Electronic Supplementary Information

Monodisperse Polysarcosine-based Highly-loaded Antibody-Drug Conjugates

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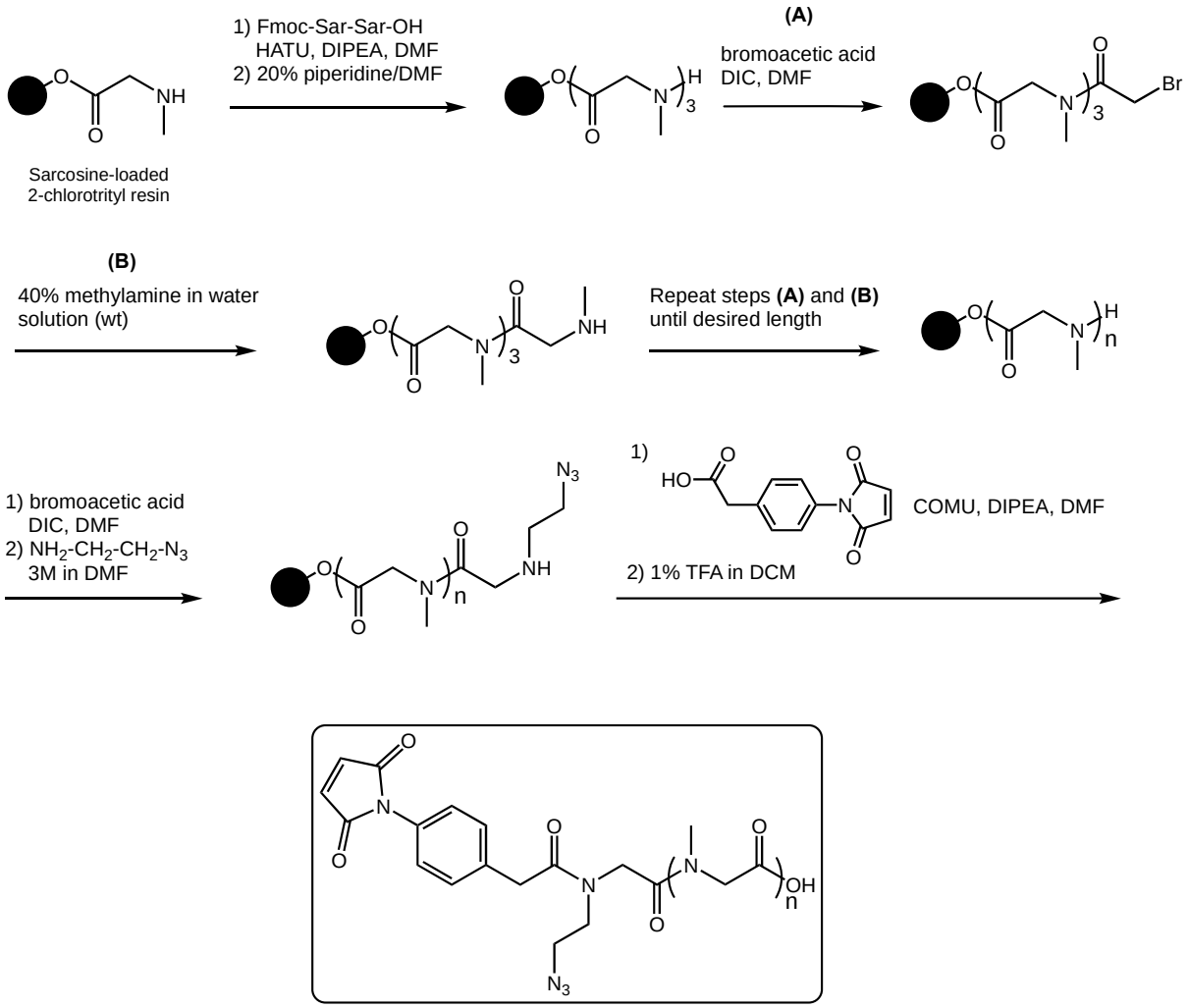
Table of supplementary figures

Table of experimental procedures

Abbreviations

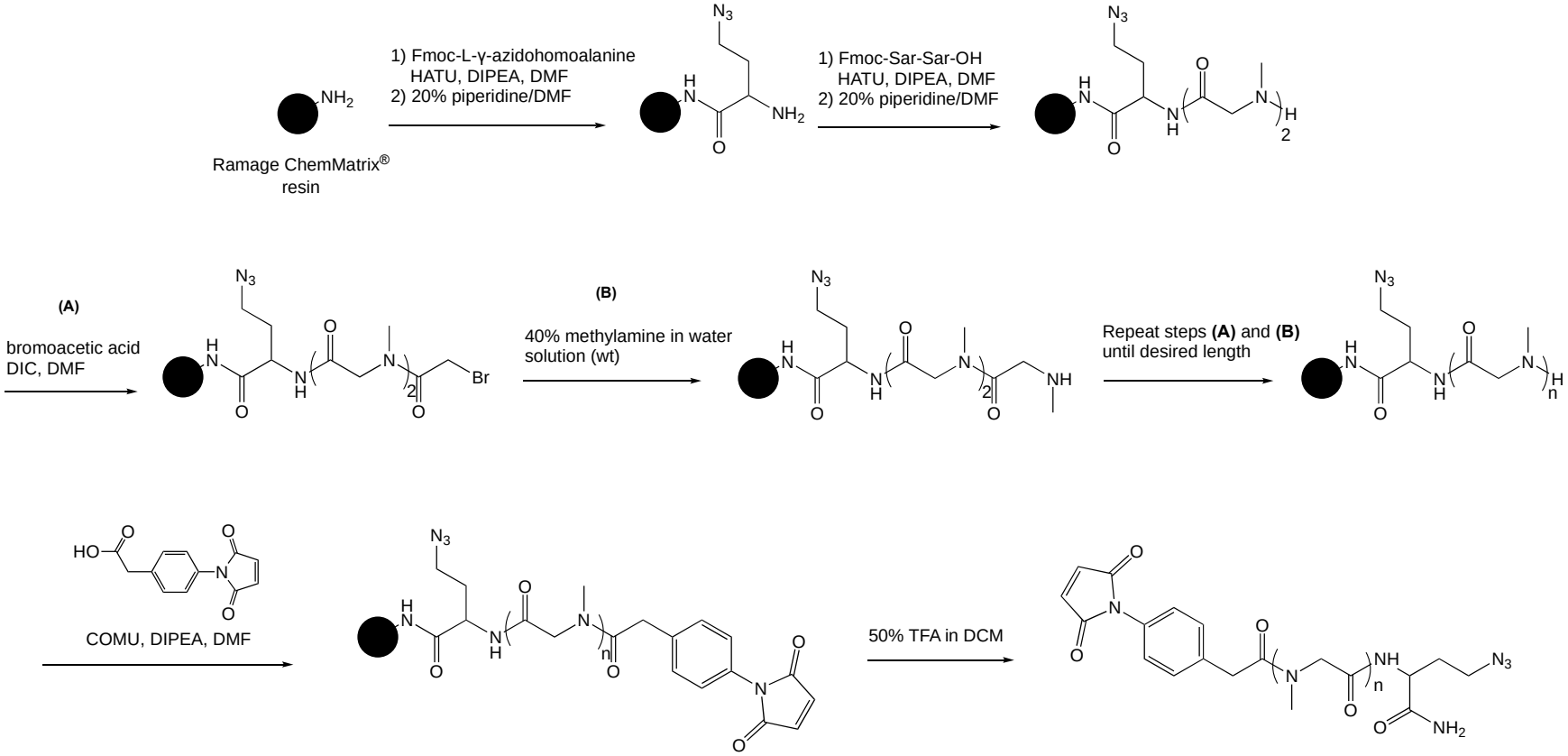
ACN: acetonitrile; AcOH: acetic acid; ADC: Antibody-drug conjugate; BAA: bromoacetic acid; COMU: (1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate; DAR: Drug-antibody ratio; DCC: N,N-Dicyclohexylcarbodiimide; DCM: dichloromethane; DIC: N,N-Diisopropylcarbodiimide; DIPEA: diisopropylethylamine; DMAP: 4-(Dimethylamino)pyridine; DME : 1,2-dimethoxyethane; DMF: dimethylformamide; DMSO: dimethyl sulfoxide; EDC: N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride; EDTA: ethylenediaminetetraacetic acid; EtOAc: ethyl acetate; EtOH: ethanol; HATU: N-[(Dimethylamino)-1H-1,2,3-triazolo-[4,5-b]pyridin-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide; HFIP: 1,1,1,3,3,3-hexafluoro-2-propanol; HIC: Hydrophobic interaction chromatography; HOBt: 1-Hydroxybenzotriazole; MeOH : methanol; MAL: maleimide; MMAE: Monomethyl Auristatin E; NMP: 1-Methyl-2-pyrrolidinone; PBS : phosphate buffer saline; PE: petroleum ether; PEG: poly(ethyleneglycol); PFP: pentafluorophenol; PK: pharmacokinetics; PSAR: poly(sarcosine); SEC: size exclusion chromatography; TCEP: Tris(2-carboxyethyl)phosphine hydrochloride; TFA: trifluoroacetic acid; THF: tetrahydrofuran.

