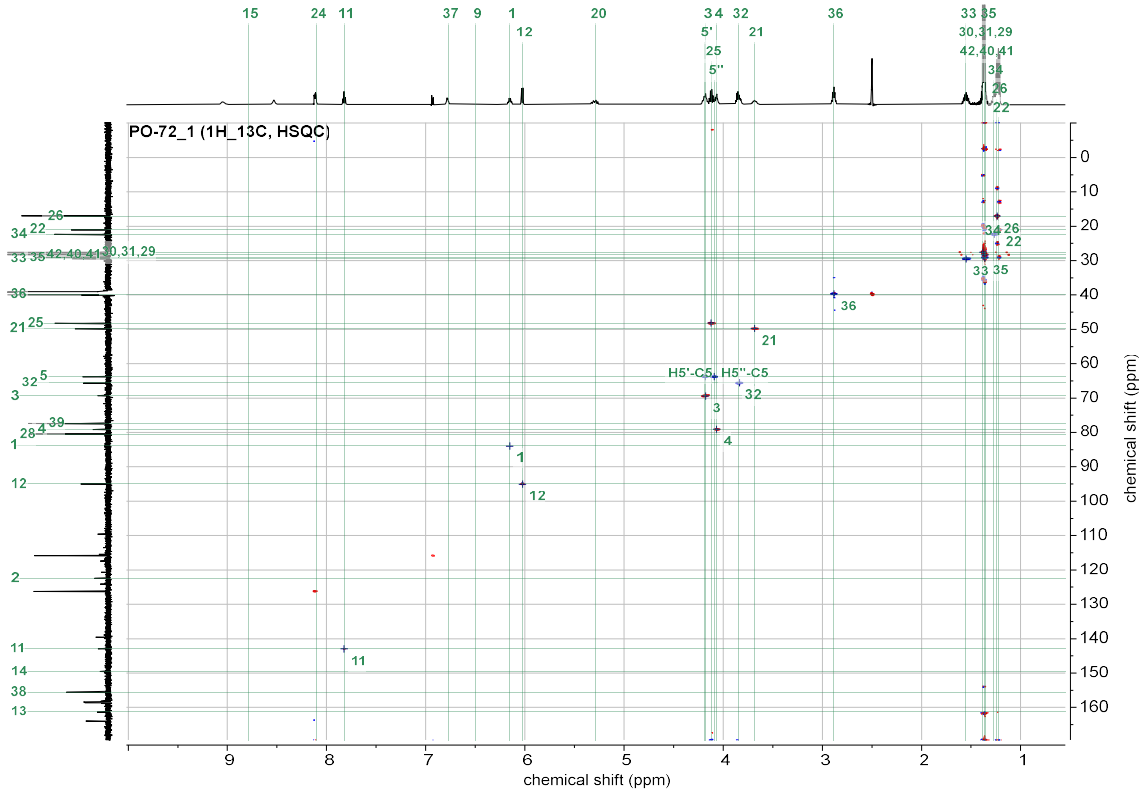


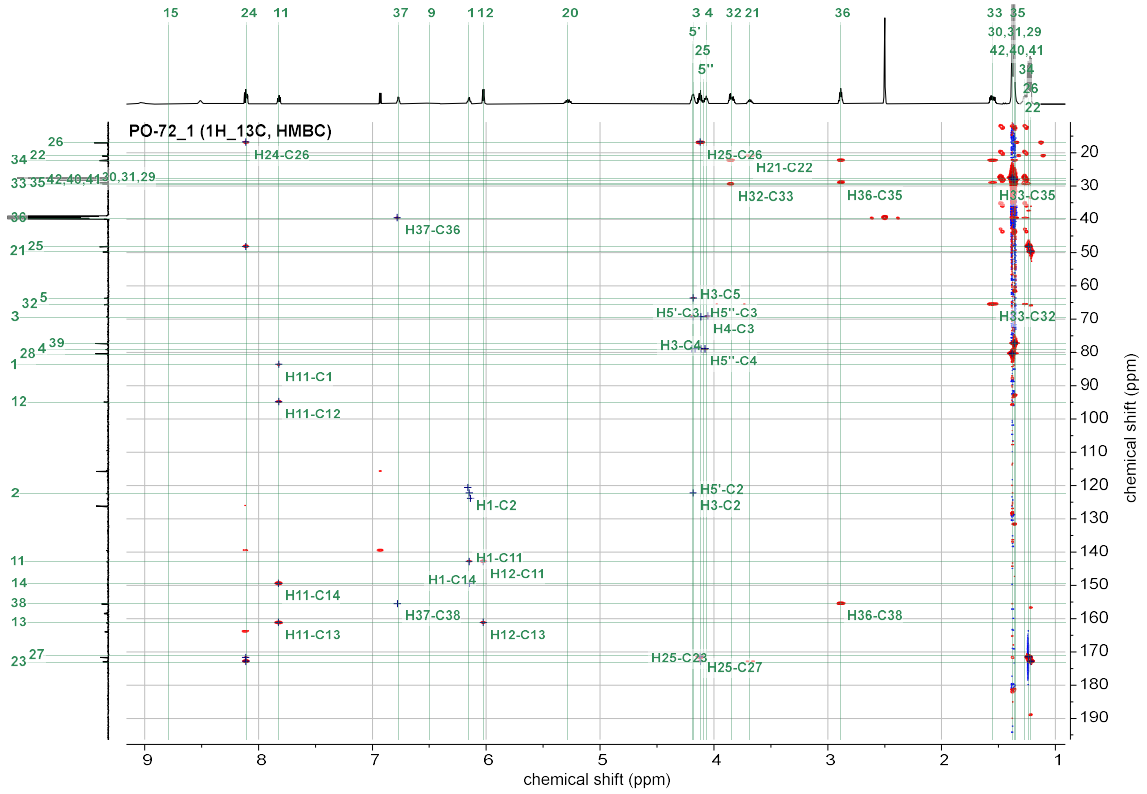
PO-72_1 (13C)



