

Supporting information

Reduction-Rebridging Strategy for the Preparation of APN-based Antibody-Drug Conjugates

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Materials

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

Instrumentation

¹H and ¹³C NMR spectra were recorded at 23°C on Bruker 400 spectrometer. Recorded shifts are reported in parts per million (δ) and calibrated using residual non-deuterated solvent. Data are represented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, br = broad), coupling constant (*J*, Hz) and integration. High resolution mass spectra (HRMS) were obtained using Agilent Q-TOF (time of flight) 6520 coupled to Agilent 1200 HPLC with Diode Array Detector. Low resolution mass spectra using Agilent MSD 1200 SL (ESI/APCI) coupled to Agilent 1200 HPLC with Diode Array Detector. IR spectra were recorded on a Nicolet 380 FT-IR spectrometer from Thermo Electron Corporation as a DCM solution or solid on a diamond plate. The semi-preparative HPLC system consisted of two Shimadzu LC-8A pumps, an SPD-10A VP detector (Shimadzu), an SCL-10A VP controller (Shimadzu), an SIL-10A autosampler, a 2 mL sample loop and a SunFire C18 column (150 mm × 19 mm i.d., 5 μm, Waters).

Synthesis

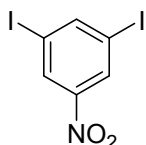
General procedure A for the preparation of compounds 4, 11, 12, 13

Sonogashira coupling. Propargyl alcohol (3 eq) was added to a solution of a diiodophenyl derivative (1 eq.) in degassed DMF (3.5 mL per 1 mmol of diiodoaniline), then TEA (4 eq.) was added the mixture. The solution was degassed and CuI (4 mol%) and PdCl₂(PPh₃)₂ (2 mol%) were added to the reaction mixture and stirred under argon for 2h. The reaction was followed by TLC. After complete conversion the reaction mixture was diluted with EtOAc, washed twice with sat. NH₄Cl, once with NaHCO₃ and once with brine, dried over MgSO₄ and concentrated. The residue was purified by flash chromatography (Cyclohexane/EtOAc: 100/0 to 0/100 gradient).

General procedure B for the preparation of meta-diAPN, ortho-diAPN, para-diAPN and compound 5

To the solution of the starting diol (1 eq.) in THF (4.5 mL per 1 mmol of the diol) was added the fine powder of MgSO_4 (30 eq.), NH_3 solution in IPA (8 eq., 2 M) and activated MnO_2 (30 eq.). The reaction mixture was stirred at r.t. for 3h and followed by HPLC. After completion the mixture was filtered through Celite and washed thoroughly with THF. The filtrate was concentrated and the residue was purified by flash chromatography (Cyclohexane/EtOAc: 100/0 to 0/100 gradient).

Compound 2

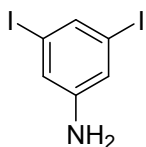


To concentrated H_2SO_4 (10.9 mL) cooled at 0°C was added in small portions 2,6-diiodo-4-nitroaniline (1 eq., 7.82 g, 20.1 mmol). After complete dissolution of the aniline, NaNO_2 (2.17 eq., 3.01 g, 43.6 mmol) was added at 0°C and stirred for 2 h at this temperature. Then, the viscous solution was poured into ice (200 g) and any solid material was filtered off. The yellow filtrate was carefully poured into a refluxed solution of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.1 eq., 0.501 g, 2.01 mmol) in EtOH (400 mL) and stirred for 2 h to reduce the diazonium salt. After cooling to room temperature, solid 3,5-diiodonitrobenzene was separated. The product was filtered off and washed with water until neutral. The product was recrystallized from EtOH to give 1,3-diiodo-5-nitrobenzene (3.7 g, 9.87 mmol, 49 %) as fine brown needles.