Scheme S1. Synthesis pathway of the monodisperse side-functionalized polysarcosine oligomers



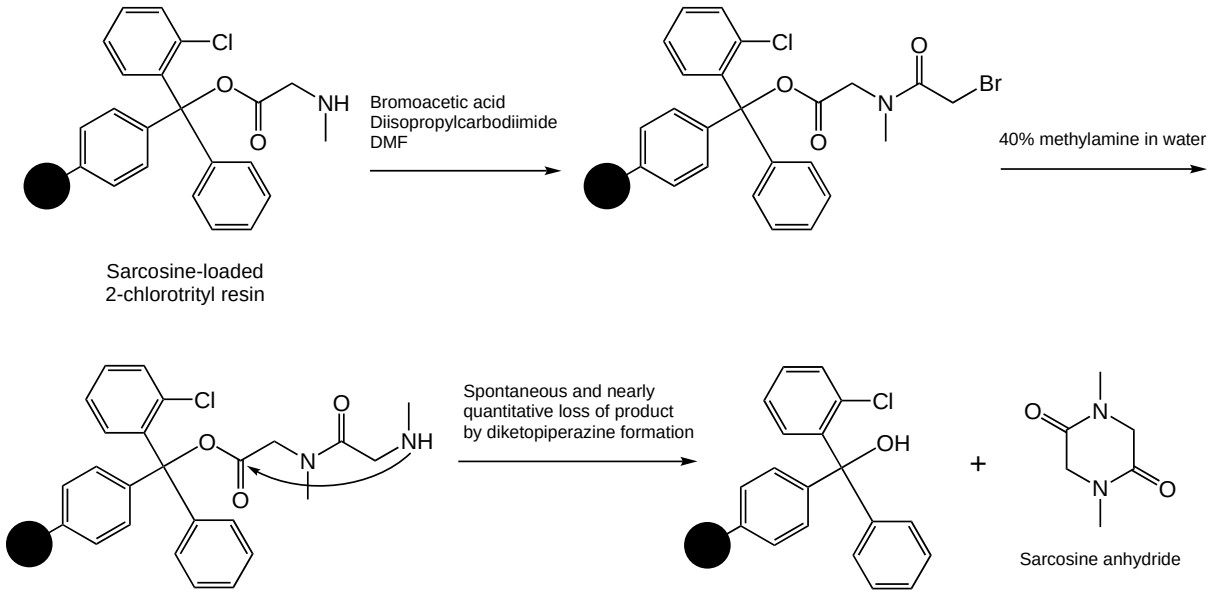
The synthesis and characterization of compounds are further detailed below.

Scheme S2. Synthesis pathway of the monodisperse polysarcosine oligomer used in the negative control PSAR12L drug-linker



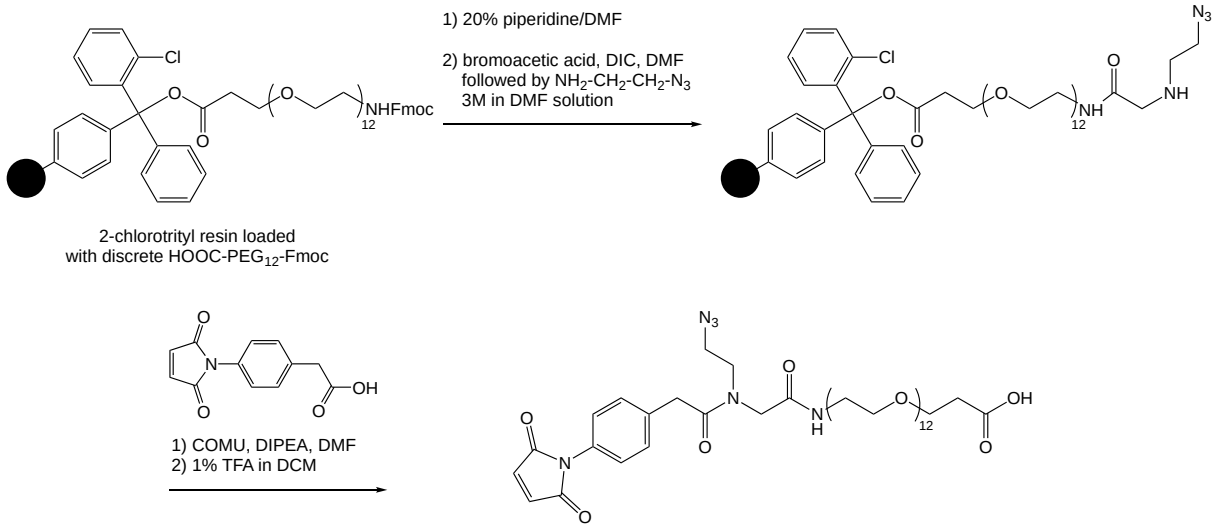
The synthesis and characterization of compounds are further detailed below.

Scheme S3. Detrimental on-resin diketopiperazine formation at the dimeric stage



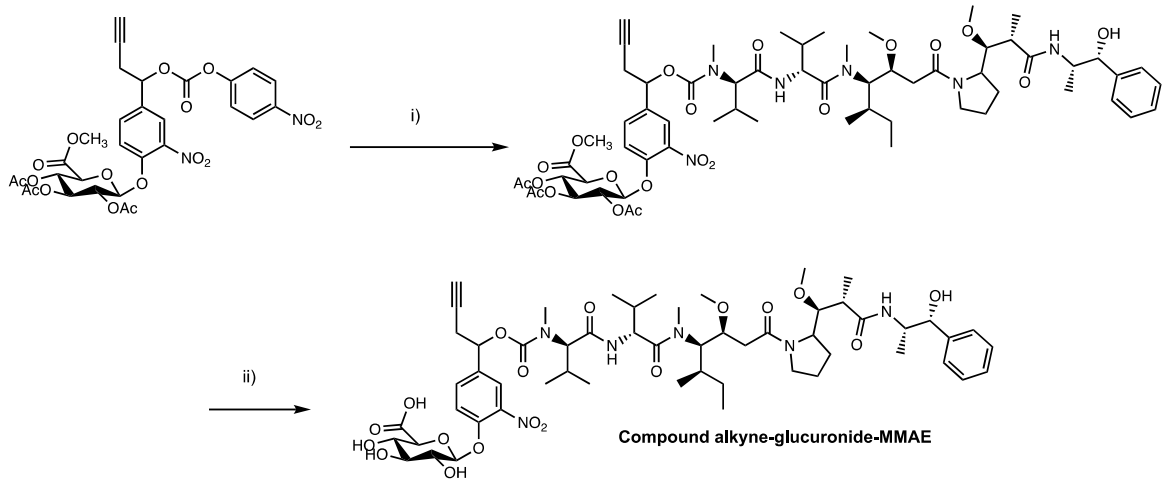
We observed an almost quantitative detrimental diketopiperazine formation at the dimeric stage during PSAR synthesis, as observed by (i) strong colour change of the resin (resin turns orange-brown within minutes), (ii) weight loss of the resin, (iii) yield loss and (iv) detection of sarcosine anhydride by mass spectrometry (m/z $[M+H]^+$ = 143.2) in the eluate after resin drainage. This phenomenon was observed with 2-chlorotrityl solid support (usually known to inhibits diketopiperazine formation because of the steric bulk of the trityl moiety) and Rink amide solid support.

Scheme S4. On-resin synthesis pathway of the side-functionalized PEG used in the negative control drug-linker PEG12



The synthesis and characterization of compounds are further detailed below.

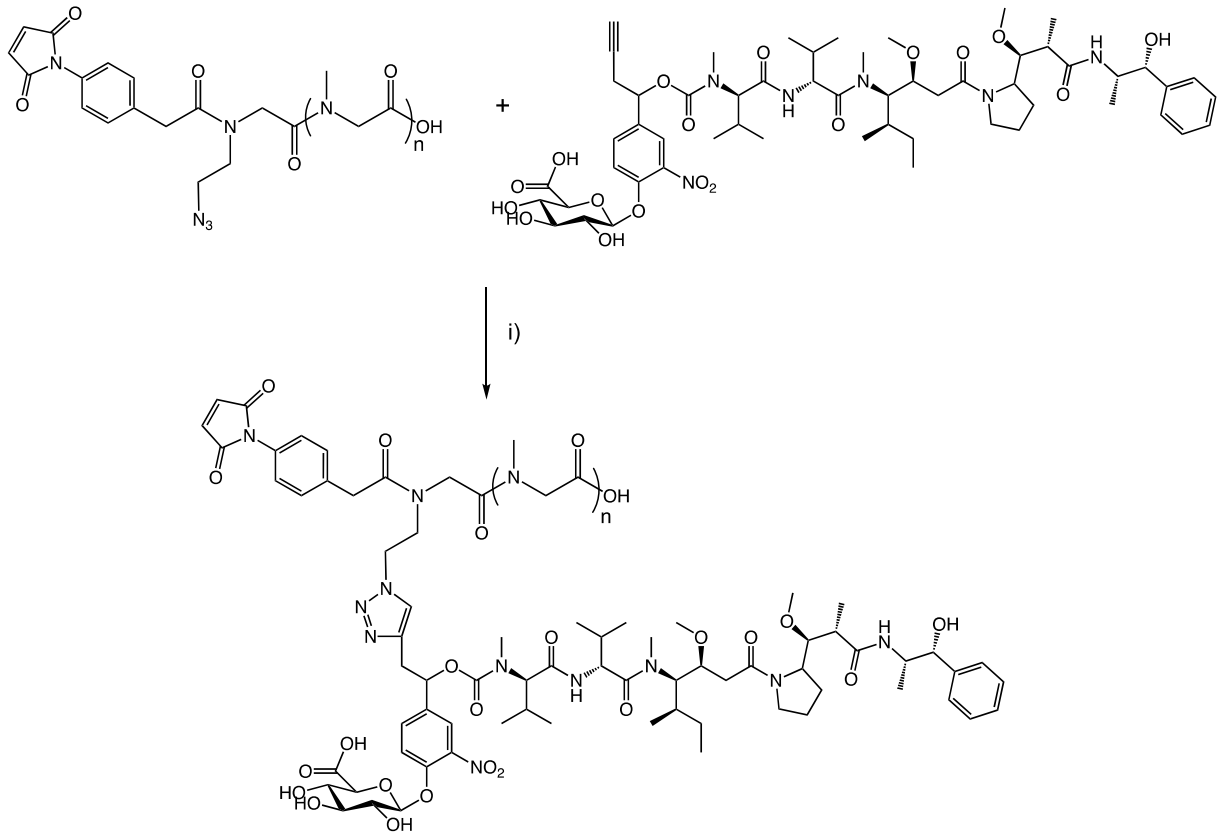
Scheme S5. Synthesis of compound alkyne-glucuronide-MMAE



i) Monomethyl auristatin E (MMAE), HOBt, pyridine, DMF, room temp, 16h; ii) LiOH, MeOH/H_2O, 0°C, 60 min.

The synthesis and characterization of compounds are further detailed below.

