Supplementary Material

Discovery and Preclinical Characterization of XMT-1660, an Optimized B7-H4-Targeted Antibody-Drug Conjugate for the Treatment of Cancer

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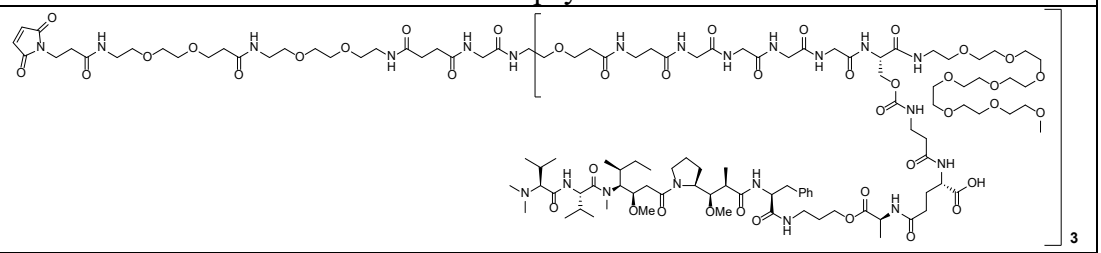
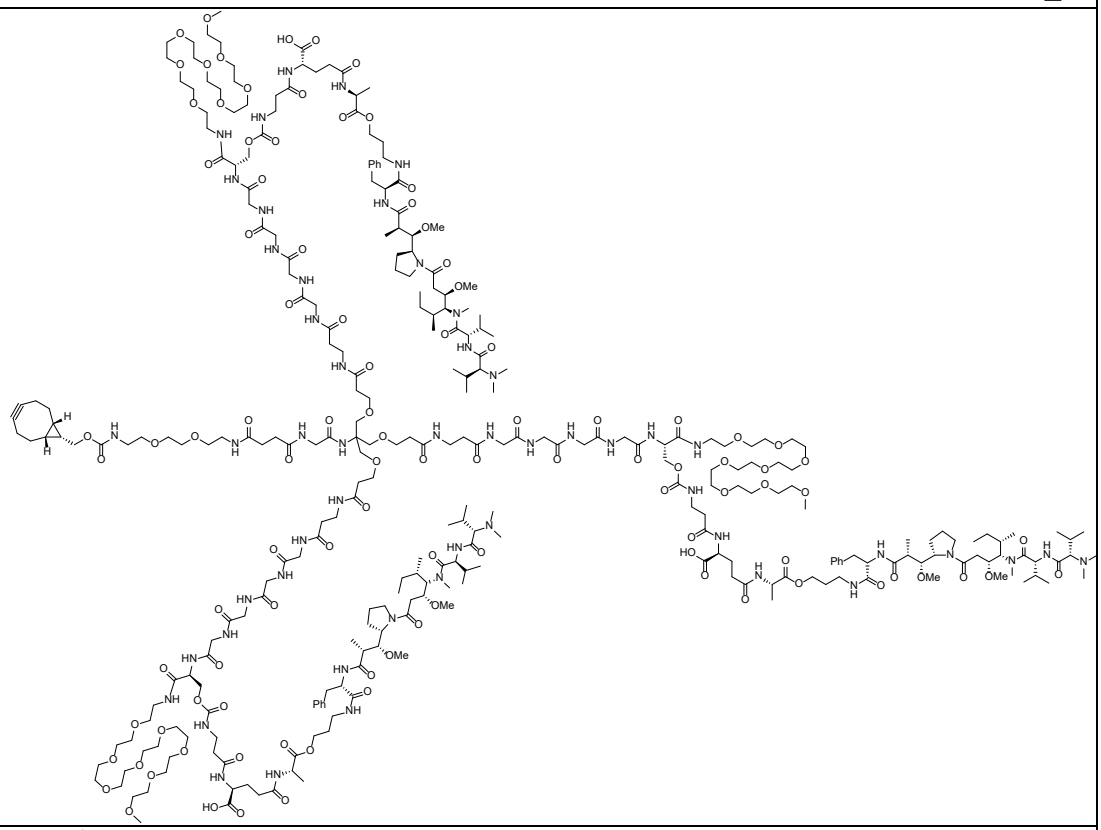
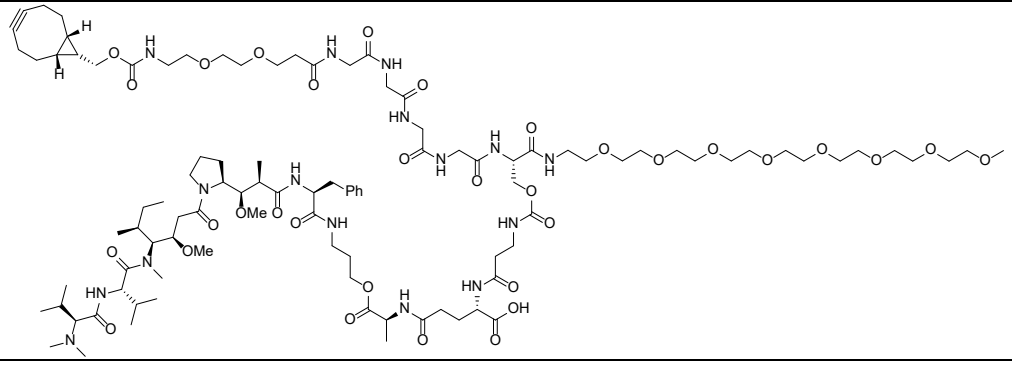
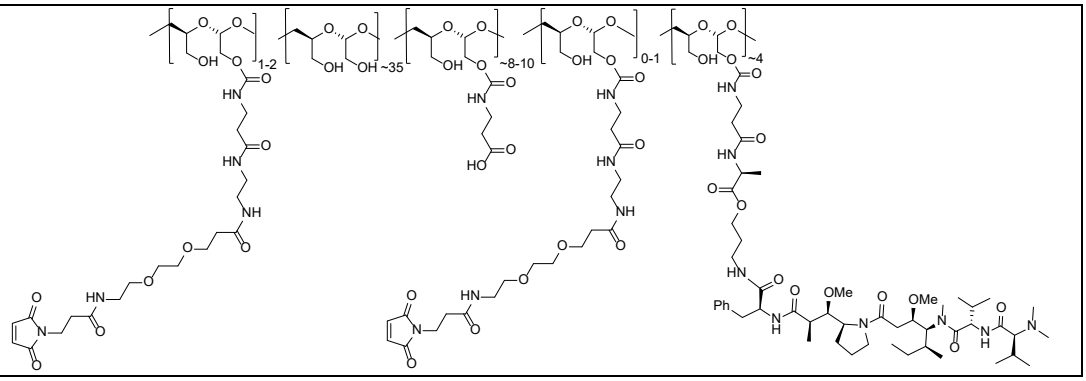
Supplementary Table 1 Structure of scaffold linker payloads used to generate ADCs

Construct	Scaffold linker payload structure
A	
B	
C	

D	

Supplementary Table 2. *In vitro* and *in vivo* key characteristics of trastuzumab Dolasynthen ADCs

	HER2 binding (Kd, nM)	**JIMT-1 Cytotoxicity (EC_{50}, nM) by toxin**	**Conjugated drug[a] AUC inf obs (day*ng/mL)**
ADC1	1.746	0.946	14400
ADC2	1.453	0.913	13300
ADC3	1.577	1.301	24900
ADC4	1.269	0.7173	27800

[a] following single dose of 0.199 mg/kg payload in tumor bearing mice

Supplementary Table 3. Binding of XMT-1660 and Unconjugated mAb to Recombinant B7-H4 Proteins from Human, Monkey, Rat, and Mouse

	Recombinant B7-H4, EC_{50}* (nM)			
Test Articles	Human	Monkey	Rat	Mouse
XMT-1660	0.21	0.21	0.15	0.38
Unconjugated mAb (XMT-1604)	0.24	0.17	0.19	0.30
Rituximab DS DAR6 (Control non-binding ADC)	>100	>100	>100	>100
Palivizumab (Control non-binding mAb)	>100	>100	>100	>100