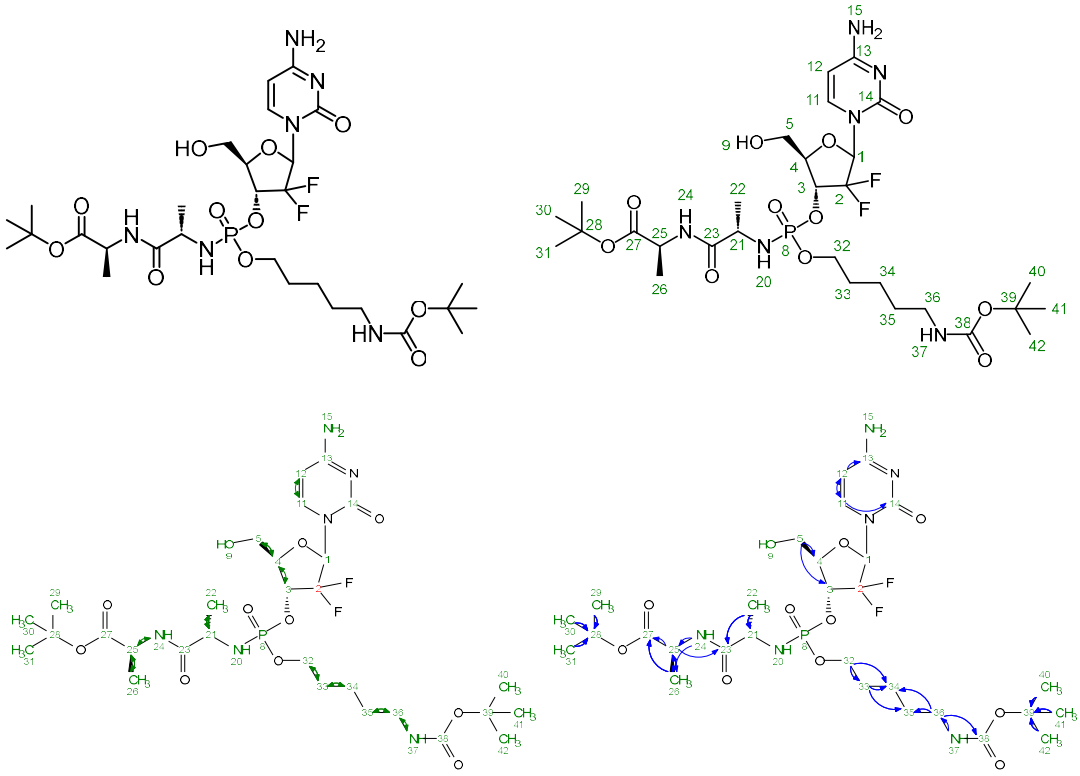

PO72_1 (31P)
8.60
8.30
8
0.53
0.47
200
150
100
50
0
-50
-100
-150
-200
-250
-300
chemical shift (ppm)
PO72_1 (31P)
8.60
8.30
-1.17
8
0.53
0.47
12
10
8
6
4
2
0
chemical shift (ppm)


15
24
11
37
9
1
12
20
3
4
5'
25
5''
32
21
36
33
35
30,31,29
42,40,41
34
26
22
PO-72-1 (1H_1H, COSY)
21-22
32-33
36-35
33-34
35
37-36
35-36
20-21
24-25
4-3
33-32
21-20
11-12
36-37
12-11
25-24
chemical shift (ppm)
chemical shift (ppm)





PO-72_2: BocNH-pentyl-Alco5(AlaAlaOtBu)-Gemcitabine *(3'-modified Gemcitabine suggested / free primary amine)*