$^1\text{H NMR}$ (400 MHz, CDCl_3 , δ ppm): 8.53 (d, $J = 1.4$ Hz, 2H), 8.38 (t, $J = 1.4$ Hz, 1H)

$^{13}\text{C NMR}$ (100 MHz, CDCl_3 , δ ppm): 151.0, 148.4, 131.7, 94.1

Compound 3

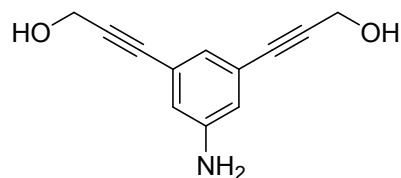


To a suspension of 1,3-diiodo-5-nitrobenzene **2** (1 eq., 8.88 g, 23.7 mmol) in anhydrous EtOH (93.1 mL) under argon was added $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ (4.02 eq., 26.8 g, 95.2 mmol). This mixture was brought to boil and a solution of NaBH_4 (0.5 eq., 0.448 g, 11.9 mmol) in EtOH (49.7 mL) was added dropwise. The reaction mixture was stirred at reflux for 20 minutes. After the reaction was cooled down to 0°C , water (74.5 mL) was added and the mixture was neutralized with NaOH (1.5 M, 30.5 mL). The aniline derivative was extracted with diethyl ether, dried over MgSO_4 and evaporated under reduced pressure to afford 3,5-diiodoaniline (4.8 g, 13.9 mmol, 59 %) as a colourless solid.

$^1\text{H NMR}$ (400 MHz, Methanol- d_4 , δ ppm): 4.87 (s, 2H), 7.03 (d, $J = 1.5$ Hz, 2H), 7.23 (t, $J = 1.5$ Hz, 1H)

$^{13}\text{C NMR}$ (100 MHz, Methanol- d_4 , δ ppm): 154.9, 136.6, 126.3, 98.2

Compound 4

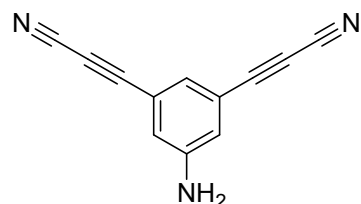


Compound 4 was prepared from 3,5-diiodoaniline in 93% yield following General procedure A.

¹H NMR (400 MHz, Methanol-d₄, δ ppm): 6.75 (t, *J* = 1.2 Hz, 1H), 6.71 (d, *J* = 1.2 Hz, 2H), 4.36 (s, 4H)

¹³C NMR (100 MHz, Methanol-d₄, δ ppm): 149.5, 125.2, 124.9, 119.0, 88.4, 85.4, 51.3

Compound 5



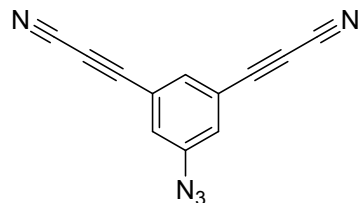
Compound 5 was prepared from 4 in 11% yield following General procedure B.

¹H NMR (400 MHz, CDCl₃, δ ppm): 7.20 (t, *J* = 1.3 Hz, 1H), 6.98 (d, *J* = 1.3 Hz, 2H), 4.02 (br. s., 2H)

¹³C NMR (100 MHz, Methanol-d₄, δ ppm): 147.1, 127.5, 121.6, 119.5, 105.0, 81.0, 63.6

MS (ESI) *m/z*: 192.1 [M+H]⁺

Compound 6



To a round bottom flask charged with 3-[3-amino-5-(2-cyanoethyl-1-yn-1-yl)phenyl]prop-2-ynenitrile **5** (1 eq., 58 mg, 0.303 mmol) was added a solution of NaNO₂ (1.1 eq., 23 mg, 0.334 mmol) in water (1 mL). The obtained reaction mixture was chilled to 0°C, then concentrated HCl (11 M, 4 mL) was added with vigorous stirring of the reaction mixture in an ice-water bath. The reaction mixture was stirred for additional 10 min. The obtained slurry solution was filtered, then a freshly prepared solution of NaN₃ (2 eq., 39.4 mg, 0.607 mmol) in water (0.5 mL) was added dropwise to the reaction mixture *via* additional funnel while maintaining the internal temperature of the reaction mixture below 5°C. Upon

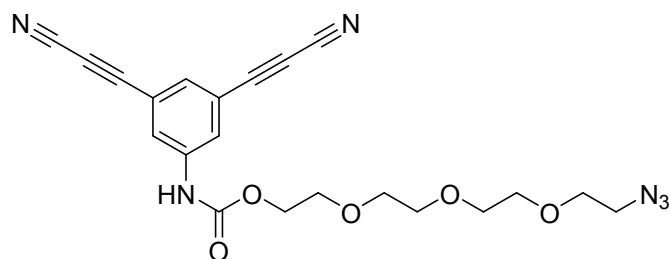
complete addition of the sodium azide solution, the reaction mixture was stirred for an additional 5 mins at 0 °C, followed by stirring at r.t. for another 5 min. The product was filtered, washed with water until neutral pH to give 3-[3-azido-5-(2-cyanoeth-1-yn-1-yl)phenyl]prop-2-ynenitrile (63 mg, 0.29 mmol, 96 %) as a pale yellow solid.