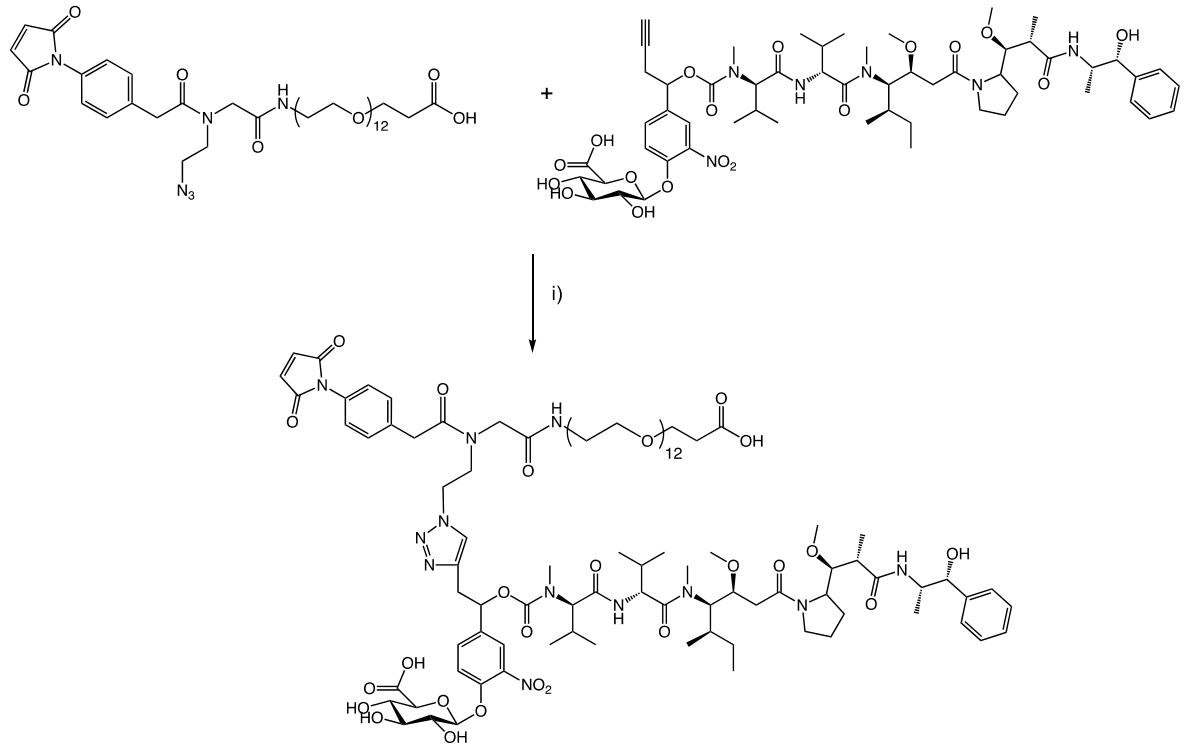
Scheme S6. Synthesis of polysarcosine-based drug-linkers (PSARn)



i) tetrakis(acetonitrile)copper(I) hexafluorophosphate, DCM, room temp, 16 hours.

The synthesis and characterization of compounds are further detailed below.

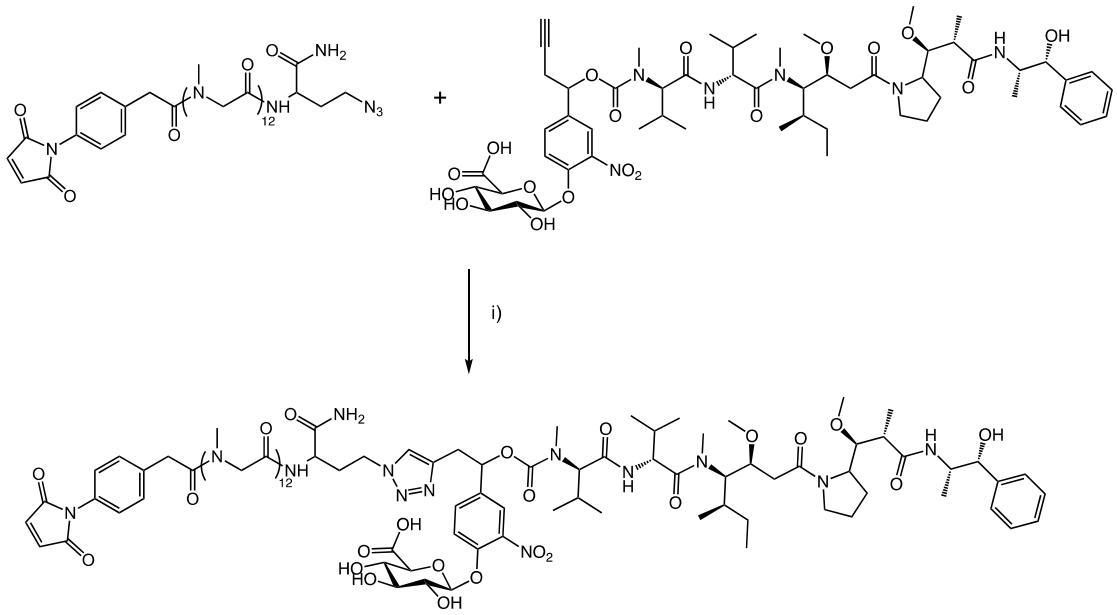
Scheme S7. Synthesis of polyethyleneglycol-based drug-linker (PEG12)



i) tetrakis(acetonitrile)copper(I) hexafluorophosphate, NMP/DCM (2:1 v/v), room temp, 16 hours.

The synthesis and characterization of compounds are further detailed below.

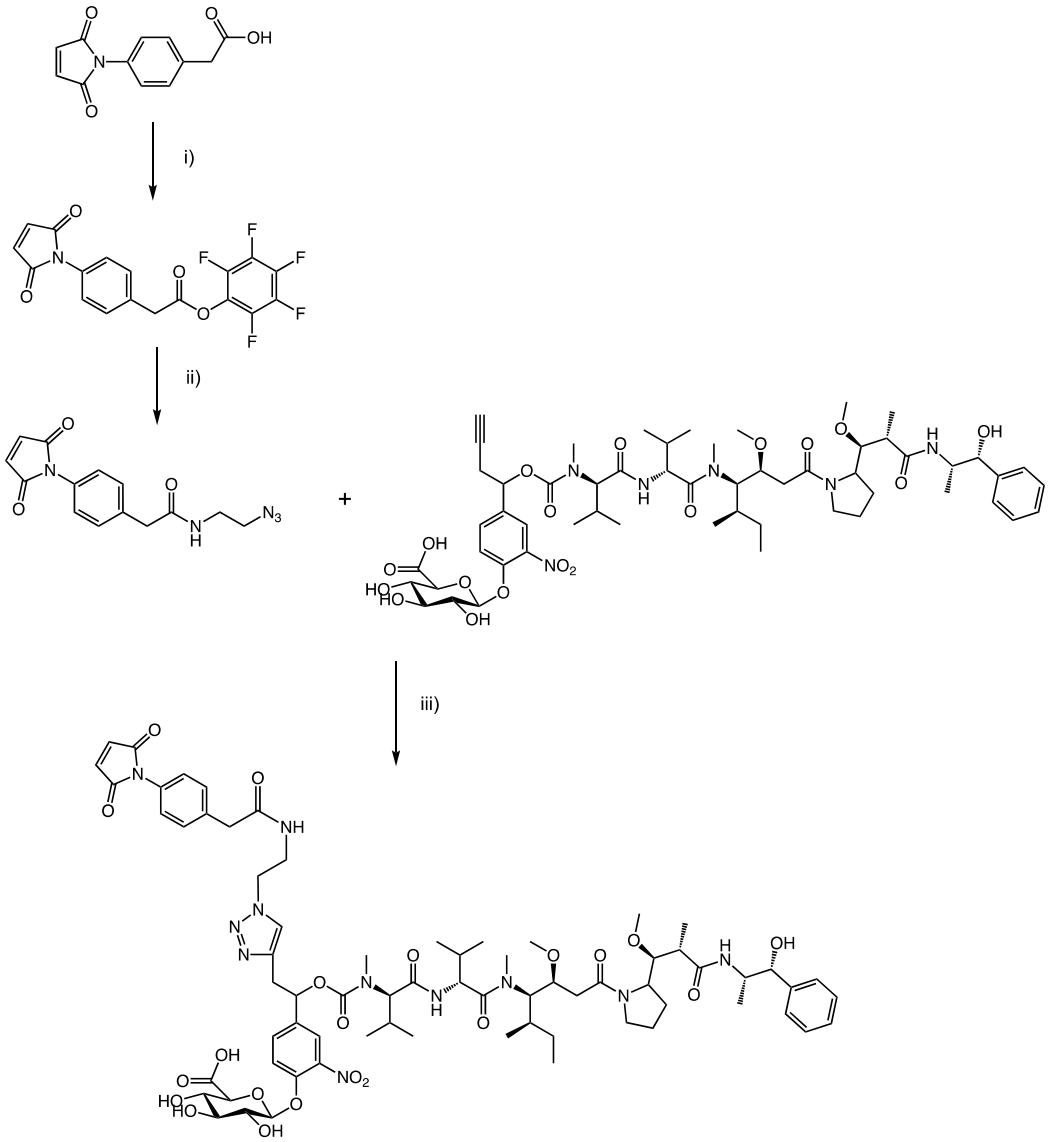
Scheme S8. Synthesis of linear negative control linker (PSAR12L)



i) tetrakis(acetonitrile)copper(I) hexafluorophosphate, NMP/DCM (2:1 v/v), room temp, 16 hours.

The synthesis and characterization of compounds are further detailed below.

Scheme S9. Synthesis of negative control linker lacking hydrophobicity masking moiety (PSAR0)



i) DCC, pentafluorophenol, DME, room temp, 2h; ii) NH_2-CH_2-CH_2-N_3, DCM, room temp, 1h; iii) tetrakis(acetonitrile)copper(I) hexafluorophosphate, NMP/DCM/ACN (1:1:1 v/v/v), room temp, 16 hours.

The synthesis and characterization of compounds are further detailed below.

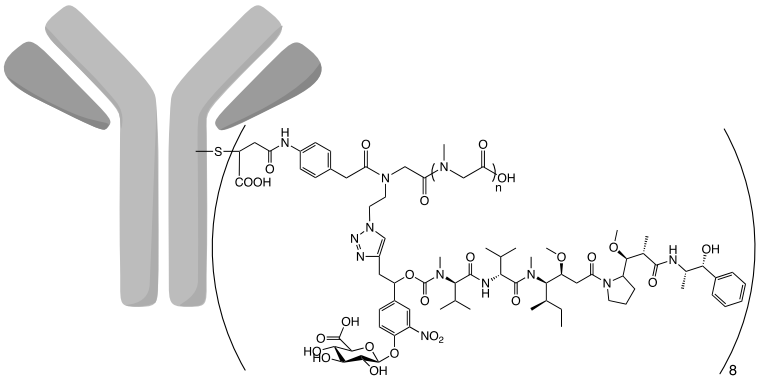
Figure S10. *In silico* logD and logS predictions of drug-linkers

Drug-linker	Linker architecture	logD (pH 7.4)	logS (pH 7.4)
PSAR12	Orthogonal	-14.3	21.2
PSAR12L	Linear	-12.0	20.7
PEG12	Orthogonal	-5	14.8
PSAR0	-	-0.1	0.2

In silico predictions of hydrophobicity (logD at pH 7.4) and water solubility (logS at pH 7.4) were calculated using the Chemicalize Online Calculation Platform from ChemAxon Ltd. Negative logD values indicate a hydrophilic, polar character of the tested compound. High logS values correlate with a greater solubility of the compound in water.