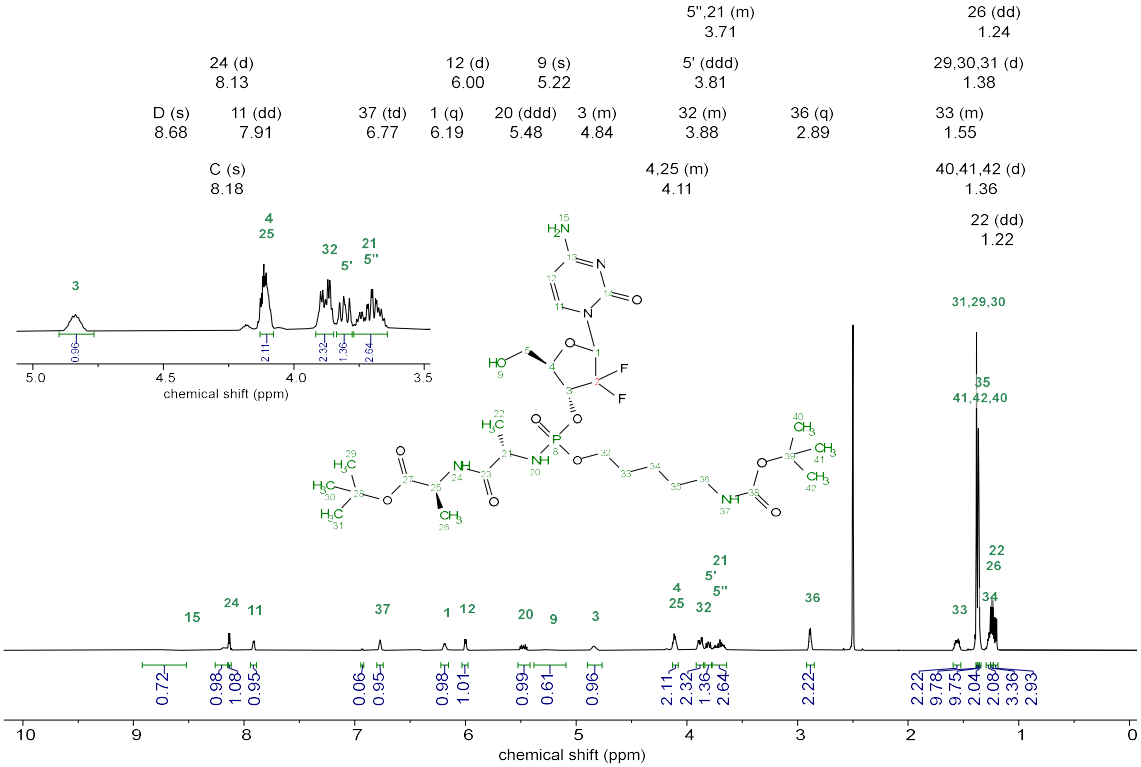
General:

- The ^{31}P-spectrum suggests two diastereomers with a ratio of 54:46. The sample contains ca. 3 mol% of its regioisomer PO-72_1
- The ^{19}F-coupled C2 is not visible in the ^{13}C or HMBC spectrum.
- The sample contains ca. 3 mol% 4-nitrophenol: **^{1}H-NMR** 11.09 (s, 1H), 8.12 (d, J = 9.2 Hz, 2H), 6.93 (d, J = 9.1 Hz, 2H).

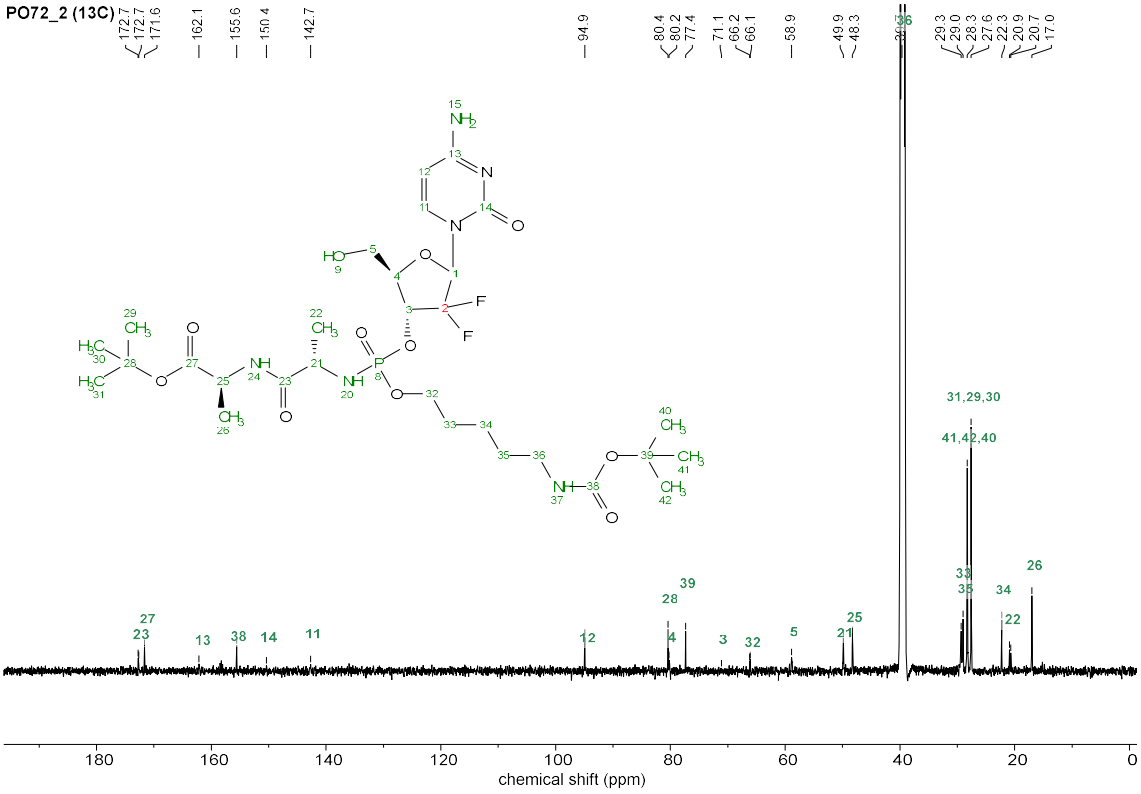
Assignment in the Gemcitabine part:

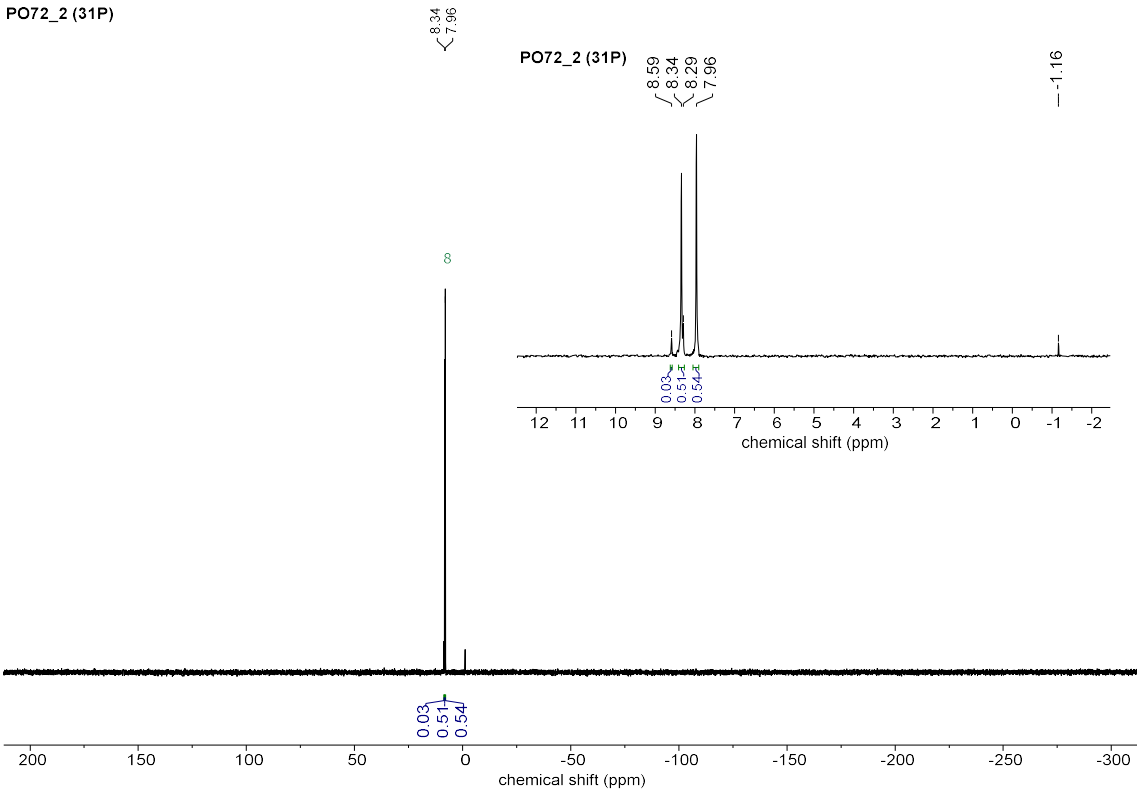
- H2 is significantly low field-shifted. The COSY with H4 is visible. Referencing with literature suggests 3'-modified Gemcitabine.
- The free -OH H9 (presumably at 5.22 ppm) does not have COSY correlations.

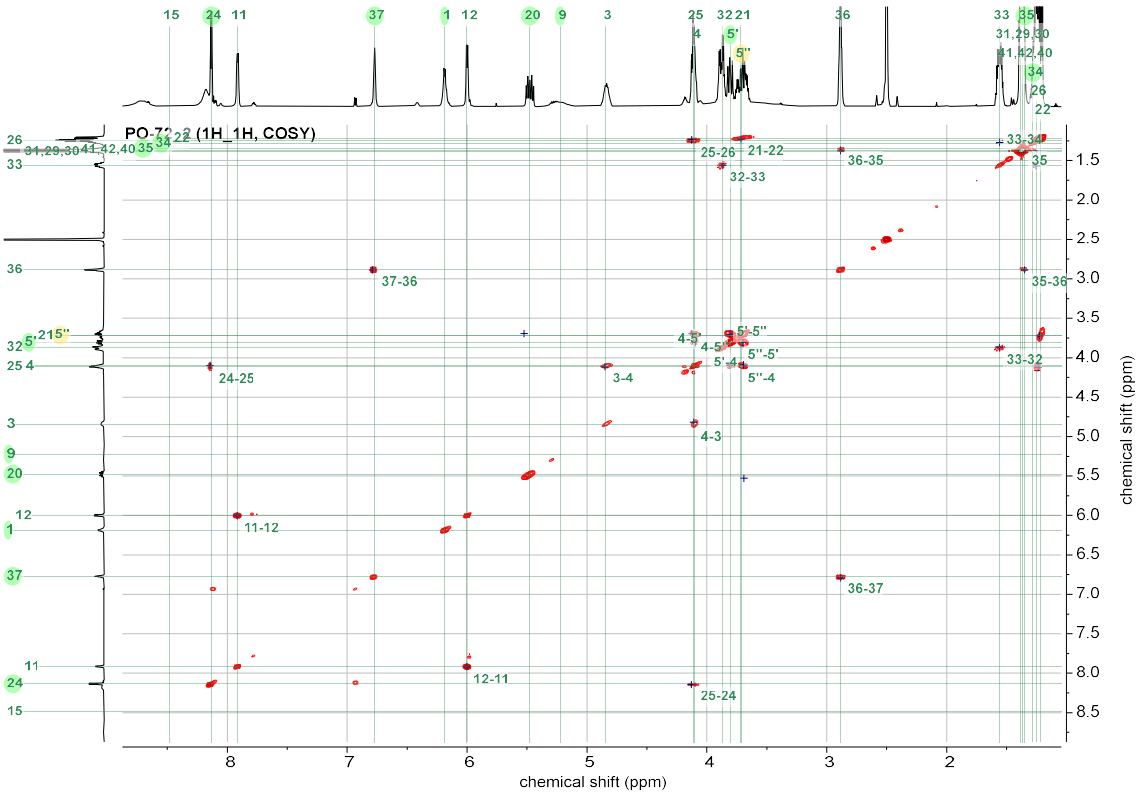
PO-72_2 (1H)