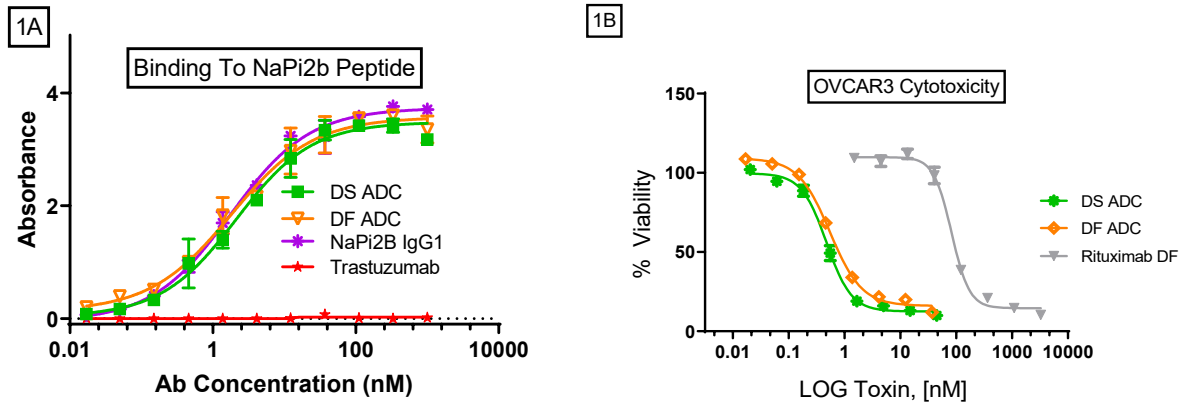
*: EC_{50} is the average value from two independent ELISA experiments.

Supplementary Table 4. Tumor Responses for the Mouse Xenograft Efficacy Studies

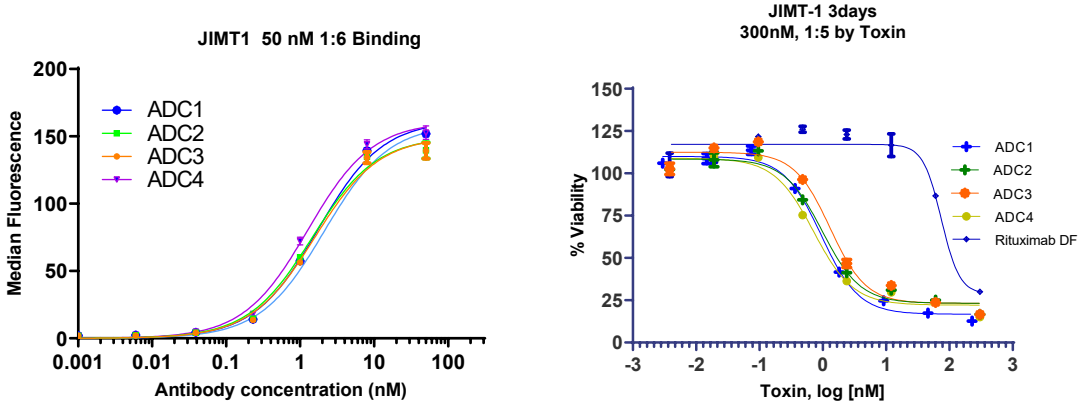
In vivo Model	Test Article	Dose (mAb/payload)	Tumor Responses
XTS-2148 HBCx-24	XMT-1660 DS DAR 6	2.3 / 0.075 mg/kg	3/10 PR & 4/10 CR[a]
XTS-2148 HBCx-24	XMT-1660 DS DAR 6	4.7 / 0.15 mg/kg	10/10 CR & 3/10 TFS[a]
XTS-2148 HBCx-24	B7-H4 DF DAR 12	2.3 / 0.15 mg/kg	3/10 PR, 6/10 CR1/10 TFS[a]
MX-1-e305	XMT-1660 DS DAR 6	4.7 / 0.15 mg/kg	10/10 CRs & 7/10 TFS[b]
MX-1-e305	XMT-1660 DS DAR 6	2.3 / 0.075 mg/kg	2/10 PRs[b]
MX-1-e305	B7-H4 DF DAR 12	2.3 / 0.15 mg/kg	1/10 CR[b]
E2817-U2110 OV2423	XMT-1660 DS DAR 6	4.6 / 0.15 mg/kg	1/8 PR & 7/8 TFS[c]
1125-111 CTG-1692	XMT-1660 DS DAR 6	4.6 / 0.15 mg/kg	1/8 PR[c]
E3817-U2203 mBR9013	XMT-1660 DS DAR6	4.6 / 0.15 mg/kg	8/8 TFS[c]

Tumor response criteria: [a] PR (Partial Regression) = number of mice presenting a tumor size lower than initial tumor size during 3 consecutive measurements. CR (Complete Regression) = number of mice presenting a 0 to 13 mm3 tumor size during at least 2 consecutive measurements. TFS (Tumor Free Survivor) = number of complete regressions recorded up to Group Day End. [b] In a PR response, the tumor volume was 50% or less of its Day 1 volume for three consecutive measurements during the study, and equal to or greater than 13.5 mm3 for one or more of these three measurements. In a CR response, the tumor volume was less than 13.5 mm3 for three consecutive measurements during the study. An animal with a CR response at the termination of a study was additionally classified as a tumor-free survivor (TFS). [c] Partial responder (PR): TV ≤ 30% of TV at Day 0 for 2 consecutive measurements Complete responder (CR): No measurable tumor for 2 consecutive measurements Tumor-free survivor (TFS): A CR that persists until study completion.

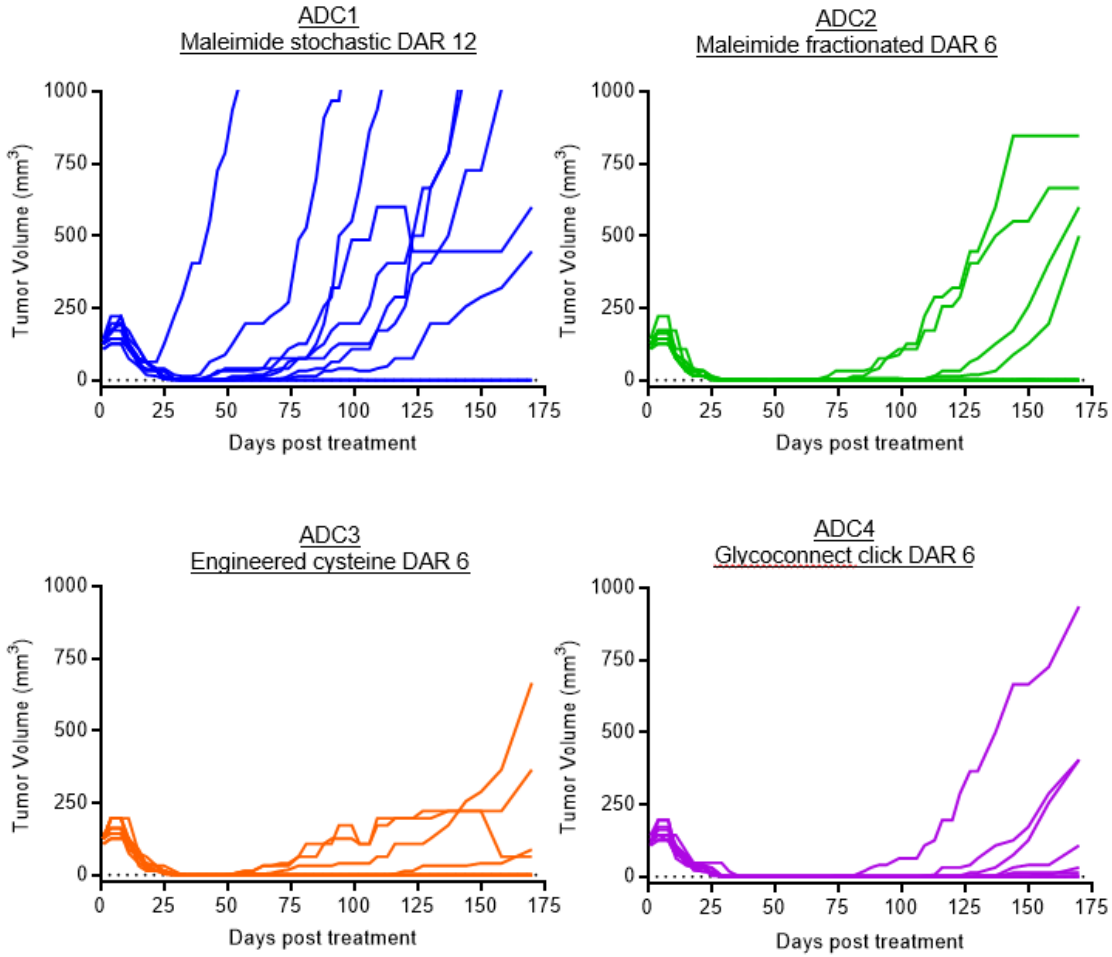
Supplementary Figure 1. *In vitro* binding and cytotoxicity of DS and DF ADCs