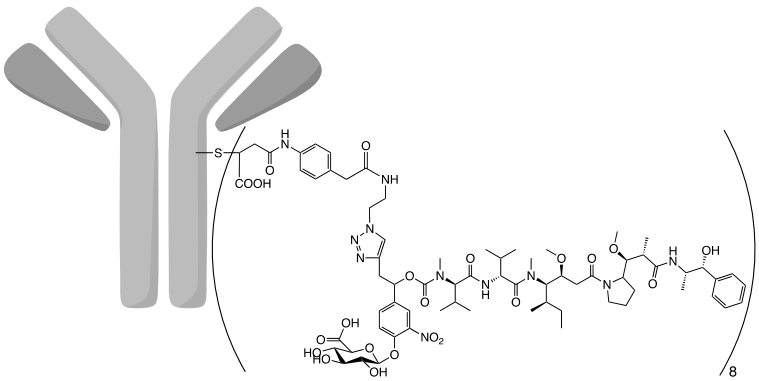
In silico calculations confirms that addition of a polar entity (PSAR or PEG) in the drug-linker architecture positively impacts physicochemical properties of the drug linker (increased hydrophilicity and water solubility of PSAR12, PSAR12L and PEG12 *versus* PSAR0). *In silico* data advantageously predict a more hydrophilic and polar character of PSAR *versus* PEG, in accordance with the observed HIC profiles of the final ADCs (PSAR12 *versus* PEG12 drug-linkers). *In silico* data are unable to predict the favourable impact of an orthogonal linker architecture on the final hydrophilicity of the conjugates as opposed to a linear architecture (drug-linkers PSAR12 and PSAR12L share similar logD and logS values).

Figure S11. Structure of ADCs

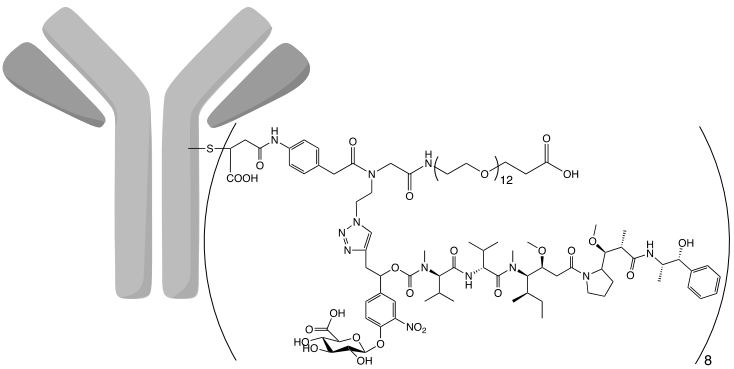


ADC-PSARn :

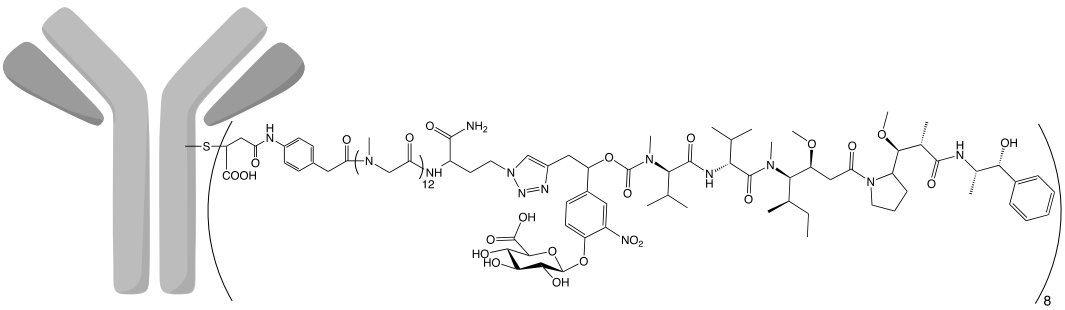
ADC-PSAR6 (n = 6)
ADC-PSAR12 (n = 12)
ADC-PSAR18 (n = 18)
ADC-PSAR24 (n = 24)



ADC-PSAR0

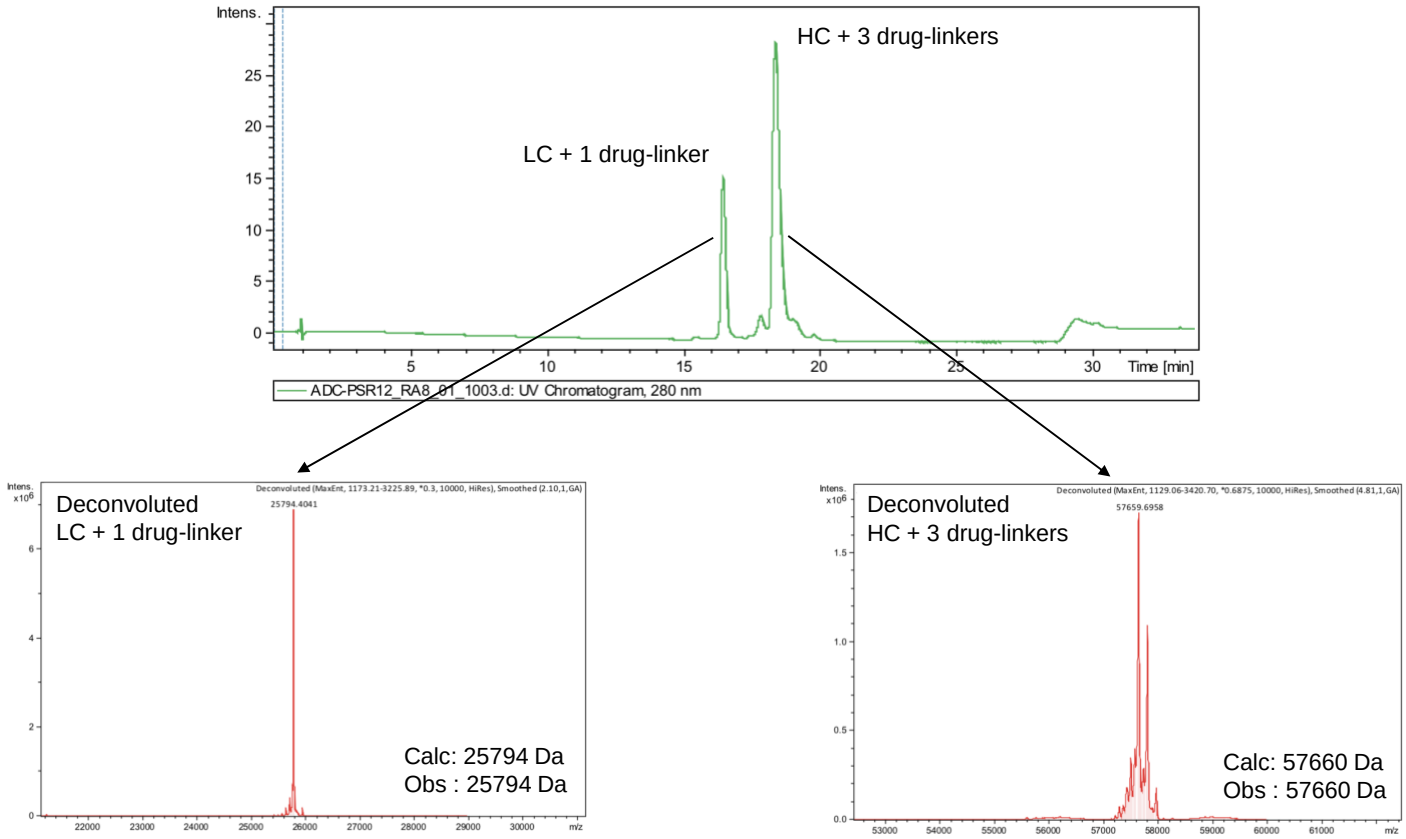


ADC-PEG12



ADC-PSAR12L

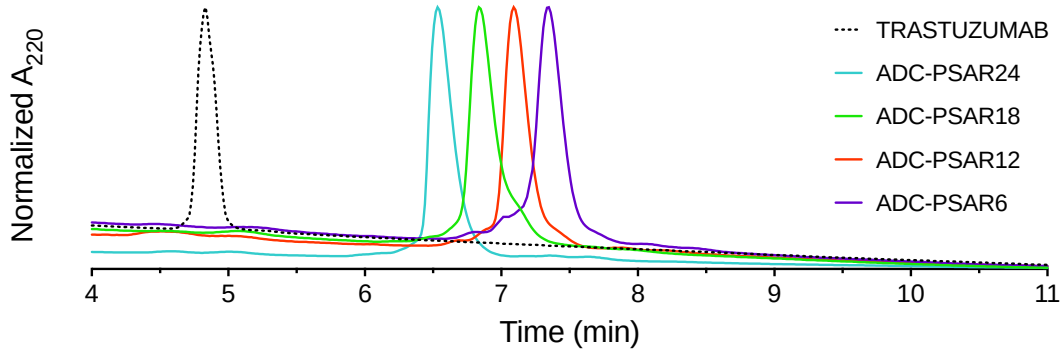
Figure S12. RPLC-QToF characterization of ADCs



Reverse phase liquid chromatography mass spectrometry (RPLC-QToF) profile of ADC-PSAR12 showed for illustration purposes. Conjugates exhibited one LC-1d (light chain with 1 drug-linker attached) and one HC-3d (heavy chain with 3 drug-linkers attached) absorbance peaks on their denaturing RPLC chromatogram (DAR8 conjugates). For mass spectrometry analysis of the heavy chain of trastuzumab, the major G0F glycoform is reported.