Syntheses of aryl azides were conducted open to the atmosphere in a well-ventilated hood away from direct light and behind a blast shield. All organic and inorganic azides should be handled and transferred either by glass pipette or plastic spoon and contact with metal should be avoided.

¹H NMR (400 MHz, CDCl₃, δ ppm): 7.60 (t, *J* = 1.3 Hz, 1H), 7.38 (d, *J* = 1.3 Hz, 2H)

¹³C NMR (100 MHz, CDCl₃, δ ppm): 142.4, 133.7, 126.2, 120.5, 104.6, 79.2, 65.1

Compound 7



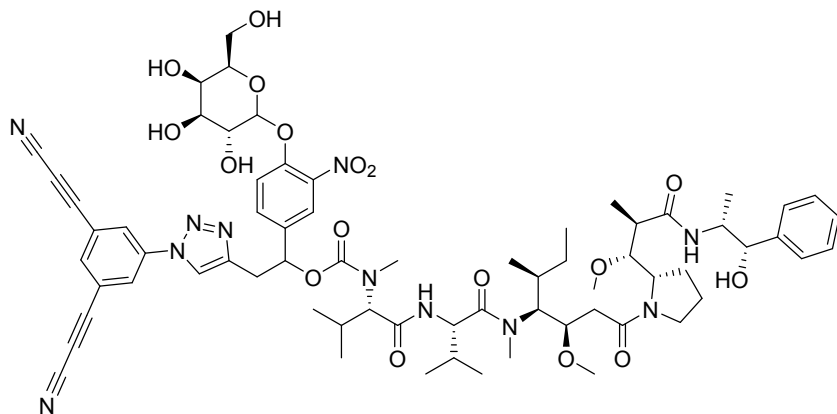
To a solution of 3-[3-amino-5-(2-cyanoeth-1-yn-1-yl)phenyl]prop-2-ynenitrile **5** (3 eq., 18.1 mg, 0.0947 mmol) in DCM (1 mL) was added a solution of triphosgene (1 eq., 9.36 mg, 0.0316 mmol) in DCM (0.5 mL). Then triethylamine (6 eq., 19.2 mg, 26.3 μL, 0.189 mmol) was added. The mixture was stirred for 5 min at r.t. and then 2-{2-[2-(2-azidoethoxy)ethoxy]ethoxy}ethan-1-ol (3 eq., 20.8 mg, 0.0947 mmol) and triethylamine (2 eq., 6.39 mg, 8.77 μL, 0.0631 mmol) were added as a solution in DCM (0.5 mL). The reaction mixture was stirred at r.t. for 2 hours. After full conversion was confirmed by HPLC the mixture was concentrated to 1 mL volume and purified by flash chromatography (cyclohexane/EtOAc 100/0 to 0/100 gradient) to give 2-{2-[2-(2-azidoethoxy)ethoxy]ethoxy}ethyl N-[3,5-bis(2-cyanoeth-1-yn-1-yl)phenyl]carbamate **7** (8 mg, 0.0183 mmol, 58 %) as a white solid.

¹H NMR (400 MHz, CDCl₃, δ ppm): 7.84 (s, 2 H), 7.59 (br. s., 1 H), 7.50 (s, 1 H), 4.42 - 4.28 (m, 2 H), 3.79 - 3.73 (m, 2 H), 3.73 - 3.63 (m, 10 H), 3.41 (t, *J* = 4.9 Hz, 2 H)

¹³C NMR (100 MHz, CDCl₃, δ ppm): 152.9, 139.6, 132.0, 125.3, 119.5, 104.8, 99.6, 80.2, 70.7, 70.6, 70.0, 69.1, 64.9, 64.3, 60.4, 50.7