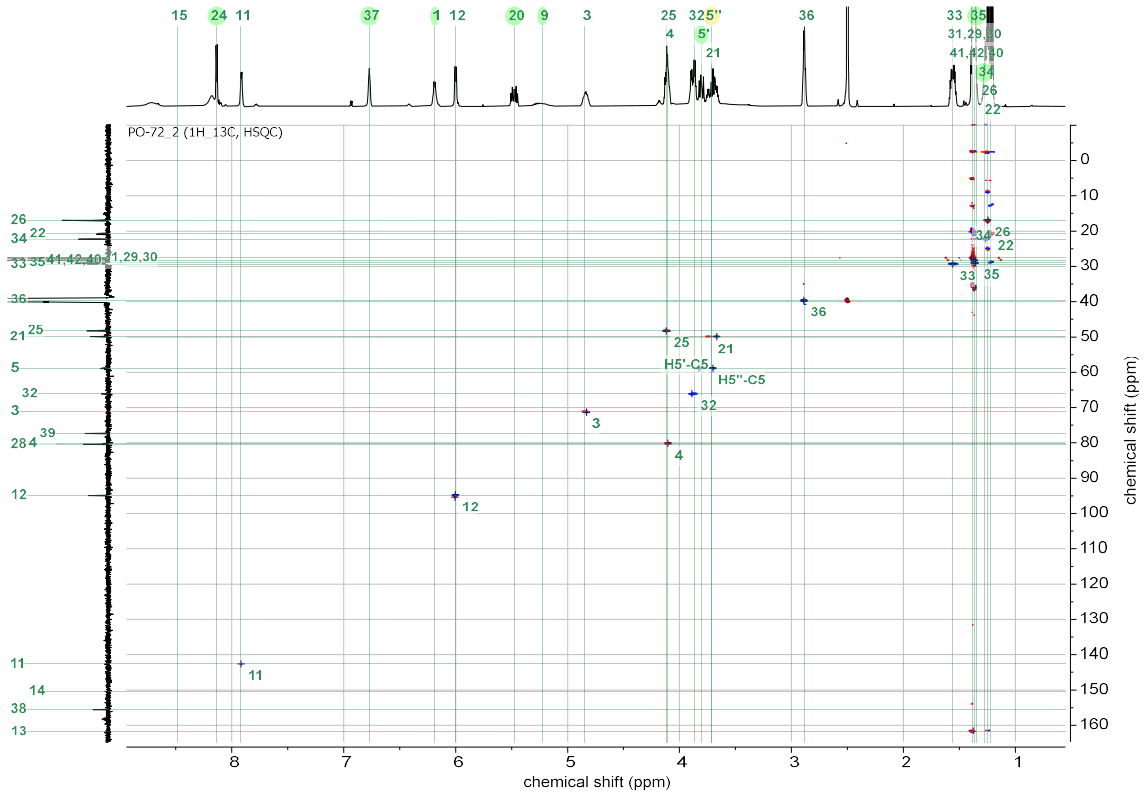


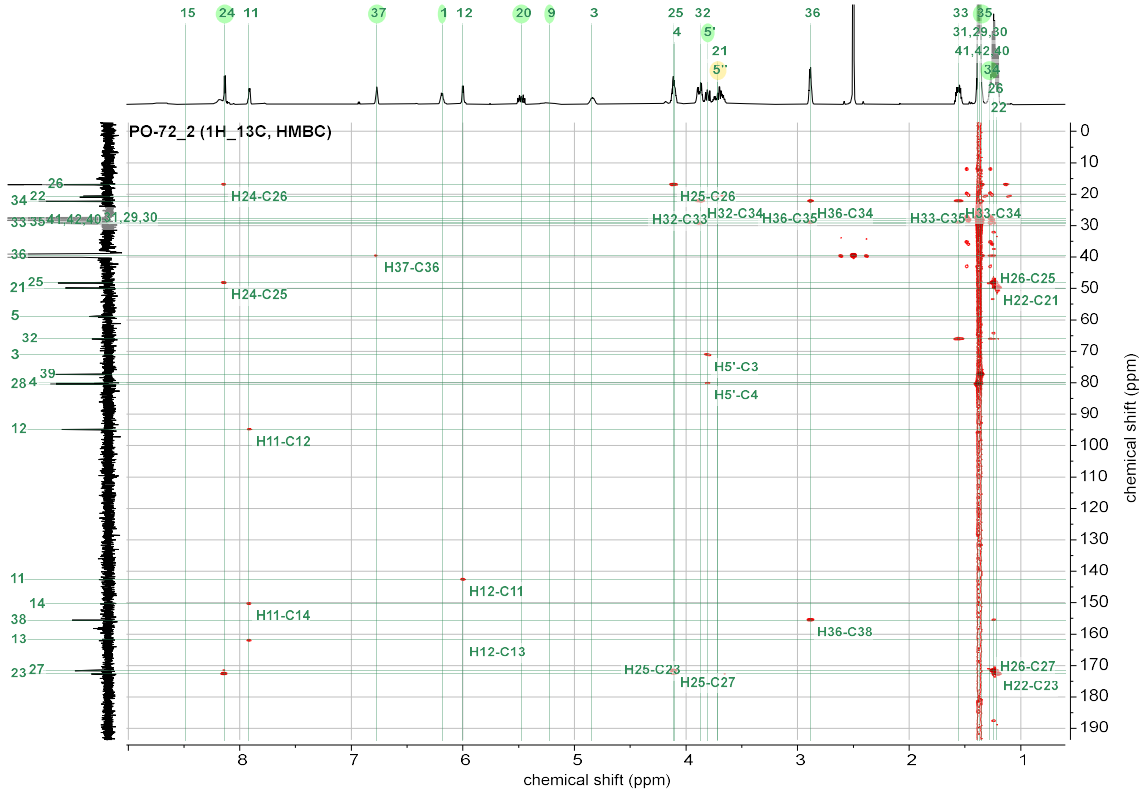
PO72_2 (13C)