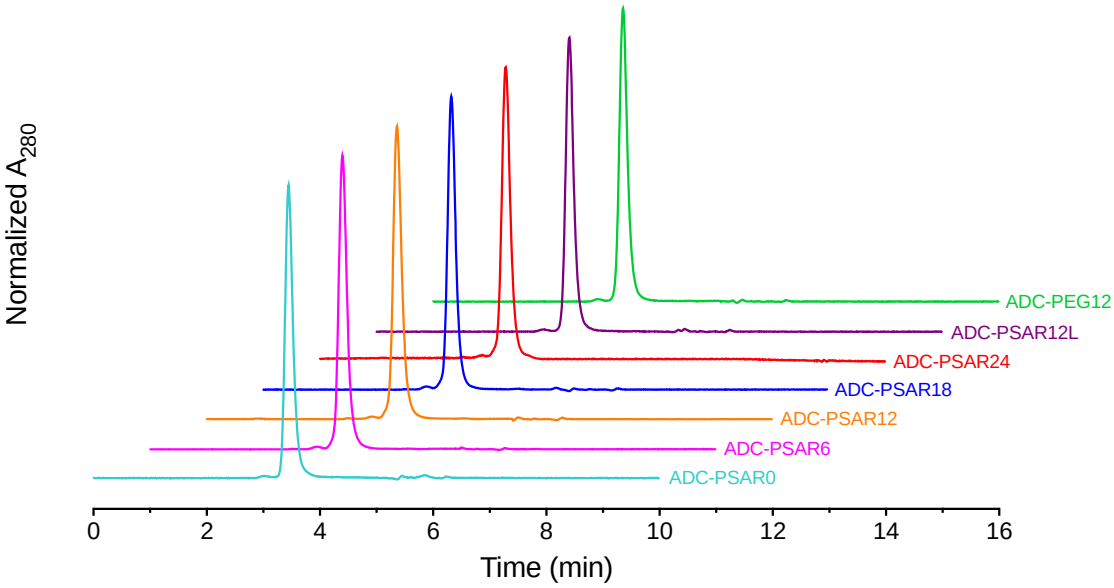
- ADC-PSAR6 (DAR8)
  - Deconvoluted LC-1d Calc: 25368 ; Obs: 25368 / Deconvoluted HC-3d Calc: 56382 ; Obs: 56380
  - Monomeric purity: 99+%
  - HIC retention time: 7.5 min
- ADC-PSAR12 (DAR8)
  - Deconvoluted LC-1d Calc: 25794 ; Obs: 25794 / Deconvoluted HC-3d Calc: 57660 ; Obs: 57660
  - Monomeric purity: 99+%
  - HIC retention time: 7.1 min
- ADC-PSAR18 (DAR8)
  - Deconvoluted LC-1d Calc: 26221 ; Obs: 26221 / Deconvoluted HC-3d Calc: 58939 ; Obs: 58939
  - Monomeric purity: 99+%
  - HIC retention time: 6.8 min
- ADC-PSAR24 (DAR8)
  - Deconvoluted LC-1d Calc: 26647 ; Obs: 26674 / Deconvoluted HC-3d Calc: 60218 ; Obs: 60218
  - Monomeric purity: 99+%
  - HIC retention time: 6.7 min
- ADC-PSAR0 (DAR8)
  - Deconvoluted LC-1d Calc: 24884 ; Obs: 24884 / Deconvoluted HC-3d Calc: 54926 ; Obs: 54926
  - Monomeric purity: 98.5%
  - HIC retention time: 8.4 min
- ADC-PSAR12L (DAR8)
  - Deconvoluted LC-1d Calc: 25793 ; Obs: 25793 / Deconvoluted HC-3d Calc: 57657 ; Obs: 57657
  - Monomeric purity: 99+%
  - HIC retention time: 8.5 min
- ADC-PEG12 (DAR8)
  - Deconvoluted LC-1d Calc: 25541 ; Obs: 25541 / Deconvoluted HC-3d Calc: 56901 ; Obs: 56900
  - Monomeric purity: 99+%
  - HIC retention time: 7.4 min

Figure S13. HIC profiles of polysarcosine-based ADCs



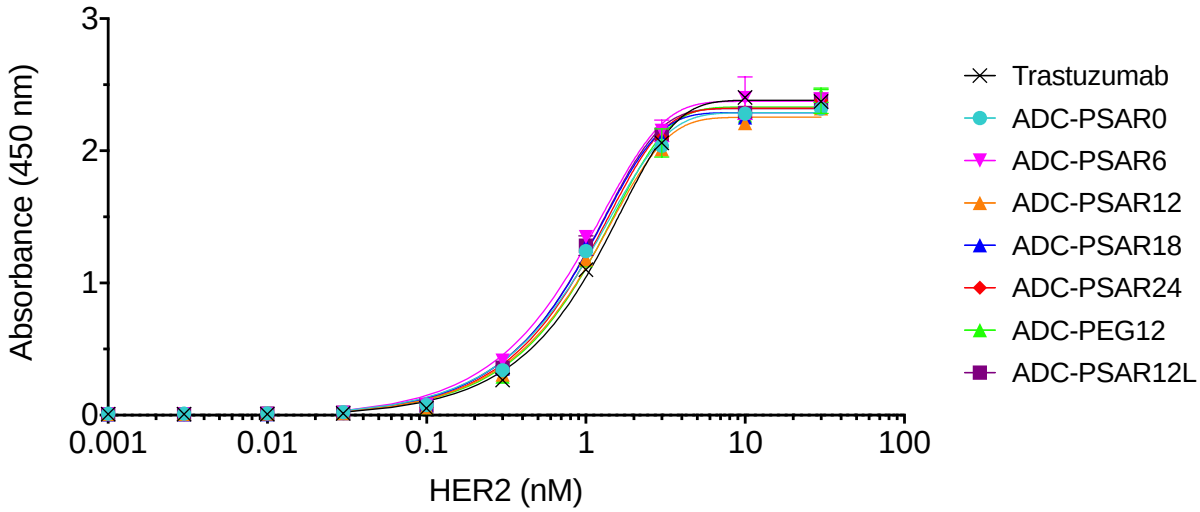
Hydrophobic Interaction Chromatography (HIC) profiles of polysarcosine-based ADCs.

Figure S14. SEC profiles of ADCs



Size exclusion chromatography (SEC) profiles of ADCs used in the present study (>95% monomeric).

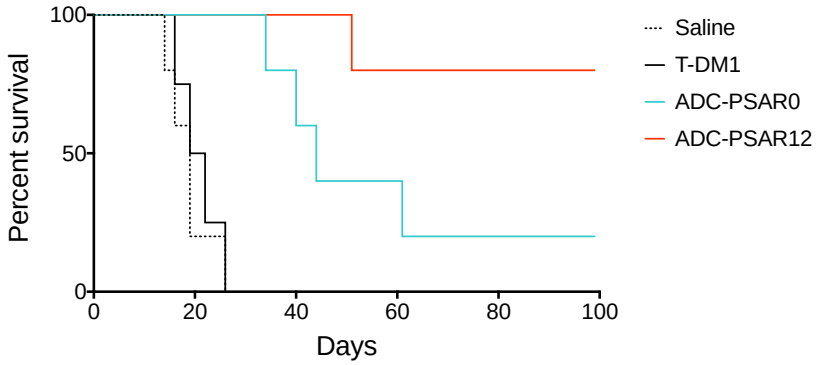
Figure S15. HER2 binding affinity profiles of ADCs



	Trastuzumab	ADC-PSAR0	ADC-PSAR6	ADC-PSAR12	ADC-PSAR18	ADC-PSAR24	ADC-PEG12	ADC-PSAR12L
EC_{50} (nM)	0.48	0.13	0.11	0.36	0.60	0.58	0.62	0.64

HER2 binding affinity profiles of ADCs used in the present study were assayed by ELISA. Grafting of 8 drug-linkers on interchain cysteines had no observable impact on antigen-binding affinity when compared to the native antibody trastuzumab. Presence or absence of PSAR/PEG hydrophobicity masking entities and linker architecture (orthogonal *versus* linear) does not impact antigen-binding affinity.

Figure S16. Survival curve of the SCID/BT-474 xenograft study



Survival curves of the first SCID/BT-474 xenograft study (Figure 3B). ADCs were injected once intravenously at a dose of 3 mg/kg. No body-weight changes were observed during the study.

Figure S17. Pharmacokinetic parameters (Sprague-Dawley rat PK study)

	TRASTUZUMAB	ADC-PSAR0	ADC-PSAR6	ADC-PSAR12	ADC-PSAR18	ADC-PSAR24	ADC-PEG12	ADC-PSAR12L
Clearance (mL/day/kg)	28.9	87.7	51.1	38.9	40.2	38.7	47.3	103.6
$AUC_{0\text{-}inf}$ (day x µM)	362.2	119.4	204.8	269.4	260.5	270.3	221.3	101.0

Reagents and general methods

All solvents and reagents were obtained from commercial sources (Sigma-Aldrich, Alfa Aesar, Fluorochem, Thermo Fisher, Carbosynth) and used without further purification unless stated otherwise. Anhydrous DMF, DCM and THF were purchased from Sigma-Aldrich. Fmoc-aminoacids and 2-chlorotrityl resin were purchased from Merck. Ramage ChemMatrix® resin was purchased from Sigma-Aldrich. Monodisperse Fmoc-PEG_{12}-COOH was purchased from PurePEG LLC. Monomethyl auristatin E (MMAE) was purchased from DCChemicals. Trastuzumab (Herceptin® IV) and T-DM1 (Kadcyla®) were purchased from Roche. On-resin synthesis was performed in empty SPE plastic tubes equipped with a 20µm polyethylene frit (Sigma-Aldrich). A Titramax 101 platform shaker (Heidolph) was used for agitation. All synthesis yields reported are based upon an initial resin loading of 1.1 mmol/g for 2-chlorotrityl resin and 0.47 mmol/g for Ramage ChemMatrix® resin (extent of labeling indicated by the manufacturers). Unless stated otherwise, all chemical reactions were carried out at room temperature under an inert argon atmosphere.

Liquid nuclear magnetic resonance spectra were recorded at room temperature on a Bruker Fourier 300HD or Bruker AV500 spectrometer, using residual solvent peak for calibration. Mass spectroscopy analysis has been performed by the Centre Commun de Spectrométrie de Masse (CCSM) of the UMR5246 CNRS institute of the University Claude Bernard Lyon 1.