MS (ESI) *m/z*: 437.3 [M+H]⁺

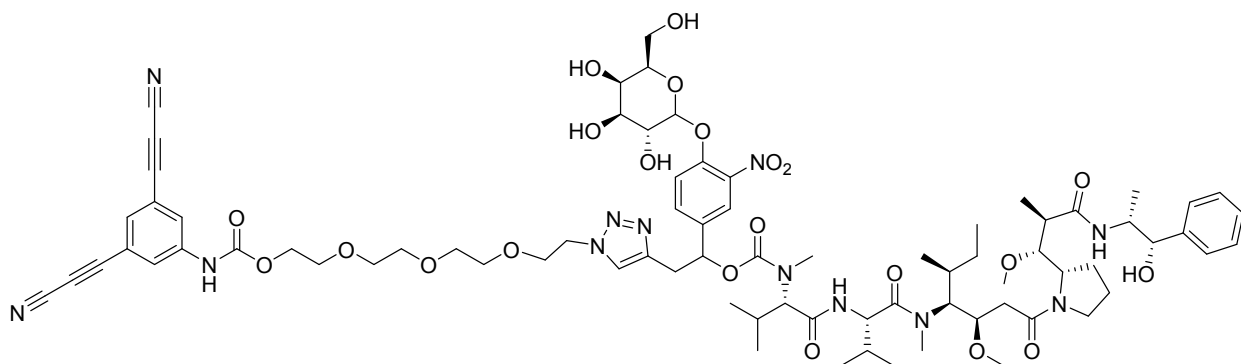
Compound 9



To a solution of **8** (11.6 mg, 0.01042 mmol) and **6** (1.3 eq., 3 mg, 0.01354 mmol) in DCM (1 mL) and MeOH (0.2mL) was added $\text{Cu}(\text{MeCN})_4\text{PF}_6$ (1.5 eq., 5.8 mg, 0.01563mmol). The reaction mixture was stirred at r.t. for 18 hours. Disappearance of starting material was observed by HPLC. Then a solution of ethylenediaminetetraacetic acid disodium salt dihydrate ($\text{EDTA}\cdot 2\text{Na}\cdot 2\text{H}_2\text{O}$) (38.7mg) in H_2O (1 mL) was added dropwise and the solution was stirred at room temperature during 2 hours for complete decomplexation of copper. The mixture was extracted with dichloromethane (3x 10 mL). The combined organic layers were dried over MgSO_4 and concentrated in vacuo. The crude product was purified by column chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 95/5 to 90/10) to give compound **9** (10.5 mg, 0.0078mmol, 75%, purity > 95%.) as a white solid.

HRESI-MS: m/z 1352.6174 (Calcd. for $\text{C}_{68}\text{H}_{87}\text{N}_{11}\text{NaO}_{17}$ 1352.6179 $[\text{M}+\text{Na}]^+$)

Compound 10

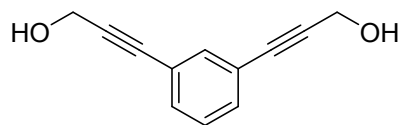


To a solution of **8** (10 mg, 0.0089 mmol) and **7** (1.8 eq., 7 mg, 0.016 mmol) in DCM (1 mL) and MeOH (0.2mL) was added $\text{Cu}(\text{MeCN})_4\text{PF}_6$ (1.5 eq., 5 mg, 0.0133mmol). The reaction mixture was stirred at r.t. for 6 hours. Disappearance of starting material was observed by HPLC. Then a solution of ethylenediaminetetraacetic acid disodium salt dihydrate ($\text{EDTA}\cdot 2\text{Na}\cdot 2\text{H}_2\text{O}$) (39mg) in H_2O (1 mL) was added dropwise and the solution was stirred at room temperature during 2 hours for complete decomplexation of copper. The mixture was extracted with dichloromethane (3x 10 mL). The combined organic layers were dried over MgSO_4 and concentrated in vacuo. The crude product was purified by

preparative-reverse phase HPLC to give compound **10** (2.46 mg, 0.0015mmol, 17 %, purity > 95%). as a white solid.