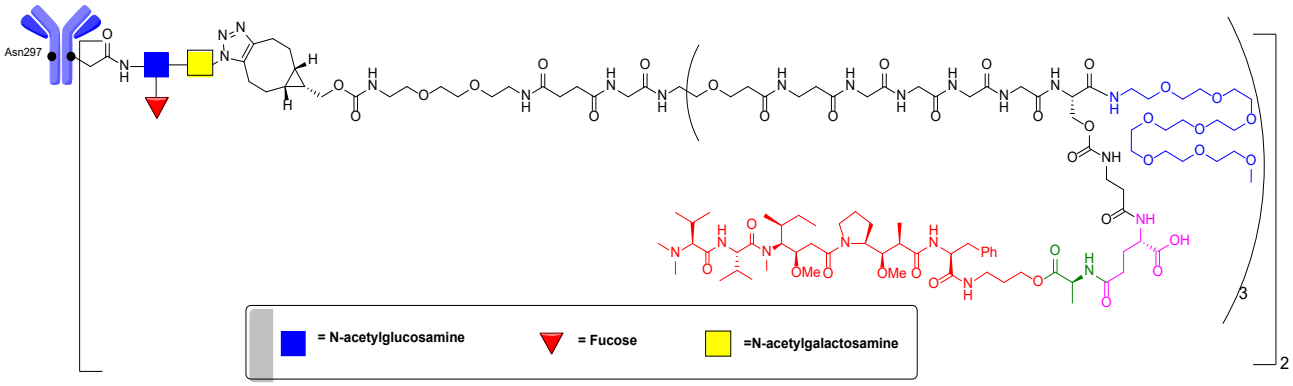
Supplementary Figure 2. *In vitro* binding and cytotoxicity of trastuzumab DS ADCs



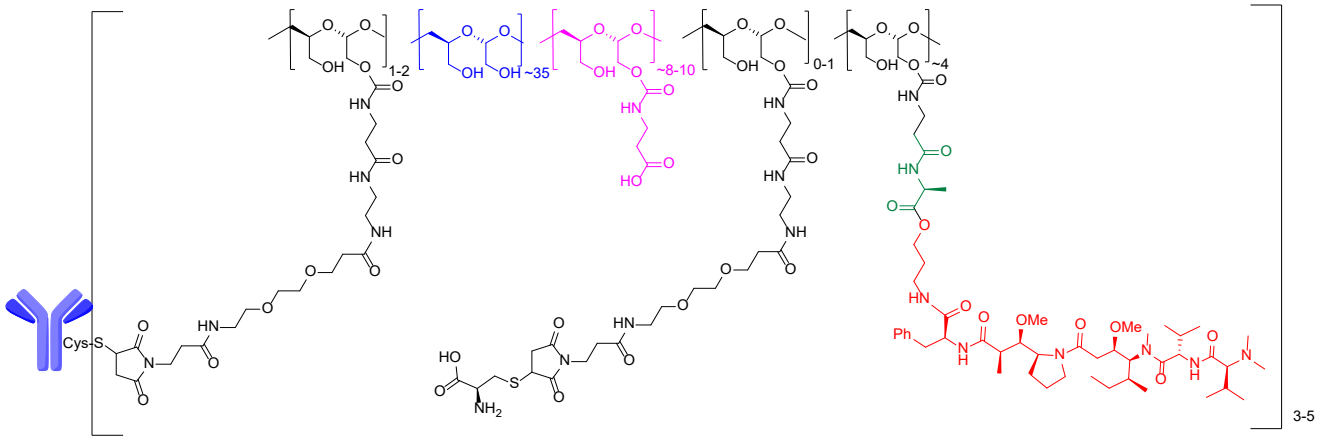
Supplementary Figure 3. JIMT-1 efficacy tumor growth inhibition following a single dose of 0.067mg/kg AF HPA



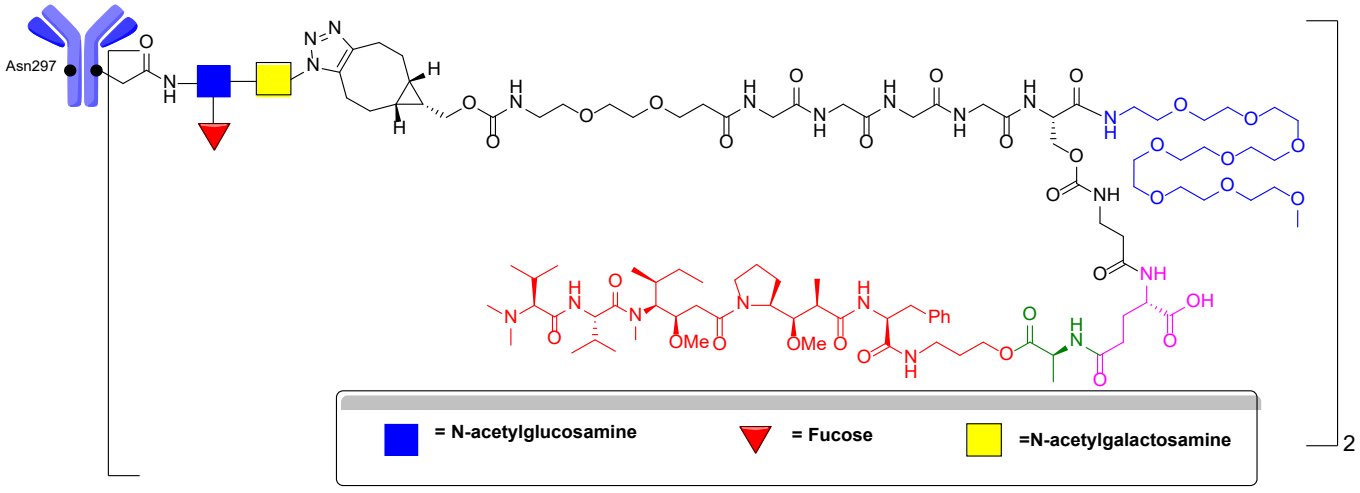
Supplementary Figure 4. Structure of B7-H4 ADCs