Normal phase flash chromatography was performed on a Teledyne Isco CombiFlash® Companion® device or Teledyne Isco CombiFlash® Rf200 device using either Interchim (spherical HP 50µm) or Biotage® ZIP® (50µm) silica cartridges. Reverse phase chromatography was performed using Biotage® SNAP Ultra C18 (25µm) cartridges or Interchim PuriFlash RP-AQ (30µm) cartridges. Chemical reactions and compound characterization were respectively monitored and analyzed by thin-layer chromatography using pre-coated 40-63µm silica gel (Macherey-Nagel), HPLC-UV (Agilent 1050) or UHPLC-UV/MS (Thermo UltiMate 3000 UHPLC system equipped with a Bruker Impact II™ Q-ToF mass spectrometer or Agilent 1260 HPLC system equipped with a Bruker MicrOTOF-QII mass spectrometer).

The protocols for experiments in mice and rats were approved by the University of Lyon Animal Ethics Committee. *In vivo* studies were performed at Antineo (Lyon, France — www.antineo.fr).

HPLC methods

HPLC Method 1: Agilent 1050 equipped with DAD detection. Mobile phase A was water and mobile phase B was acetonitrile. Column was an Agilent Zorbax SB-Aq 4.6x150mm 5µm (room temperature). Gradient was 5%B to 95%B in 20 min, followed by a 5 min hold at 95%B. Flow rate was 1.5 mL/min. UV detection was monitored at 214 nm.

HPLC Method 2: Agilent 1050 equipped with DAD detection. Mobile phase A was water and mobile phase B was acetonitrile. Column was an Agilent Zorbax SB-Aq 4.6x150mm 5µm (room temperature). Gradient was 0%B to 50%B in 30 min, followed by a 5 min hold at 50%B. Flow rate was 1.0 mL/min. UV detection was monitored at 214 nm.

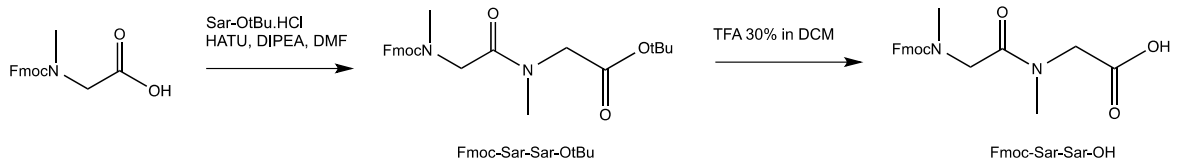
HPLC Method 3: Same as HPLC Method 1 but contains 0.1% TFA into the mobile phase A.

HPLC Method 4: Same as HPLC Method 2 but contains 0.1% TFA into the mobile phase A.

Organic chemistry procedures

1. Synthesis of side-functionalized monodisperse polysarcosine oligomers

1.1. Synthesis of Fmoc-Sar-Sar-OH



Fmoc-Sar-Sar-OtBu

Fmoc-Sar-Sar-OH

1.1.1. Synthesis of Fmoc-Sar-Sar-OtBu

Fmoc-Sar-OH (2000 mg / 6.42 mmol) and HATU (2443 mg / 6.42 mmol) were dissolved in 28 mL of anhydrous DMF in a round-bottom flask. DIPEA (2491 mg / 19.27 mmol) was added and the mixture was stirred for 3 min at room temperature. Sarcosine tert-butyl ester hydrochloride (1167 mg / 6.42 mmol) was then added and the reaction mixture was stirred at room temperature for 90 min. Volatiles were removed under vacuum and the residue was diluted with water and extracted 3 times with EtOAc. The organic phase was dried over $MgSO_4$, filtered and evaporated under vacuum to afford a solid crude. The crude was taken up in EtOAc/DCM 80:20 (v/v) and white insolubles were removed *via* filtration. The filtrate was purified by chromatography on silica gel (petroleum ether/EtOAc, gradient from 60:40 to 20:80) to afford **Fmoc-Sar-Sar-OtBu** (2310 mg / 82%) as a white solid. HRMS *m/z* (ESI^+): Calc $[M+H]^+$ = 439.2227 ; Exp $[M+H]^+$ = 439.2234 ; Error = -1.5 ppm. HPLC Method 1 retention time = 13.3 min. TLC eluting with 100% EtOAc: Rf=0.8.

1.1.2. Tert-butyl ester removal

Fmoc-Sar-Sar-OtBu (2310 mg / 5.27 mmol) was dissolved in 20 mL of DCM and 8.5 mL of TFA was slowly added. The solution was stirred at room temperature until entire tert-butyl ester deprotection was observed by HPLC (approximately 2 hours). Volatiles were then removed under vacuum and the residue was triturated with diethyl ether to afford **Fmoc-Sar-Sar-OH** (1690 mg / 84%) as a white solid. ^1H NMR (500 MHz, DMSO-d_6, 100°C) δ (ppm) 2.84 (s, 3H), 2.93 (s, 3H), 4.01 (s, 2H), 4.05 (s, 2H), 4.25 (t, *J* = 6.3 Hz, 1H), 4.34 (d, *J* = 6.4 Hz, 2H), 7.33 (t, *J* = 7.4 Hz, 2H), 7.41 (t, *J* = 7.4 Hz, 2H), 7.63

(d, *J* = 7.4 Hz, 2H), 7.85 (d, *J* = 7.5 Hz, 2H). HRMS *m/z* (ESI^+): Calc $[M+H]^+$ = 383.1601 ; Exp $[M+H]^+$ = 383.1602 ; Error = 0.0 ppm. HPLC Method 1 retention time = 6.2 min. TLC eluting with DCM/MeOH 85:15 (v/v): Rf=0.65.

1.2. Synthesis of side-functionalized monodisperse polysarcosine oligomers

1.2.1. Resin loading with Fmoc-Sar-OH

Typically, 1000 mg of 2-chlorotrityl chloride resin beads (100-200 mesh, 1% DVB, 1.1 mmol/g, Novabiochem) were swollen in 10 mL of anhydrous DCM for 10 min. Fmoc-Sar-OH (1.2 eq), previously dissolved in 10 mL of dry DCM, was added onto the resin. DIPEA (5 eq) was added and the reaction vessel was agitated for 2 hours at room temperature. After draining, the resin was washed with DCM (3 times), DMF (2 times), DCM (3 times) and MeOH (2 times). The resin was dried under high vacuum overnight. Substitution level was assessed from the weight gain of the resin and/or from Fmoc cleavage test (absorbance measurement at 301 nm) and was found to be quasi-quantitative (usually 0.95-1.1 mmol/g). Resin was stored at -20°C until further use.

1.2.2. Fmoc-Sar-Sar-OH coupling procedure

Resin was treated with 20% piperidine in DMF (1 mL per 100 mg of resin) for 2 times 15 min at room temperature. The resin was then washed with DMF (4 times) and DCM (4 times). To the resin was added a solution of Fmoc-Sar-Sar-OH (3 eq), HATU (2.85 eq) and DIPEA (6 eq) in DMF (1 mL per 100 mg of resin). The reaction vessel was agitated for 2 hours and the resin was extensively washed with DMF (5 times) and DCM (5 times). The resin was treated with 20% piperidine in DMF (1 mL per 100 mg of resin) for 2 times 15 min at room temperature. The resin was then washed with DMF (4 times) and DCM (4 times). The resin was dried under vacuum and stored at -20°C until further use.

1.2.3. Elongation of polysarcosine

Elongation of the polysarcosine oligomer was performed until the desired length was obtained, by alternating bromoacetylation and amine displacement steps. The bromoacetylation step was performed by adding 10 eq of bromoacetic acid and 13 eq of diisopropylcarbodiimide in DMF (2 mL per 100 mg of resin). The mixture was agitated for 30 min, drained and washed with DMF (4 times). For the amine displacement step, a 40% (wt) methylamine in water solution was added (1.5 mL per 100 mg of resin) and the vessel was shaken for 30 min, drained and washed with DMF (4 times) and DCM (4 times).

1.2.4. Introduction of the azido group

10 eq of bromoacetic acid and 13 eq of diisopropylcarbodiimide in DMF (2 mL per 100 mg of resin) were added onto the resin and the mixture was agitated for 30 min, drained and washed with DMF (4 times). A 3 molar solution of 2-azidoethan-1-amine[1] in DMF was added (1 mL per 100 mg of resin) and the vessel was shaken for 45 min, drained and washed with DMF (4 times) and DCM (4 times).

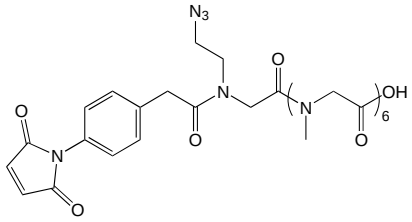
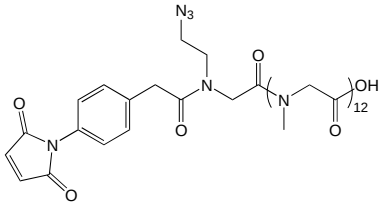
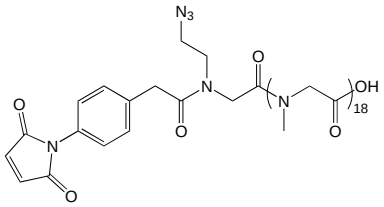
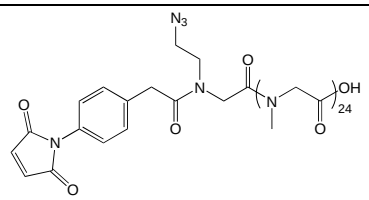
1.2.5. 4-maleimidophenylacetic acid coupling

To the resin was added a solution of commercially available 4-maleimidophenylacetic acid (5 eq), COMU (4.9 eq) and DIPEA (4.9 eq) in DMF (1 mL per 100 mg of resin). The reaction vessel was agitated for 90 min and the resin was washed with DMF (3 times) and DCM (3 times). Cleavage of the compound of interest from the resin was performed using a 1% TFA in DCM (v/v) solution under agitation for 5 minutes (repeated twice). Resin was filtered and volatiles were removed under reduced pressure to afford a solid crude.

1.2.6. Purification

PSAR compounds were purified on Interchim® RP-AQ (30µm) cartridges. Mobile phase A was water + 0.1% TFA and mobile phase B was acetonitrile + 0.1% TFA. The gradient ranged from 0 to 30% B.