HRESI-MS: m/z 1571.7280 (Calcd. for $C_{77}H_{104}N_{12}NaO_{22}$ 1571.7286 $[M+Na]^+$)

Compound 11



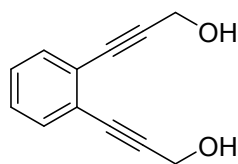
Compound **11** was prepared from 1,3-diiodobenzene in 55% yield following General procedure A.

1H NMR (400 MHz, Methanol- d_4 , δ ppm): 7.47 (s, 1H), 7.36 - 7.43 (m, 2H), 7.29 - 7.36 (m, 1H), 4.41 (s, 4H)

^{13}C NMR (100 MHz, Methanol- d_4 , δ ppm): 135.3, 132.5, 129.8, 124.8, 89.7, 84.5, 51.2

MS (ESI) m/z : 187.0 $[M+H]^+$

Compound 12



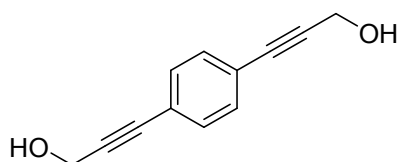
Compound **12** was prepared from 1,2-diiodobenzene in 40% yield following General procedure A.

1H NMR (400 MHz, Methanol- d_4 , δ ppm): 7.38 - 7.53 (m, 2H), 7.25 - 7.38 (m, 2H), 4.48 (s, 4H);

^{13}C NMR (100 MHz, Methanol- d_4 , δ ppm): 135.6, 131.9, 129.2, 95.6, 86.6, 53.9

MS (ESI) m/z : 187.1 $[M+H]^+$

Compound 13



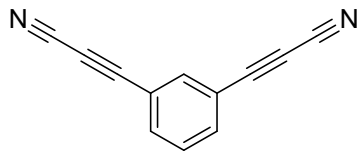
Compound **13** was prepared from 1,2-diiodobenzene in 35% yield following General procedure A.

1H NMR (400 MHz, METHANOL- d_4 , δ ppm): 7.39 (s, 4H), 4.41 (s, 4H);

^{13}C NMR (100 MHz, METHANOL- d_4 , δ ppm): 132.6, 124.3, 101.4, 84.9, 51.2

MS (ESI) m/z : 187.1 $[M+H]^+$.

***meta*-diAPN**



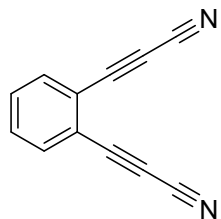
***meta*-diAPN** was prepared from **11** in 35% yield following General procedure B.

¹H NMR (400 MHz, Methanol-d₄, δ ppm): 8.10 (d, *J* = 1.50 Hz, 1H), 7.93 (dd, *J* = 1.50, 8.00 Hz, 1H), 7.63 (t, *J* = 8.00 Hz, 2H)

¹³C NMR (100 MHz, Methanol-d₄, δ ppm): 139.3, 137.8, 131.2, 120.0, 105.7, 81.7, 64.2

MS (ESI) *m/z*: 177.1 [M+H]⁺

***ortho*-diAPN**



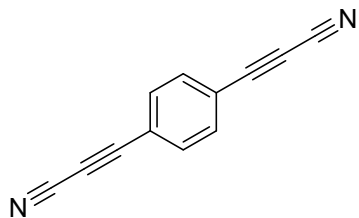
***ortho*-diAPN** was prepared from **12** in 42% yield following General procedure B.

¹H NMR (400 MHz, Methanol-d₄, δ ppm): 7.89 (dd, *J* = 3.30, 5.80 Hz, 2H), 7.73 (dd, *J* = 3.30, 5.80 Hz, 2H);

¹³C NMR (100 MHz, Methanol-d₄, δ ppm): 136.0, 133.5, 126.5, 105.5, 80.2, 67.2

MS (ESI) *m/z*: 177.0 [M+H]⁺

***para*-diAPN**



***para*-diAPN** was prepared from **13** in 19% yield following General procedure B.

¹H NMR (400 MHz, Methanol-d₄, δ ppm): 7.94 (s, 4H)

¹³C NMR (100 MHz, Methanol-d₄, δ ppm): 135.0, 121.6, 105.5, 82.0, 65.9

MS (ESI) *m/z*: 177.0 [M+H]⁺

General procedure for the reduction-bridging of the antibody

To the solution of trastuzumab (10 mg/mL, 100 μ L) in PBS (100 mM with 5 mM EDTA, pH 7.4) was added a solution of TCEP (5 eq., 3.44 μ L of 10 mM solution in H₂O) and the mixture was incubated at 37 °C for 2 hours. A solution of payload (5 eq., 1.72 μ L of 20 mM solution in DMSO) was added and the mixture was incubated at 25 °C for 12 hours. The conjugates were purified using size-exclusion spin columns (Bio-Spin® Columns with Bio-Gel® P-30, Bio-Rad).