XMT-1660 B7-H4 DS DAR 6

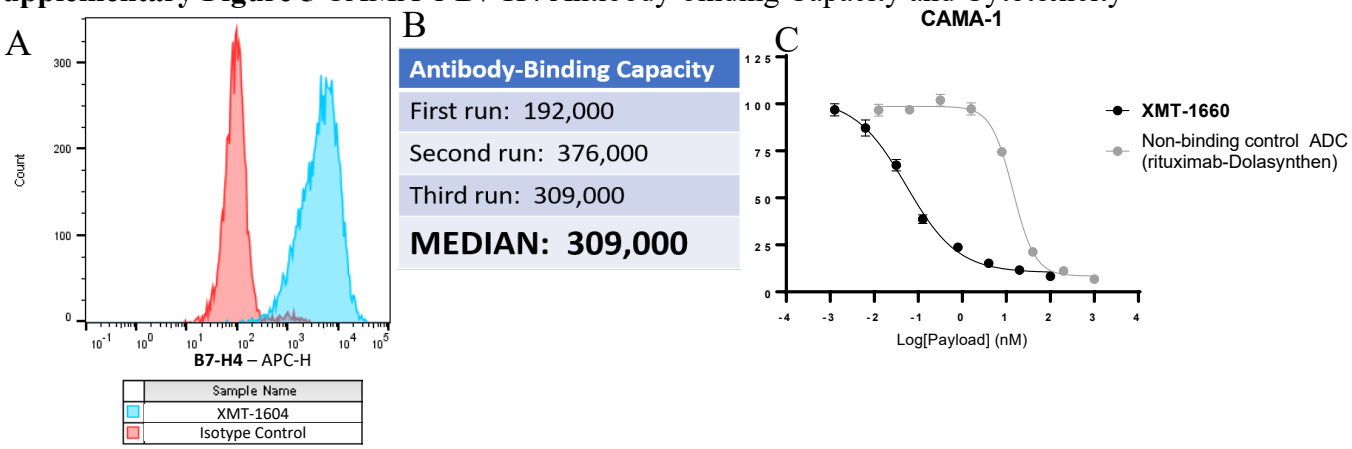


B7-H4 DF DAR 12



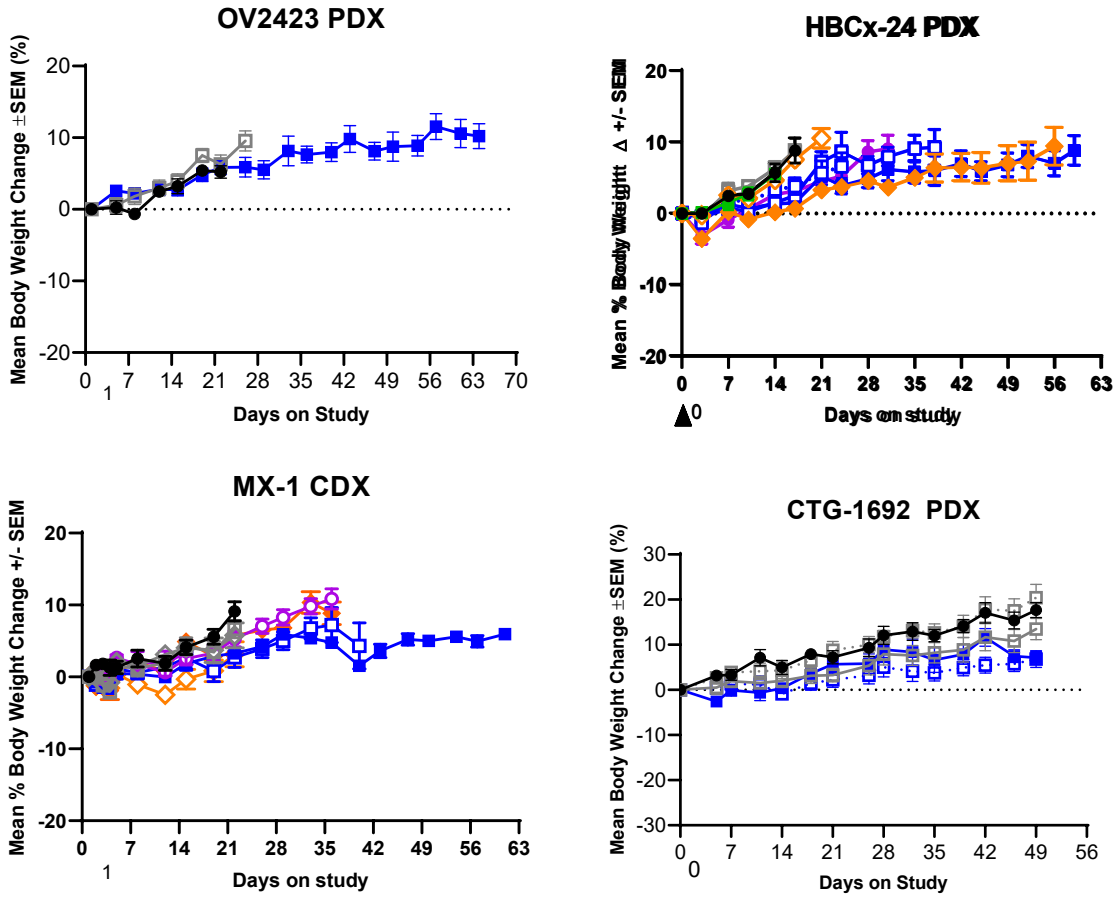
B7-H4 DS DAR 2

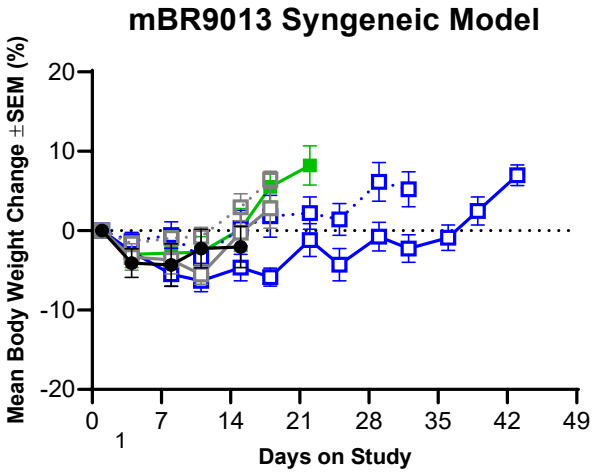
Supplementary Figure 5 CAMA-1 B7-H4 Antibody-binding Capacity and Cytotoxicity



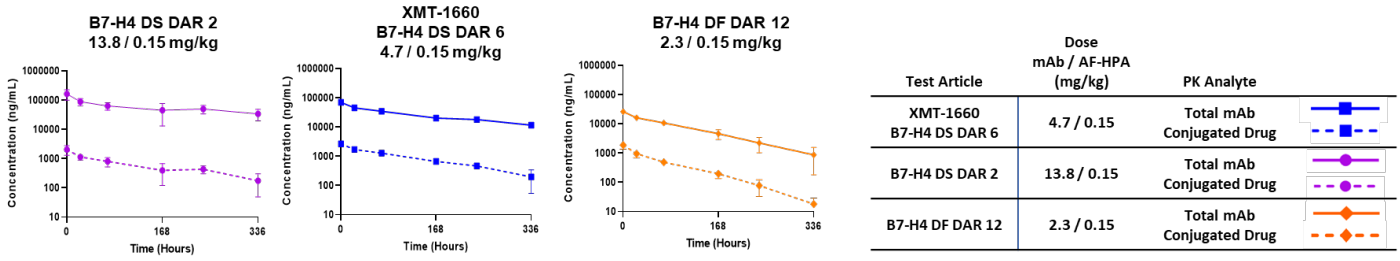
A, Flow cytometry with unconjugated B7-H4 antibody on CAMA-1 cells B, Quantitative assessment of antibody-binding capacity on CAMA-1 cells using the Quantum Simple Cellular Kit (see Supplemental Methods). C, Cytotoxicity of XMT-1660 against CAMA-1 cells.

Supplementary Figure 6. Body Weight Changes for In vivo efficacy studies



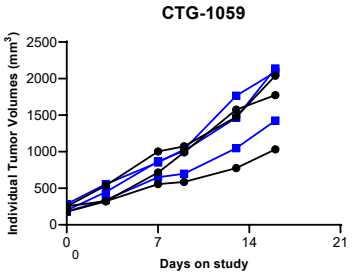


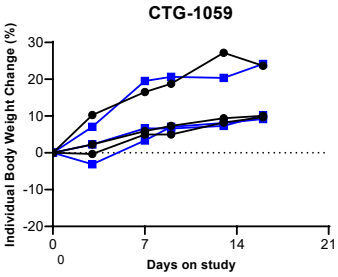
Supplementary Figure 7 Mouse Pharmacokinetics following single dose ADC administration in MX-1 xenograft tumor model

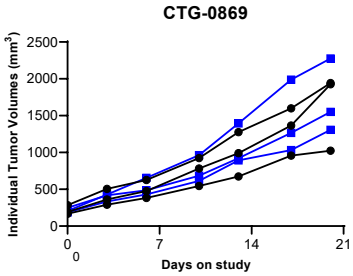


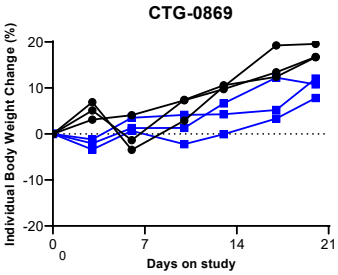
Plasma levels of Total mAb (solid line) and conjugated drug (dashed line) in MX-1 tumor-bearing animals following a single IV dose of 0.15 mg/kg payload. As micro sampling techniques were used, free drug was not analyzed.