1.2.7. Compounds

Compound Name	Structure	Yield	MS (ESI$^+$)	HPLC Method 4 retention time
PSAR6-N_3-phenyl-MAL (amorphous beige solid)		81%	Calc $[M+H]^+$ = 784.3373 Exp $[M+H]^+$ = 784.3342 Error = 3.9 ppm	18.1 min
PSAR12-N_3-phenyl-MAL (beige solid)		74%	Calc $[M+2H]^{2+}$ = 605.7836 Exp $[M+2H]^{2+}$ = 605.7833 Error = 0.5 ppm	17.2 min
PSAR18-N_3-phenyl-MAL (beige solid)		62%	Calc $[M+2H]^{2+}$ = 818.8950 Exp $[M+2H]^{2+}$ = 818.8957 Error = -0.9 ppm	16.4 min
PSAR24-N_3-phenyl-MAL (beige solid)		60%	Calc $[M+3H]^{3+}$ = 688.3400 Exp $[M+3H]^{3+}$ = 688.3415 Error = -2.3 ppm	16.5 min

2. Synthesis of monodisperse polysarcosine oligomer used in the negative control PSAR12L drug-linker

2.1. Resin loading with Fmoc-L-γ-azidohomoalanine-OH

Typically, 500 mg of Ramage ChemMatrix® beads (0.47 mmol/g, Sigma-Aldrich) were swollen in 5 mL of DCM for 15 min. Resin was treated with 20% piperidine in DMF (1 mL per 100 mg of resin) for 2 times 15 min at room temperature. The resin was then washed with DMF (4 times) and DCM (4 times). To the resin was added a solution of Fmoc-L-γ-azidohomoalanine-OH (3 eq), HATU (2.9 eq) and DIPEA (6 eq) in DMF (1 mL per 100 mg of resin). The reaction vessel was agitated for 1.5 hours and the resin was extensively washed with DMF (5 times) and DCM (5 times). Unreacted sites were

acetylated using a capping solution made of acetic anhydride/DIPEA/DMF (1:2:3 v/v) (vessel shaken for 30 min). The solution was drained and the resin was washed with DMF (4 times) and DCM (4 times). Resin was treated with 20% piperidine in DMF (1 mL per 100 mg of resin) for 2 times 15 min at room temperature. The resin was then washed with DMF (4 times) and DCM (4 times).

2.2. Fmoc-Sar-Sar-OH coupling procedure

To the resin was added a solution of Fmoc-Sar-Sar-OH (4 eq), HATU (3.9 eq) and DIPEA (8 eq) in DMF (1 mL per 100 mg of resin). The reaction vessel was agitated for 2 hours and the resin was extensively washed with DMF (4 times) and DCM (4 times). The resin was treated with 20% piperidine in DMF (1 mL per 100 mg of resin) for 2 times 15 min at room temperature. The resin was then washed with DMF (4 times) and DCM (4 times).

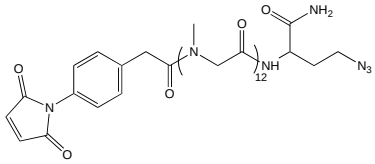
2.3. Elongation of polysarcosine compounds

Elongation of the polysarcosine oligomer was performed until the desired length was obtained, by alternating bromoacetylation and amine displacement steps, as already described above.

2.4. 4-maleimidophenylacetic acid coupling

4-maleimidophenylacetic acid was coupled following the procedure described above. Cleavage of the compound from the resin was performed using 5 mL of a TFA/DCM (50:50) solution for 30 minutes under agitation at room temperature. The process was repeated once and the pooled filtrates were evaporated under reduced pressure to give a solid crude that was purified on Interchim® RP-AQ (30µm) cartridges as described in the above section.

2.5. Compound

Compound Name	Structure	Yield	MS (ESI+)	HPLC Method 4 retention time
N_3-PSAR12-phenyl-MAL (beige solid)		82%	Calc $[M+2H]^{2+}$ = 605.2916 Exp $[M+2H]^{2+}$ = 605.2936 Error = -3.2 ppm	12.5 min

3. Synthesis of side-functionalized PEG used in the negative control drug-linker PEG12

3.1. Resin loading with Fmoc-PEG_{12}-COOH

200 mg of 2-chlorotrityl chloride resin beads (100-200 mesh, 1% DVB, 1.1 mmol/g, Novabiochem) were swollen in 4 mL of anhydrous DCM for 10 min. Fmoc-PEG_{12}-COOH (PurePEG LLC., 1.2 eq), previously dissolved in 2 mL of dry DCM, was added onto the resin. DIPEA (3 eq) was added and the reaction vessel was agitated for 1 hour at room temperature. 300µL of MeOH was added to quench unreacted resin. After 10 min of shaking, the solution was drained and the resin was washed with DMF (3 times) and DCM (3 times). The resin was treated with 20% piperidine in DMF (1 mL per 100 mg of resin) for 2 times 15 min at room temperature. The resin was then washed with DMF (4 times) and DCM (4 times).

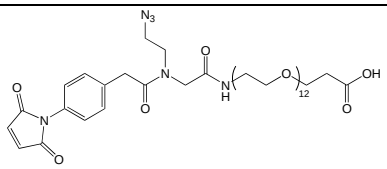
3.2. Introduction of the azido group

The bromoacetylation step was performed by adding 10 eq of bromoacetic acid and 13 eq of diisopropylcarbodiimide in DMF (2 mL per 100 mg of resin). The mixture was agitated for 30 min, drained and washed with DMF (4 times). For the amine displacement step, a 3 molar solution of 2-azidoethan-1-amine[1] in DMF was added (1 mL per 100 mg of resin) and the vessel was shaken for 45 min, drained and washed with DMF (4 times) and DCM (4 times).

3.3. 4-maleimidophenylacetic acid coupling

4-maleimidophenylacetic acid was coupled following the procedure described above. Cleavage of the compound of interest from the resin was performed using a 1% TFA in DCM (v/v) solution under agitation for 5 minutes (repeated twice). Resin was filtered and volatiles were removed under reduced pressure to afford an oily crude that was purified on Interchim® RP-AQ (30µm) cartridges as described in the above section.

3.4. Compound

Compound Name	Structure	Yield	MS (ESI$^+$)	HPLC Method 4 retention time
PEG12-N_3-phenyl-MAL (slightly yellow oil)		33%	$[M+H]^+$ = 957.5	18.1 min

4. Synthesis of compound alkyne-glucuronide-MMAE

4.1 Step 1

68.4 mg (0.099 mmol) of starting carbonate[2], 71.3 mg (0.099 mmol) of MMAE and 13.5 mg (0.099 mmol) of HOBt were dissolved in 2.4 mL of anhydrous DMF/pyridine (80:20 v/v). After stirring at room temperature for 16 hours, volatiles were evaporated and the residue was purified by chromatography on silica gel (DCM/MeOH, gradient from 98:2 to 95:5) to afford 98 mg (78%) of the protected intermediate as a white solid. $[M+H]^+$ = 1267.6. TLC eluting with DCM/MeOH 95:5 (v/v): Rf=0.7.

4.2 Step 2

110.8 mg (0.087 mmol) of starting material was dissolved in MeOH (10 mL) at 0°C. LiOH monohydrate (36.7 mg / 0.87 mmol) was dissolved in water (1 mL) and was slowly added to the reaction vessel. After stirring at 0°C for 70 min, the mixture was neutralized with acetic acid (68.2 mg / 1.14 mmol) and

concentrated under reduced pressure. The resulting material was taken up in a water/MeOH/DMF solution (1:1:1 v/v) and purified on a 30g Biotage® SNAP Ultra C18 (25µm) cartridge. Mobile phase A was water + 0.1% TFA and mobile phase B was acetonitrile + 0.1% TFA. The gradient ranged from 10 to 60% B. Compound **alkyne-glucuronide-MMAE** (mixture of two diastereoisomers) was obtained as a white solid (95 mg / 96%). LC-HRMS *m/z* (ESI^+): Calc $[M+H]^+$ = 1127.5758 ; Exp $[M+H]^+$ = 1127.5757 ; Error = 0.1 ppm. HPLC Method 3 retention time = 10.3 min.

5. Synthesis of polysarcosine-based drug-linkers (PSARn) by click chemistry

Alkyne-glucuronide-MMAE (1 eq; obtained as described above), PSARn-N_3-phenyl-MAL (1.1 eq; obtained as described above) and tetrakis(acetonitrile)copper(I) hexafluorophosphate (3 eq) were combined in a reaction vessel. DCM was added to reach a final alkyne-glucuronide-MMAE concentration of 12 mM. The reaction was stirred 16 hours at room temperature under argon in the dark. After removal of the volatiles under reduced pressure, the residue was taken up in DMF and purified on a 30g Biotage® SNAP Ultra C18 (25µm) cartridge. Mobile phase A was water + 0.1% TFA and mobile phase B was acetonitrile + 0.1% TFA. The gradient ranged from 10 to 50% B.

Compound **PSAR6** was obtained as a white solid (3.3 mg / 20%). LC-HRMS *m/z* (ESI^+): Calc $[M+2H]^{2+}$ = 955.9566 ; Exp $[M+2H]^{2+}$ = 955.9533 ; Error = 3.4 ppm. HPLC Method 3 retention time = 9.2 min.

Compound **PSAR12** was obtained as a white solid (9.0 mg / 33%). LC-HRMS *m/z* (ESI^+): Calc $[M+2H]^{2+}$ = 1169.0679 ; Exp $[M+2H]^{2+}$ = 1169.0621 ; Error = 4.9 ppm. HPLC Method 3 retention time = 8.7 min.

Compound **PSAR18** was obtained as a white solid (11.5 mg / 40%). LC-HRMS *m/z* (ESI^+): Calc $[M+2H]^{2+}$ = 1382.1792 ; Exp $[M+2H]^{2+}$ = 1382.1803 ; Error = -0.7 ppm. HPLC Method 3 retention time = 8.6 min.

Compound **PSAR24** was obtained as a white solid (15 mg / 44%). LC-HRMS *m/z* (ESI^+): Calc $[M+4Na]^{4+}$ = 820.1309 ; Exp $[M+4Na]^{4+}$ = 820.1324 ; Error = -1.8 ppm. HPLC Method 3 retention time = 8.4 min.