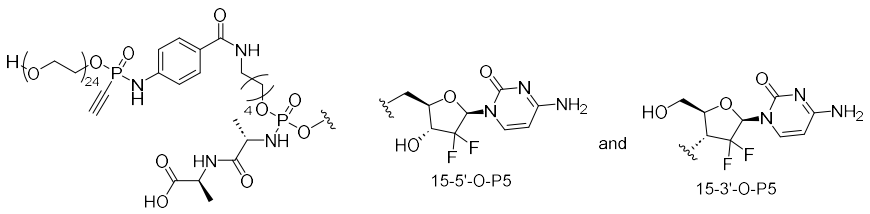

PO72_2 (31P)
8.34
7.96
8
0.03
0.51
0.54
200 150 100 50 0 -50 -100 -150 -200 -250 -300
chemical shift (ppm)
PO72_2 (31P)
8.59
8.34
8.29
7.96
-1.16
0.03
0.51
0.54
12 11 10 9 8 7 6 5 4 3 2 1 0 -1 -2
chemical shift (ppm)


15 24 11 37 1 12 20 9 3 25 4 32 5' 5'' 21 36 33 35 31,29,30 41,42,40 34 26 22
PO72_2 (1H_1H, COSY)
25-26
21-22
32-33
36-35
33-34
35
37-36
35-36
24-25
3-4
4-3
5'-5''
4-5'
5''-5'
5'-4
5''-4
33-32
11-12
36-37
12-11
25-24
8 7 6 5 4 3 2
chemical shift (ppm)
1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0 7.5 8.0 8.5
chemical shift (ppm)


PO-72_2 (1H_13C, HSQC)
chemical shift (ppm)
chemical shift (ppm)


PO-72_2 (1H_13C, HMBC)
H24-C26
H25-C26
H32-C33
H32-C34
H36-C35
H36-C34
H33-C35
H33-C34
H37-C36
H24-C25
H26-C25
H22-C21
H5'-C3
H5'-C4
H11-C12
H12-C11
H11-C14
H36-C38
H12-C13
H25-C23
H25-C27
H26-C27
H22-C23
chemical shift (ppm)
chemical shift (ppm)

5.24.2. 15-P5



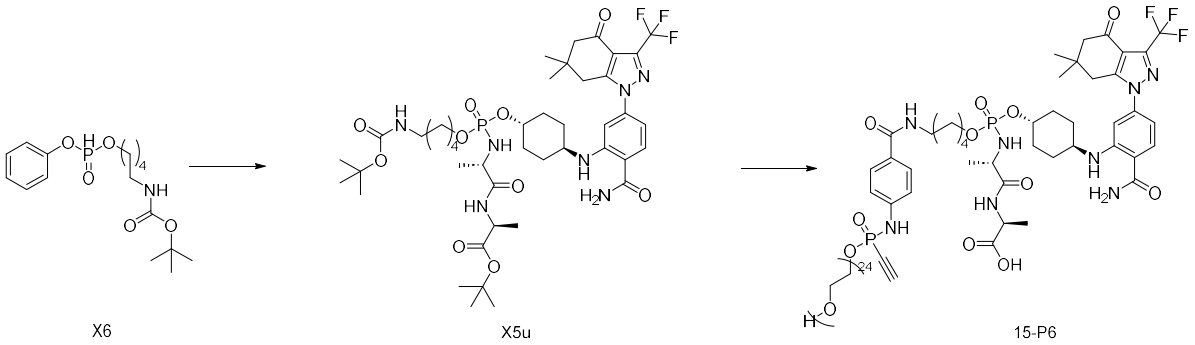
The title compound was synthesized in accordance with general procedure 6 either from a regioisomeric mixture of **X5t** or from regioisomeric pure 5'-O-**X5t** and 3'-O-**X5t**.

A regioisomeric mixture of the title compound was synthesized in accordance with general procedure 6 from 8 mg **X5t** (0.011 mmol, 1 eq) and 14.2 mg P5(PEG24)-COOH (0.011 mmol, 1eq.). The product was obtained as yellowish oil after preparative HPLC and lyophilization. 4 mg of the title compound were isolated (2.2 µmol, 20.7%). MS for $C_{77}H_{137}F_2N_7O_{36}P_2^{2+}$ $[M+2H]^{2+}$ calcd.: 917.9268, found: 917.9184.

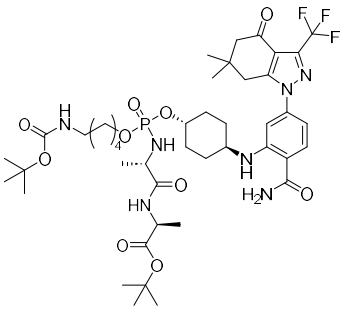
15-5'-O-P5 was synthesized in accordance with general procedure 6 from 16 mg 5'-O-**X5t** (0.022 mmol, 1 eq). and 28.4 mg P5(PEG24)-COOH (0.022 mmol, 1eq.). The product was obtained as yellowish oil after preparative HPLC and lyophilization (15.8 mg, 0.009 mmol, 39%) over two steps. HR-MS for $C_{77}H_{137}F_2N_7O_{36}P_2^{2+}$ $[M+2H]^{2+}$ calcd.: 917.9268, found: 917.8909.