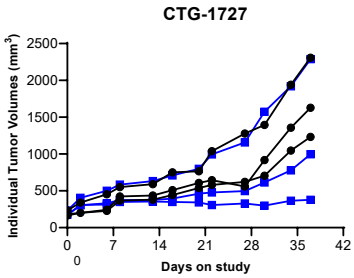
Supplementary Figure 8 Tumor Volume and Body Weight Changes for the Breast Cancer PDX Panel

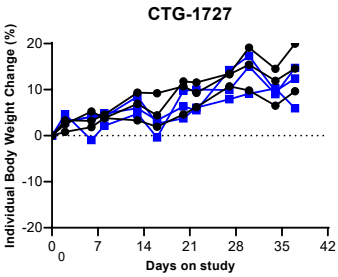


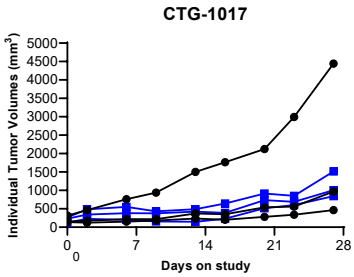


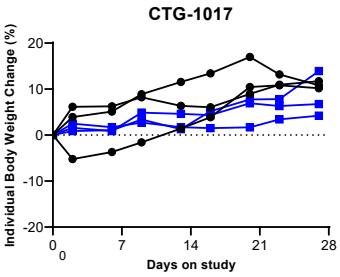


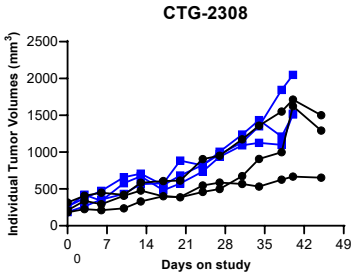


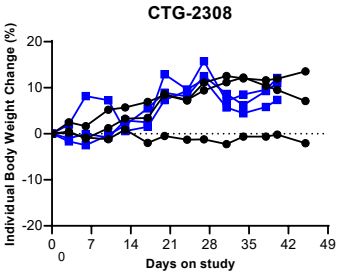


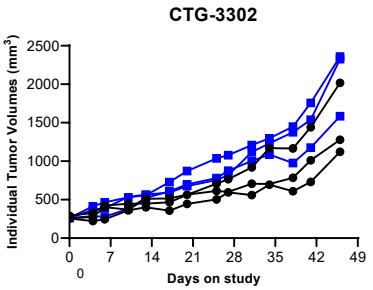








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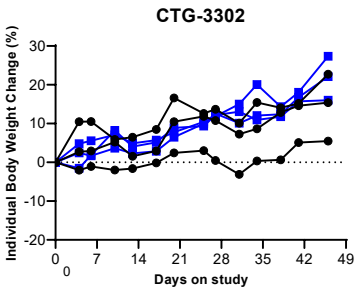
1000
500
0
0 7 14 21 28 35 42 49
0
Days on study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Individual Body Weight Change (%)
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-10
-20
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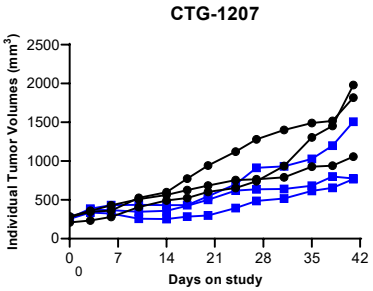
0
Days on study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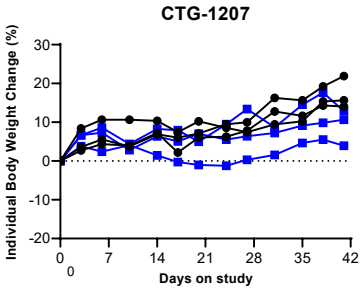
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Individual Body Weight Change (%)
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-10
-20
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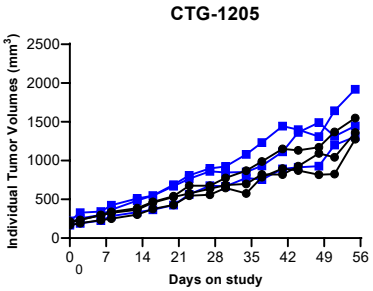
Days on study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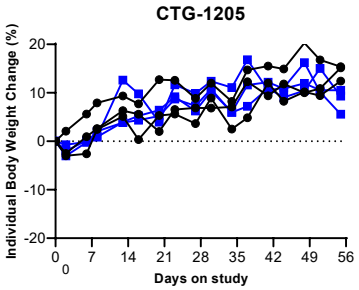
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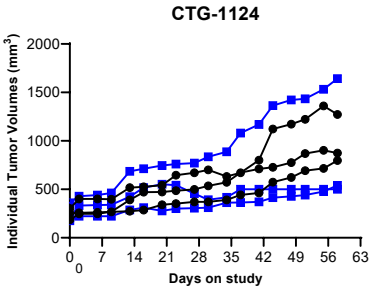
Days on study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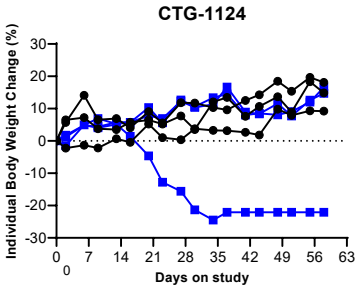
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-30
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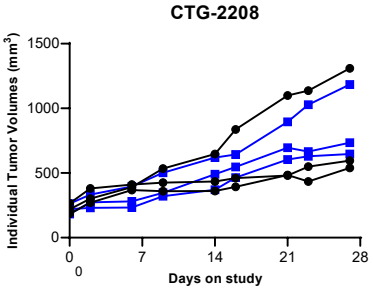
Days on study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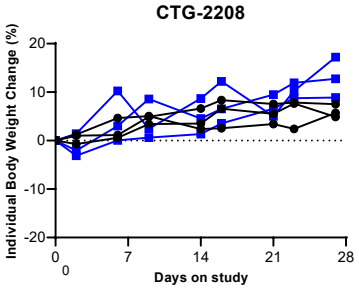
Days on study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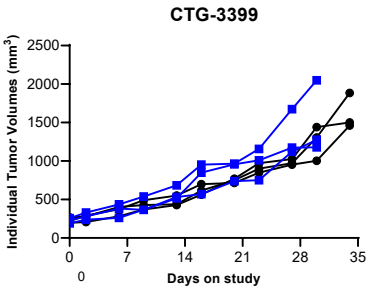
-20
0 7 14 21 28
0
Days on study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1000
500
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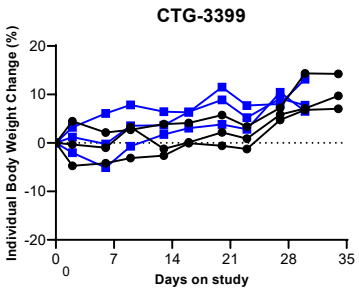
0
Days on study


CTG-3399
Individual Body Weight Change (%)
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10
0
-10
-20
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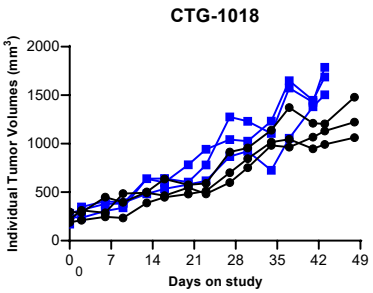
0
Days on study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Individual Tumor Volumes (mm3)
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1000
500
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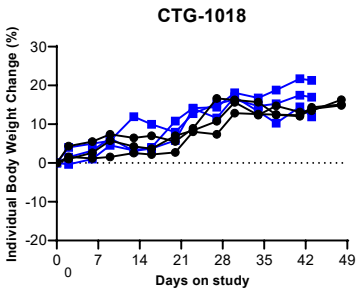
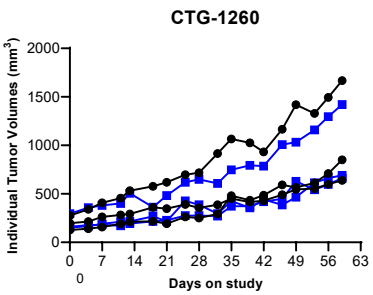
0
Days on study


CTG-1018
Individual Body Weight Change (%)
30
20
10
0
-10
-20
0 7 14 21 28 35 42 49
0
Days on study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Individual Tumor Volumes (mm3)
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1000
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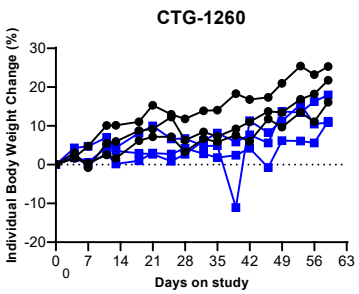
0
Days on study