SDS-PAGE

Reducing glycine-SDS-PAGE was performed on 4–15% Mini-PROTEAN® TGX™ Gel (Bio-Rad ref 4561084) following standard lab procedures. Samples (10 μ L at \sim 7 μ M in total mAb) were mixed with 10 μ L of loading buffer (1:19 β -mercaptoethanol : 2x Laemmli Sample Buffer, Bio-Rad ref 1610737) and heated at 95 °C for 5 minutes. The gel was run at constant voltage (200 V) for 40 min using TRIS 0.25 M - Glycine 1.92 M - SDS 1% as a running buffer. Gels were stained with Coomassie Blue (InstantBlue™, Expedeon).

Native mass spectrometry experiments

Native MS analyses were carried-out in positive mode, on an ESI-TOF (LCT, Micromass, Altrincham, UK) upgraded by MS Vision (MS Vision, Almere, Netherlands) coupled with an automated chip-based nanoESI infusion source (Triversa Nanomate, Advion Ithaca, USA). Instrumental parameters were tuned to ensure transmission of high molecular weight species and preservation of potential non-covalent interactions disruption. The acceleration voltage was set to 120 V and the pressure in the interface region of the mass spectrometer was 6.0 mbar for T-ADPN-Gal-MMAE and T-ADPN-PEG₄-Gal-MMAE analysis. Acquisitions were performed during 2 min with a scan time of 4 s after external calibration with cesium iodide 2 mg/mL. MS data interpretations were performed using Mass Lynx V4.1 (Waters, Manchester, UK).

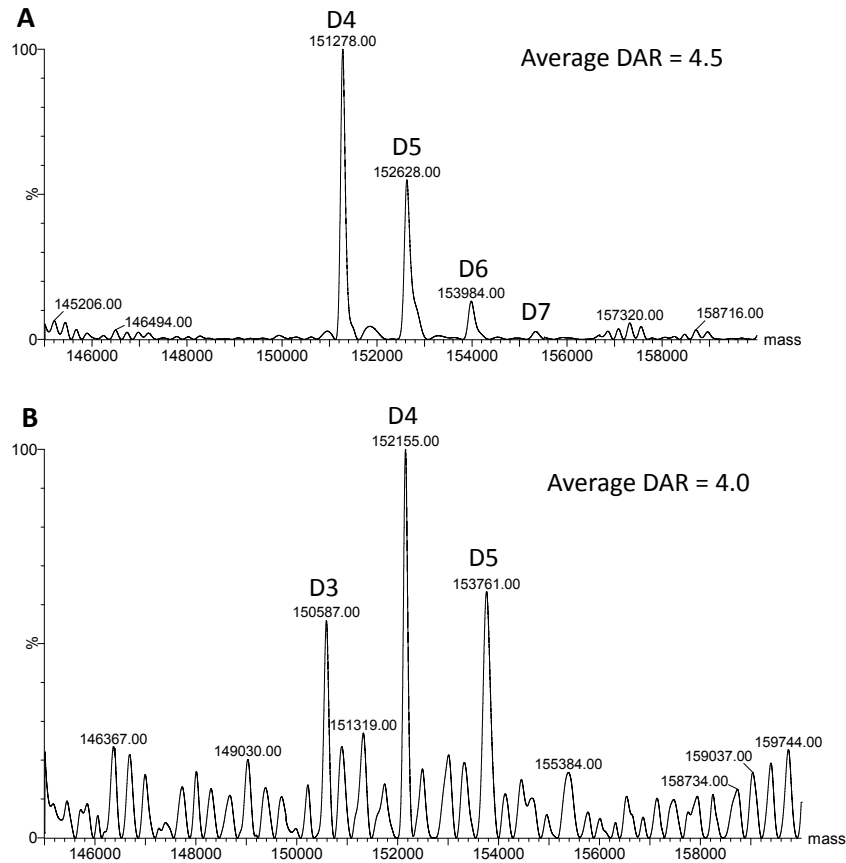


Figure S1. Deconvoluted native mass spectra of T-ADPN-Gal-MMAE (A) and T-ADPN-PEG₄-Gal-MMAE (B).