6. Synthesis of polyethyleneglycol-based drug-linker PEG12 by click chemistry

Alkyne-glucuronide-MMAE (obtained as described above) and PEG12-N_3-phenyl-MAL (obtained as described above) were reacted and purified as described in the above section 4, using NMP/DCM (2:1 v/v) as reaction solvent.

Compound **PEG12** was obtained as a slightly yellow oil (10.4 mg / 45%). LC-HRMS *m/z* (ESI^+): Calc $[M+2H]^{2+}$ = 1042.5211 ; Exp $[M+2H]^{2+}$ = 1042.5218 ; Error = -0.7 ppm. HPLC Method 3 retention time = 8.0 min.

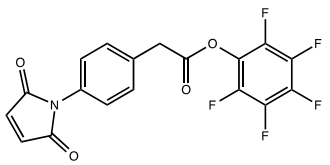
7. Synthesis of linear negative control drug-linker PSAR12L by click chemistry

Alkyne-glucuronide-MMAE (obtained as described above) and N_3-PSAR12-phenyl-MAL (obtained as described above) were reacted and purified as described in the above section 4, using NMP/DCM (2:1 v/v) as reaction solvent.

Compound **PSAR12L** was obtained as a white solid (16.0 mg / 39%). LC-HRMS *m/z* (ESI^+): Calc $[M+2H]^{2+}$ = 1168.5759 ; Exp $[M+2H]^{2+}$ = 1168.5792 ; Error = -2.8 ppm. HPLC Method 3 retention time = 6.8 min.

8. Synthesis of negative control drug-linker PSAR0

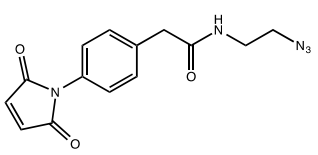
8.1 Synthesis of perfluorophenyl 2-(4-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)phenyl)acetate



Commercially available 4-maleimidophenylacetic acid (299 mg / 1.29 mmol), DCC (267 mg / 1.29 mmol) and pentafluorophenol (238 mg / 1.29 mmol) were dissolved in 15 mL of anhydrous DME in a reaction vessel. After 2 hours of stirring at room temperature, insolubles were removed by filtration and the filtrate was purified by chromatography on silica gel (petroleum ether/EtOAc, gradient from 80:20 to 20:80) to afford title compound (400 mg / 78%) as a white solid. ^1H NMR (300 MHz, $CDCl_3$) δ (ppm)

4.01 (s, 2H), 6.87 (s, 2H), 7.40 (d, J = 8.7 Hz, 2H), 7.47 (d, J = 8.7 Hz, 2H). HRMS *m/z* (ESI^+): Calc $[M+H]^+$ = 398.0446 ; Exp $[M+H]^+$ = 398.0448 ; Error = -0.4 ppm.

8.2 Synthesis of N-(2-azidoethyl)-2-(4-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)phenyl)acetamide



Previous intermediate (78 mg / 0.20 mmol) was dissolved in 1 mL of anhydrous DCM in a reaction vessel. 2-azidoethan-1-amine[1] (33.8 mg / 0.40 mmol) was added and the reaction was stirred 1 hour at room temperature. 1N HCl solution was then added and the mixture was extracted 3 times with DCM. The organic phase was dried over $MgSO_4$, filtered and evaporated under vacuum to afford a solid crude that was purified by chromatography on silica gel (petroleum ether/EtOAc, gradient from 60:40 to 0:100) to afford title compound (18 mg / 31%) as a white solid. MS (ESI^+): $[M+H]^+$ = 300.1 ; HPLC Method 1 retention time = 8.4 min. TLC eluting with 100% EtOAc: Rf=0.65.

8.3 Synthesis of negative control drug-linker PSAR0 by click chemistry

Alkyne-glucuronide-MMAE (obtained as described above) and N-(2-azidoethyl)-2-(4-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)phenyl)acetamide (obtained as described above) were reacted and purified as described in the above section 4, using ACN/NMP/DCM (1:1:1 v/v/v) as reaction solvent.

Compound **PSAR0** was obtained as an off-white solid (10.3 mg / 48%). LC-HRMS *m/z* (ESI^+): Calc $[M+2H]^{2+}$ = 713.8425 ; Exp $[M+2H]^{2+}$ = 713.8415 ; Error = 1.3 ppm. HPLC Method 3 retention time = 10.2 min.

Preparation of ADCs

A solution of trastuzumab (10 mg/mL in PBS 7.4 + 1 mM EDTA) was treated with 14 molar equivalent of tris(2-carboxyethyl)phosphine (TCEP) for 2 hours at 37°C. The fully reduced antibody was buffer-exchanged with potassium phosphate 100 mM pH 7.4 + 1 mM EDTA by three rounds of dilution/centrifugation using Amicon 30K centrifugal filters device (Merck Millipore). 10 molar equivalents of drug-linker (from a 12 mM DMSO stock solution) was added to the antibody (residual DMSO <10% v/v). The solution was incubated 30 min at room temperature. The conjugate was buffer-exchanged/purified with PBS 7.4 using PD MiniTrap G-25 columns (GE Healthcare) and was sterile-filtered (0.20µm PES filter). Conjugates were diluted at 5 mg/mL with PBS 7.4 and were incubated at 37°C for 48h to promote complete hydrolysis of the succinimidyl moiety. Final protein concentration was assessed spectrophotometrically at 280 nm using a Colibri microvolume spectrometer device (Titertek Berthold). Mass recovery (yield) of the conjugation/purification procedure was routinely >90%.

Characterization of ADCs

RPLC-QToF

Thermo UltiMate 3000 UHPLC system + Bruker Impact II™ Q-ToF mass spectrometer. Mobile phase A was water + 0.1% formic acid and mobile phase B was acetonitrile + 0.1% formic acid. Column was an Agilent PLRP-S 1000Å 2.1x150mm 8µm (80°C). Gradient was 10%B to 50%B in 25 min. Flow rate was 0.4 mL/min. UV detection was monitored at 280 nm. The Q-ToF mass spectrometer was used in the m/z range 500-3500 (ESI^+). Data were deconvoluted using the MaxEnt algorithm included in the Bruker Compass® software. ADC samples were diluted with H_2O for injection (approx. 1.5 mg/mL final ADC concentration).

SEC

SEC was performed on an Agilent 1050 HPLC system having an extra-column volume below 15µL (equipped with short sections of 0.12mm internal diameter peek tubing and a micro-volume UV flow cell). Column was an Agilent AdvanceBio SEC 300Å 4.6x150mm 2.7µm (maintained at room temperature). Mobile phase was 100 mM sodium phosphate and 200 mM sodium chloride (pH 6.8). 10% acetonitrile (v/v) was added to the mobile phase to minimize secondary hydrophobic interactions with the stationary phase and prevent bacterial growth. Flow rate was 0.35 mL/min. UV detection was monitored at 280 nm.

HIC

Hydrophobic interaction chromatography (HIC) was performed on an Agilent 1050 HPLC system. Column was a Tosoh TSK-GEL BUTYL-NPR 4.6x35mm 2.5 µm (25°C). Mobile phase A was 1.5 M $(NH_4)_2SO_4$ + 25 mM potassium phosphate pH 7.0. Mobile phase B was 25 mM potassium phosphate pH 7.0 + 15% isopropanol (v/v). Linear gradient was 0%B to 100%B in 10 min, followed by a 3 min hold at 100%B. Flow rate was 0.75 mL/min. UV detection was monitored at 220 and 280 nm.

HER2-binding affinity assay

HER2-binding affinity of ADCs used in the present study was assessed by ELISA, as previously described.[3] 96-well EIA/RIA plates (Corning cat#3590) were coated overnight at 4°C with trastuzumab or ADCs (5 µg/mL in PBS, pH=7.4, 100 µL/well). The plates were washed 2 times with PBS-T (PBS + 0.05% Tween® 20) and blocked with 0.1% BSA in PBS-T for 1 hour at room temperature. Serial dilution of recombinant HER2-His Tag protein (Sino Biological cat#10004-H08H) were subsequently added and the plates were incubated for 2 hours at room temperature. After 5 washes with PBS-T, HRP-conjugated anti-6xHis antibody (Takara cat#631210) was added for 1 hour at room temperature to detect bound HER2. After 5 washes with PBS-T, TMB substrate solution (Thermo-Fisher cat#N301) was added. Peroxidase activity was stopped with 0.18M H_2SO_4 and absorbance read at 450 nm (reference wavelength 650 nm). Sigmoidal fittings were performed using GraphPad Prism 7 software.

PK studies in mice

ADCs were injected at 3 mg/kg in female SCID mice (4–6 weeks old — Charles River) *via* the tail vein (five animals per group, randomly assigned). Blood was drawn into citrate tubes via retro-orbital bleeding at various time points, processed to plasma and stored at -80°C until analysis. ADC concentration was assessed using a human IgG ELISA kit (Stemcell™ Technologies) according to the manufacturer's protocol. Standard curves of Trastuzumab were used for quantification. Pharmacokinetics parameters (clearance and AUC) were calculated by two-compartmental analysis using Microsoft® Excel® software incorporating PK functions (add-in developed by Usansky et al., Department of Pharmacokinetics and Drug Metabolism, Allergan, Irvine, USA).

PK studies in rats

ADCs were injected at 3 mg/kg in female Sprague-Dawley rats (4–6 weeks old — Charles River) *via* the tail vein (three animals per group, randomly assigned). Blood was drawn into citrate tubes via retro-orbital bleeding at various time points, processed to plasma and stored at -80°C until analysis. ADC concentration was assessed using a human IgG ELISA kit (Stemcell™ Technologies) according to the manufacturer's protocol. Standard curves of Trastuzumab were used for quantification. Pharmacokinetics parameters (clearance and AUC) were calculated by two-compartmental analysis using Microsoft® Excel® software incorporating PK functions (add-in developed by Usansky et al., Department of Pharmacokinetics and Drug Metabolism, Allergan, Irvine, USA).

Xenograft studies

BT-474 breast cancer cells were implanted subcutaneously in female SCID mice (4-5 weeks old). When tumors had grown to approximately 150 mm^3, ADCs were dosed once intravenously (tail vein) at a dose of 3 mg/kg (first study, n = 5 animals/group) or 2.5 mg/kg (second study, n = 6 animals/group). Median tumor growth was assessed by measuring individual tumor volumes every 3-7 days using a caliper device (formula $(L \times W^2)/2$). Mice were sacrificed when the tumor volume exceeded 1000 mm^3. No mice body-weight changes were observed during these studies.

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