CTG-1260
Individual Body Weight Change (%)
30
20
10
0
-10
-20
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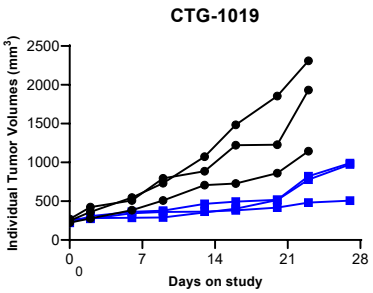
0
Days on study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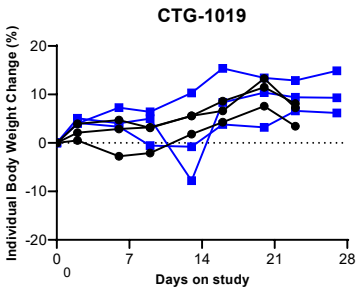
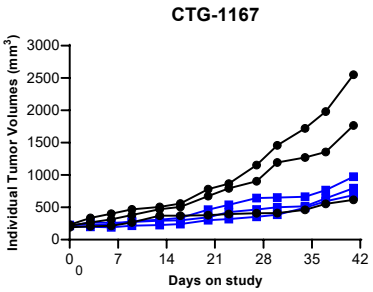
2000
1500
1000
500
0
0 7 14 21 28
0
Days on study


CTG-1019
Individual Body Weight Change (%)
20
10
0
-10
-20
0 7 14 21 28
0
Days on study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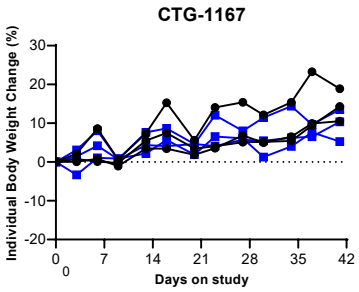
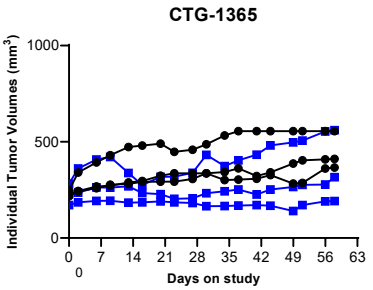
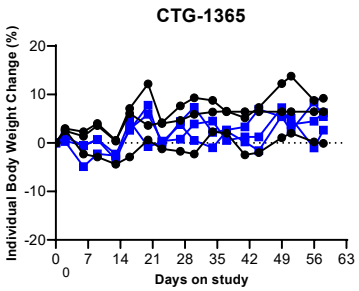
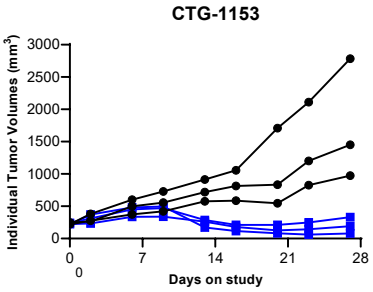
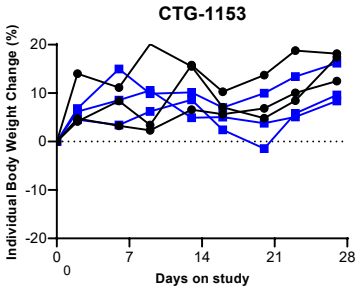
3000
2500
2000
1500
1000
500
0
0 7 14 21 28 35 42
0
Days on study

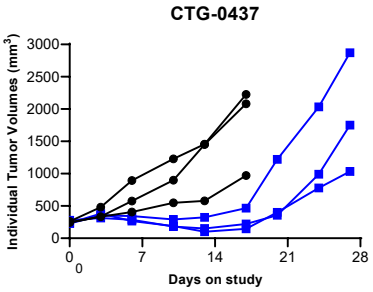

CTG-1167
Individual Body Weight Change (%)
30
20
10
0
-10
-20
0 7 14 21 28 35 42
0
Days on study

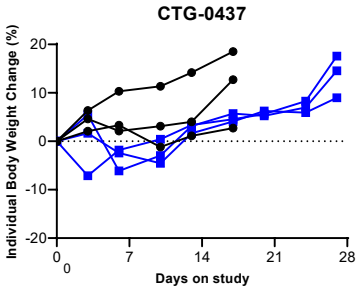

CTG-1365
Individual Tumor Volumes (mm3)
1000
500
0
0 7 14 21 28 35 42 49 56 63
0
Days on study

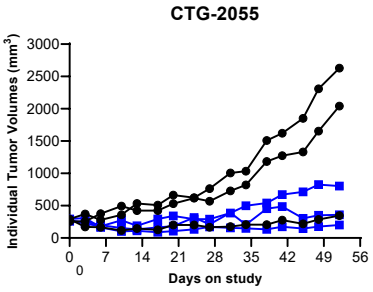

CTG-1365
Individual Body Weight Change (%)
20
10
0
-10
-20
0 7 14 21 28 35 42 49 56 63
0
Days on study

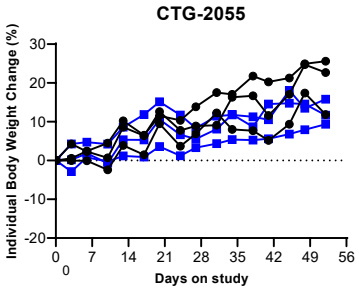

CTG-1153
Individual Tumor Volumes (mm3)
3000
2500
2000
1500
1000
500
0
0 7 14 21 28
0
Days on study

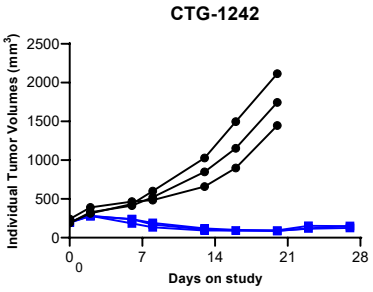

CTG-1153
Individual Body Weight Change (%)
20
10
0
-10
-20
0 7 14 21 28
0
Days on study

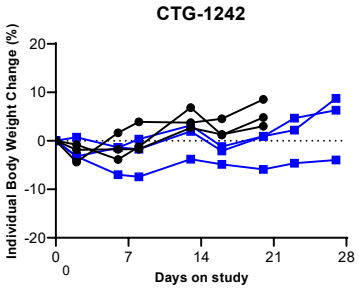

CTG-0437
Individual Tumor Volumes (mm3)
3000
2500
2000
1500
1000
500
0
0 7 14 21 28
0
Days on study

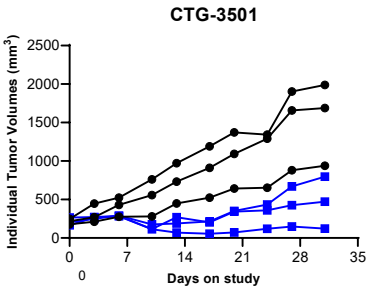

CTG-0437
Individual Body Weight Change (%)
20
10
0
-10
-20
0 7 14 21 28
0
Days on study

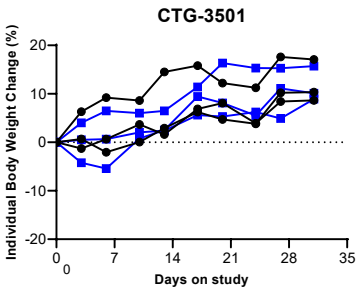

CTG-2055
Individual Tumor Volumes (mm3)
3000
2500
2000
1500
1000
500
0
0 7 14 21 28 35 42 49 56
0
Days on study

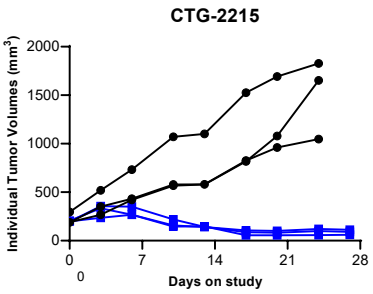

CTG-2055
Individual Body Weight Change (%)
30
20
10
0
-10
-20
0 7 14 21 28 35 42 49 56
0
Days on study

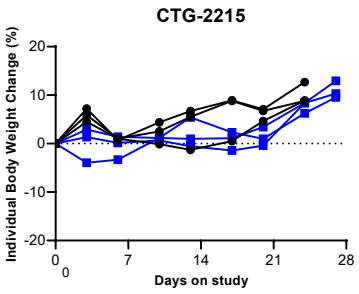

CTG-1242
Individual Tumor Volumes (mm3)
2500
2000
1500
1000
500
0
0 7 14 21 28
0
Days on study