15-3'-O-P5 was synthesized in accordance with general procedure 6 from 7.4 mg 3'-O-**X5t** (0.010 mmol, 1 eq). and 13 mg P5(PEG24)-COOH (0.010 mmol, 1eq.). The product was obtained as yellowish oil after preparative HPLC and lyophilization (10.6 mg, 0.006 mmol, 58%) over two steps. HR-MS for $C_{77}H_{137}F_2N_7O_{36}P_2^{2+}$ $[M+2H]^{2+}$ calcd.: 917.9268, found: 917.8978.

5.25. Linker 15-P6: Scheme

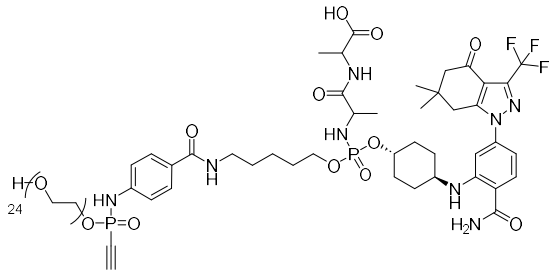


5.25.1. X5u



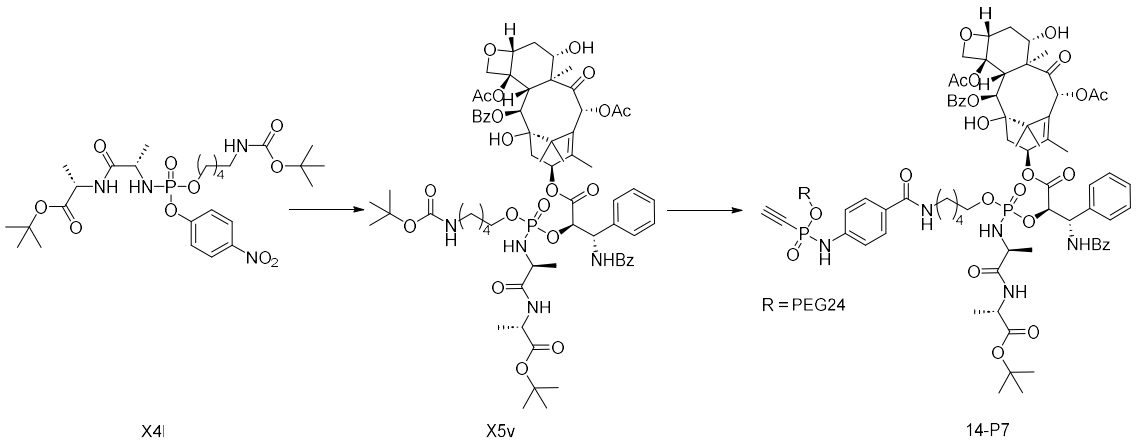
The title compound was synthesized in accordance with the general procedure 4 from 11.5 mg SNX-2112 (0.025 mmol) and 85 mg **X6** (0.248 mmol, 10 eq.), 107 mg L-alanine-L-alanine *tert*.-butyl ester (0.495 mmol, 20 eq.), 150 mg carbon tetrachloride (0.991 mmol, 40 eq.) and 50 mg triethylamine (0.462, 20 eq.). The product was obtained as white solid after preparative HPLC and lyophilization (6.1 mg, 0.007 mmol, 26.5%). HR-MS for $C_{43}H_{66}F_3N_7O_{10}P^+$ $[M+H]^+$ calcd.: 928.4555, found: 928.46185.

5.25.2. 15-P6

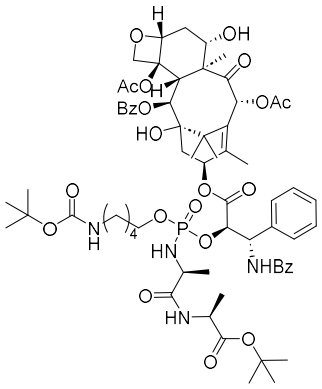


The title compound was synthesized in accordance with general procedure 6 from 6.1 mg **X5u** (0.007 mmol, 1 eq). and 9 mg P5(PEG24)-COOH (0.007 mmol, 1eq.). The product was obtained as yellowish oil after preparative HPLC and lyophilization (2.7 mg, 1.4 µmol, 20%). MS for $C_{91}H_{153}F_3N_8O_{35}P_2^{2+}$ $[M+2H]^{2+}$ calcd.: 1018.5, found: 1018.4.