***In vitro* evaluation of the cytotoxicity**

HER2-positive SK-BR-3 and HER2-negative MDA-MB-231 cells were grown in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum, Penicillin (100 units/mL), and Streptomycin (100 µg/mL). Cell lines were maintained in a 5% CO₂ humidified atmosphere at 37 °C. The day before experiment, cells were seeded in 96-well plates at 3000 cells/well in 100 µL fresh complete medium. Cells were incubated with product T-DM1, T-diAPN-Gal-MMAE or T-diAPN-PEG₄-Gal-MMAE during 96 h. The CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) was used to determine the cell viability according to manufacturer's instructions. Absorbance was measured at 490 nm using a 96-well plate reader (Flx-Xenius XM, Safas, Monaco). The IC₅₀ values were determined in GraphPad Prism 5 software.

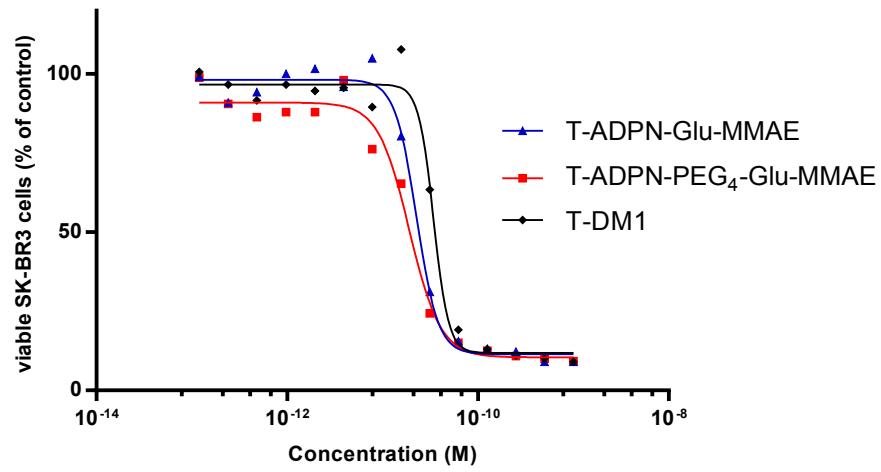


Figure S2. *In vitro* cytotoxic activity of ADCs on HER2-positive cancer cell line (SK-BR-3).

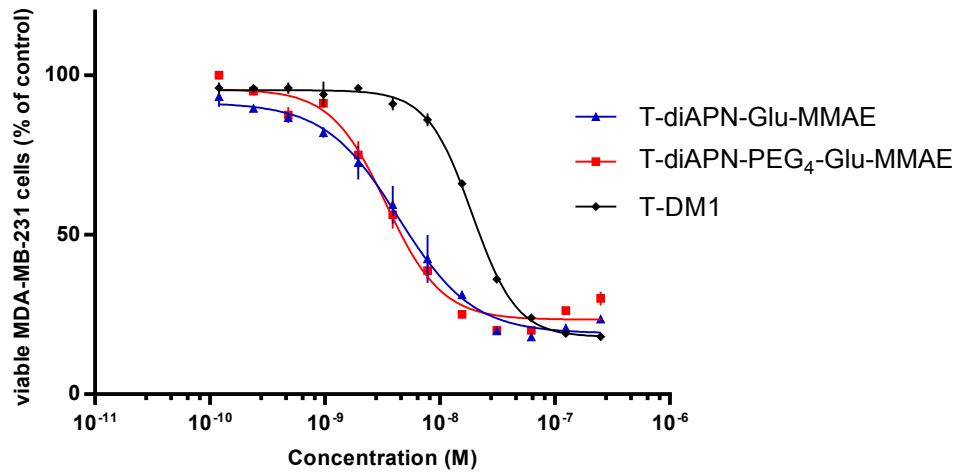


Figure S3. *In vitro* cytotoxic activity of ADCs on HER2-negative cancer cell line (MDA-MB-231).