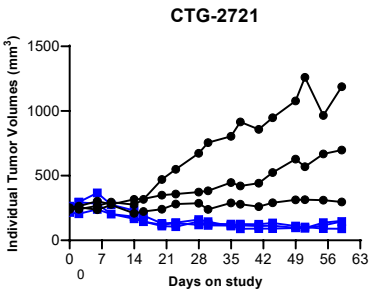

CTG-1242
Individual Body Weight Change (%)
20
10
0
-10
-20
0 7 14 21 28
0
Days on study

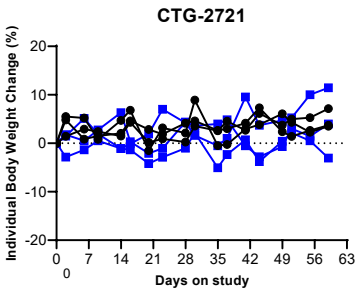

CTG-3501
Individual Tumor Volumes (mm3)
2500
2000
1500
1000
500
0
0 7 14 21 28 35
0
Days on study

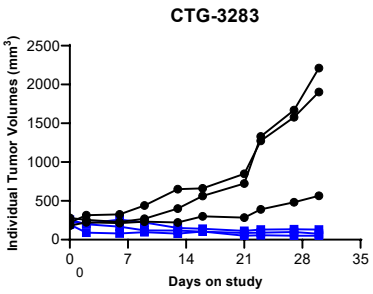

CTG-3501
Individual Body Weight Change (%)
20
10
0
-10
-20
0 7 14 21 28 35
0
Days on study

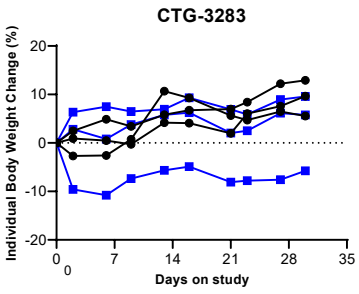

CTG-2215
Individual Tumor Volumes (mm3)
2000
1500
1000
500
0
0 7 14 21 28
0
Days on study

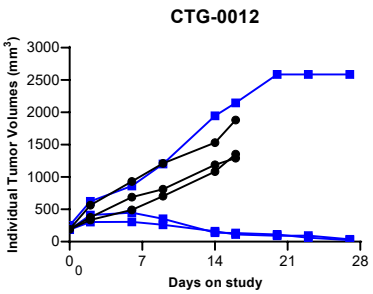

CTG-2215
Individual Body Weight Change (%)
20
10
0
-10
-20
0 7 14 21 28
0
Days on study

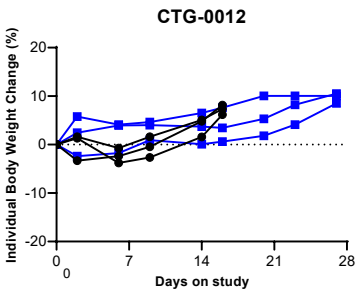

CTG-2721
Individual Tumor Volumes (mm3)
1500
1000
500
0
0 7 14 21 28 35 42 49 56 63
0
Days on study

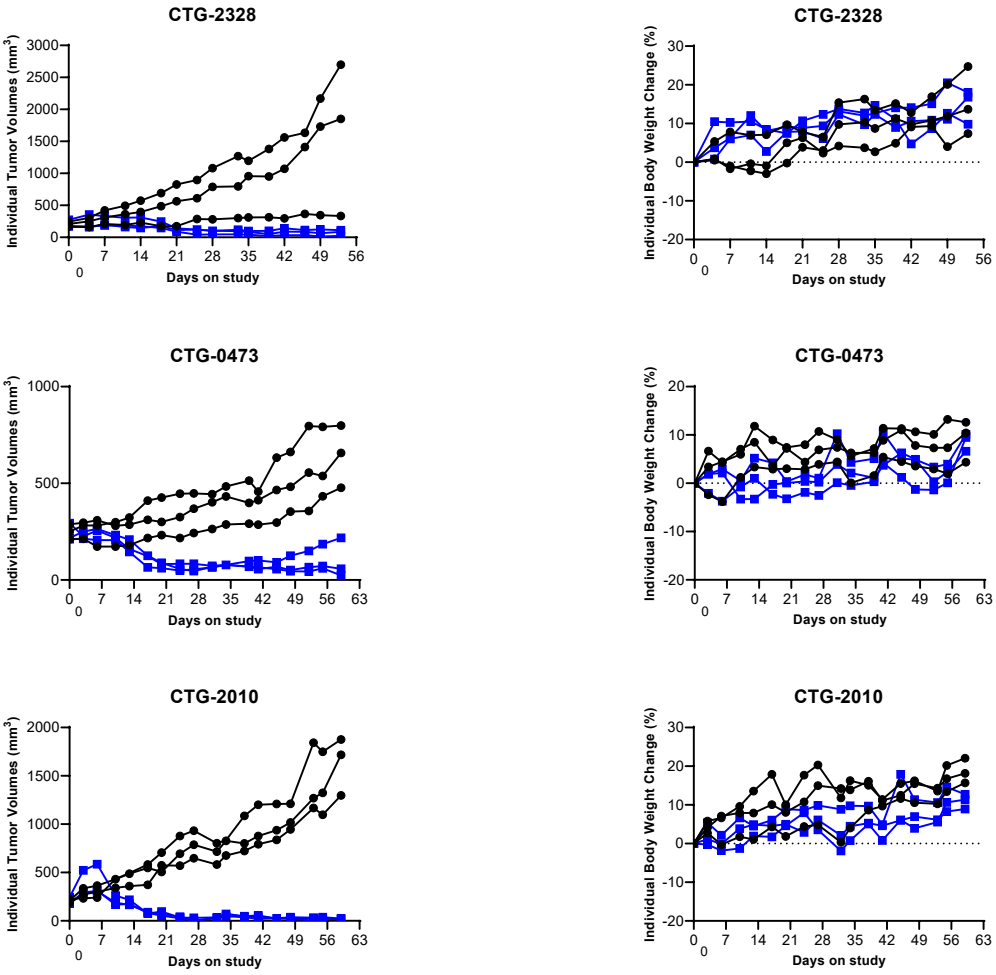

CTG-2721
Individual Body Weight Change (%)
20
10
0
-10
-20
0 7 14 21 28 35 42 49 56 63
0
Days on study


CTG-3283
Individual Tumor Volumes (mm3)
2500
2000
1500
1000
500
0
0 7 14 21 28 35
0
Days on study

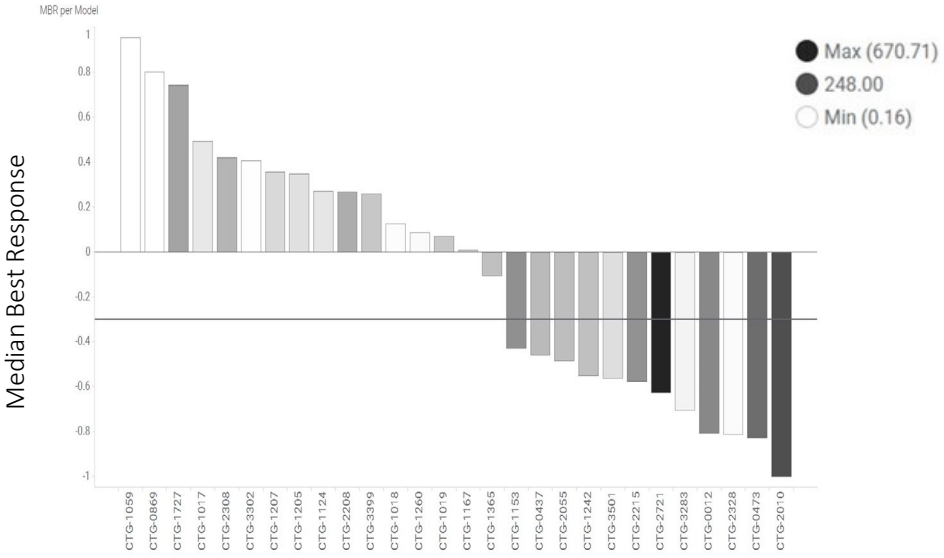

CTG-3283
Individual Body Weight Change (%)
20
10
0
-10
-20
0 7 14 21 28 35
0
Days on study