5.26. Linker 15-P7: Scheme

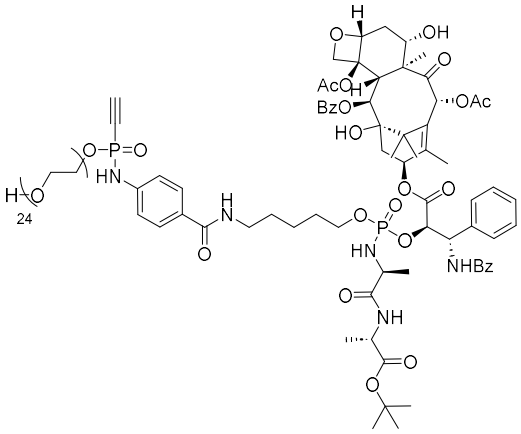


5.26.1. X5v



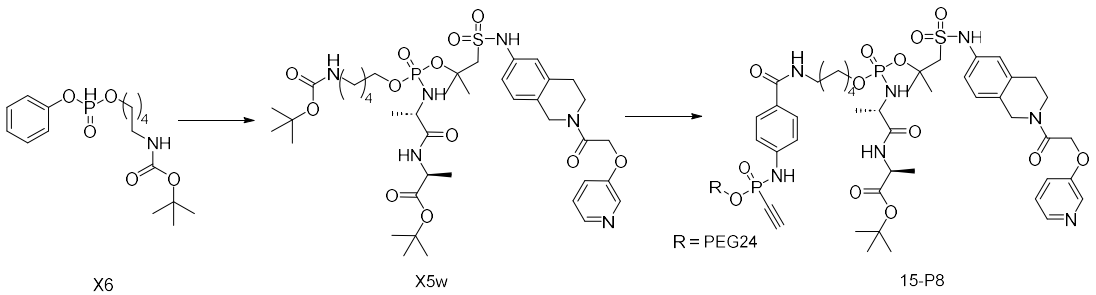
The title compound was synthesized in accordance with the general procedure 3 from 5 mg Paclitaxel (0.006 mmol) and 10.6 mg **X4l** (0.018 mmol, 3.0 eq.). The product was obtained as white solid after preparative HPLC and lyophilization (4.6 mg, 0.0035 mmol, 58%). MS for $C_{67}H_{91}N_4O_{21}P^{2+}$ $[M+H]^+$ calcd.: 1317.57, found: 1317.70.

5.26.2. 14-P7

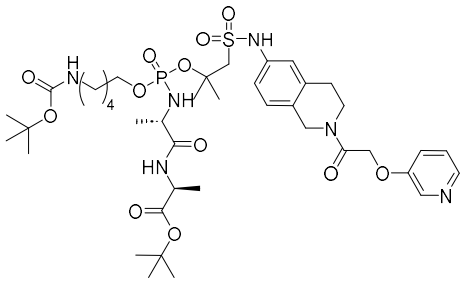


The title compound was synthesized in accordance with general procedure 5 from 3.5 mg **X5v** (0.003 mmol, 1 eq) and 4.4 mg P5(PEG24)-COOH (0.004 mmol, 1.2 eq.). The product was obtained as colorless oil after preparative HPLC and lyophilization (3.6 mg, 0.002 mmol, 55%) over two steps. HR-MS for $C_{119}H_{186}N_5O_{46}P_2^{3+}$ $[M+3H]^+$ calcd.: 828.0621, found: 828.0642.

5.27. Linker 15-P8: Scheme

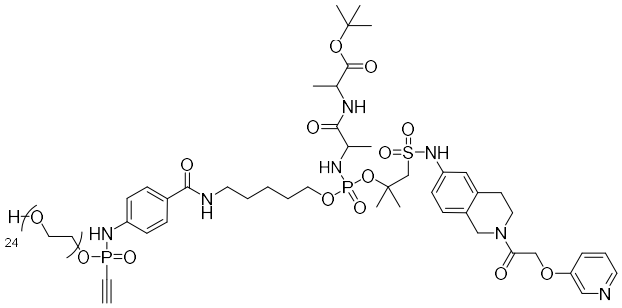


5.27.1. X5w



The title compound was synthesized in accordance with the general procedure 4 from 9 mg LSN3154567 (0.021 mmol) and 74 mg **X6** (0.210 mmol, 10 eq.), 62 mg L-alanine-L-alanine *tert*.-butyl ester hydrochloride (0.231 mmol, 11 eq.), 38 mg carbon tetrachloride (0.231 mmol, 11 eq.) and 50 mg triethylamine (0.462, 22 eq.). The product was obtained as white solid after preparative HPLC and lyophilization (6.7 mg, 0.008 mmol, 36 %). MS for $C_{40}H_{64}N_6O_{12}PS^+$ $[M+H]^+$ calcd.: 883.40, found: 883.29.

5.28. 15-P8



The title compound was synthesized in accordance with general procedure 5 from 6.7 mg **X5w** (0.007 mmol, 1 eq). and 12.7 mg P5(PEG24)-COOH (0.010 mmol, 1.2 eq.). The product was obtained as yellow oil after preparative HPLC and lyophilization (6.7 mg, 0.0034 mmol, 45%). MS for $C_{92}H_{159}N_7O_{37}P_2S^{2+}$ $[M+2H/2]^+$ calcd.: 1023.99, found: 1023.85.

6. References

1 Ochtrop, P. *et al.* Compact hydrophilic electrophiles enable highly efficacious high DAR ADCs with excellent in vivo PK profile. *Chemical Science* (2023). https://doi.org:10.1039/D2SC05678J

2 Kasper, M.-A. *et al.* Cysteine-Selective Phosphonamidate Electrophiles for Modular Protein Bioconjugations. *Angewandte Chemie International Edition* **58**, 11625-11630 (2019). https://doi.org:https://doi.org/10.1002/anie.201814715

3 Kasper, M.-A. *et al.* Ethynylphosphonamidates for the Rapid and Cysteine-Selective Generation of Efficacious Antibody–Drug Conjugates. *Angewandte Chemie International Edition* **58**, 11631-11636 (2019). https://doi.org:https://doi.org/10.1002/anie.201904193