CTG-0012
Individual Tumor Volumes (mm3)
3000
2500
2000
1500
1000
500
0
0 7 14 21 28
0
Days on study

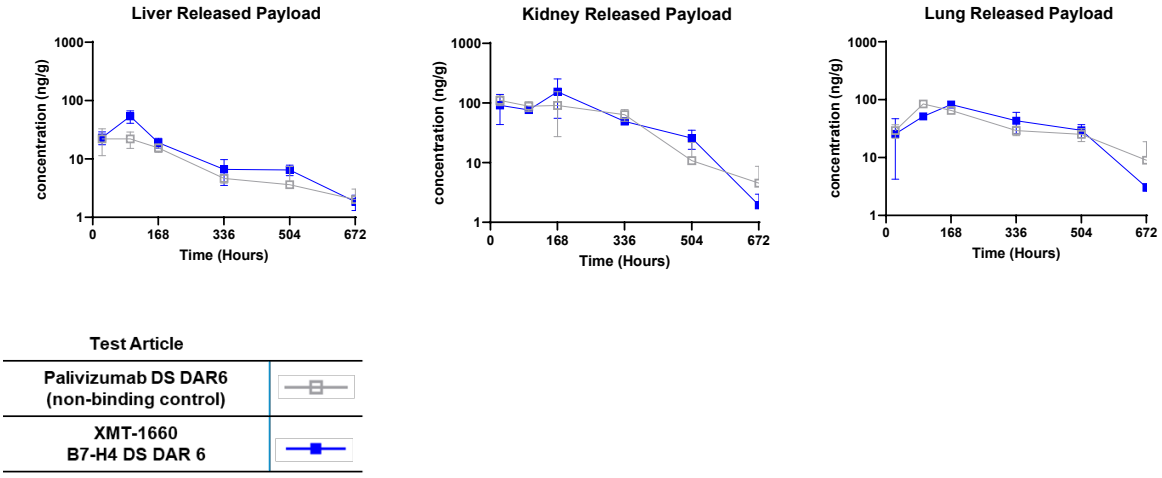

CTG-0012
Individual Body Weight Change (%)
20
10
0
-10
-20
0 7 14 21 28
0
Days on study



Supplementary Figure 9. Waterfall plot of MBR in the Breast Cancer PDX Panel with mRNA Level Shown.



Supplementary Figure 10 Biodistribution of XMT-1660 in the Rat



Male Sprague Dawley Rats received a single 9 mg/kg (mAb) IV dose of either XMT-1660 or a control DS DAR 6 ADC. At each timepoint n=3/group were euthanized, and tissues were sampled for bioanalysis. Plots depict "Released Payload" (Free AF-HPA and Free AF) and show comparable distribution of Released Payload between XMT-1660 and non-targeting control ADC in Liver, Kidney & Lung

Supplementary Materials and Methods

Antibody-binding capacity of unconjugated B7-H4 antibody (XMT-1604) on CAMA-1 breast cancer cells was determined with the Quantum Simple Cellular Kit (Bangs Laboratories, Inc., Cat# 819). Beads and CAMA-1 cells were stained with XMT-1604–AF647 (~1.1 fluorophores per antibody) at a final concentration of 1 µg/ml. Beads and cells were run on MACSQuant 10 on the same day using the same instrument settings. FlowJo was used to generate Geometric Mean channel values for each sample. QuickCal, v. 3,0 Data Analysis Template (provided by Bangs Laboratories) was used to calculate antibody binding capacity of CAMA-1. Three biological replicates were performed.

XMT-1604 Amino Acid Sequence

Heavy Chain:

EVQLVESGGGLIQPGGSLRLSCAASGFIVSRNYMNWVRQAPGKGLEWVSVIYGSGRTDSADSVK
GRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARDADYGLDVWGQGTTVTVSSASTKGPSVFPL
APSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL
GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEV
TCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNG
QPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

Light Chain:

EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSG
SGTDFTLTISRLEPEDFAVYYCQQYGSSPLYTFGQGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASV
VCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACE
VTHQGLSSPVTKSFNRGEC

Syntheses of Constructs A-C

ABBREVIATIONS

ACN Acetonitrile

Alloc Allyloxycarbonyl

AcOH Acetic acid

BOC tert-butyloxycarbonyl

CDI Bis(1H-imidazol-1-yl)methanone

HOAt 3H-[1,2,3]triazolo[4,5-b]pyridin-3-ol

EDC.HCl 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride

EDTA Ethylenediaminetetraacetic acid

DCM Dichloromethane

DIEA *N,N*-Diisopropylethylamine

DMAP *N,N*-Dimethylpyridin-4-amine

DMF Dimethylformamide

NMP N-Methylpyrrolidone

GENERAL INFORMATION

^{1}H NMR spectra were recorded on either Bruker 400 or 500 MHz NMR spectrometers using deuterated solvents as stated. Temperatures are given in degrees Celsius (°C); operations were carried out at room temperature or ambient temperature, that is, in the range 18−25 °C. Chemical shifts are expressed in parts per million (ppm, δ units). The reference was set to either TMS peak or the residual non-deuterated solvent peak. Coupling constants are given in units of hertz (Hz). Splitting patterns describe apparent multiplicities and are designated as s (singlet), d (doublet), dd (doublet−doublet), t (triplet), q (quartet), m (multiplet), and br s (broad singlet).

Purity was determined as follows:

Instrument: Waters Acquity UPLC I-class coupled to Bruker Compact QTOF. The method used was: Mobile Phase A: Water + 0.1% Formic Acid; Mobile Phase B: Acetonitrile + 0.1% Formic Acid; Column: Agilent InfinityLab Poroshell 120 EC-C18, 2.1 x 50 mm, 1.9 µm. Gradient: time 0 min 5%B, time 12 min 36.5% B, time 14 min 65% B time 16 min 95% B held at 95%B for 2 min; flow rate 0.45 mL/min, Column temperature: 50 deg °C, DAD: 200 - 500 nm, MS detection: m/z 50-3000, ESI+ only, end plate offset 500 V, Capillary 3500 V, Nebulizer 3.0 bar, Dry gas 7.5 l/min, Dry temp 250 °C; DAD trace (200-500 nm) is used to determine area % purity. MS traces are used for mass confirmation.

Reverse-phase semi-prep chromatography was performed with Gilson systems using a Gemini 5 µm C18 110A, LC column 100 x 21.2mm reverse-phase HPLC column in water/MeCN with 0.1% TFA, 0.1% ammonium acetate, or 0.1% formic acid as mobile phase or on a medium pressure Teledyne ISCO EZ Prep. Most of the reactions described were monitored by thin-layer chromatography on 0.25 mm E. Merck silica gel plates (60F-254), visualized with UV light or LC−MS. Flash column chromatography was performed on an ISCO system Teledyne ISCO Combiflash Nextgen 300+ (4700 Superior Street, Lincoln, NE) unless otherwise mentioned using silica gel cartridges.

8,8-bis((2-carboxyethoxy)methyl)-3,6-dioxo-1-phenyl-2,10-dioxa-4,7-diazadodecane-12-carboxylic acid was purchased from eNovation Chemicals LLC, Bridgewater, NJ.

2,5-dioxopyrrolidin-1-yl3-(2-(2-(3-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1yl)propanamido)ethoxy)ethoxy)propanoate was purchased from QuantaBiodesign Ltd,

Boc-Glu(OtBu)-OH, di-tert-butyl dicarbonate, (S)-benzyl 2-((tert-butoxycarbonyl)amino)-3-hydroxypropanoate, 3-methoxy-3-oxopropan-1-ammonium chloride, Boc-Asp(OAll)-OH were purchased from Sigma-Aldrich

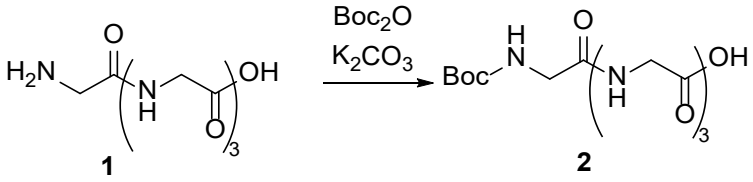
2-(2-(2-(2-aminoacetamido)acetamido)acetamido)acetic acid was purchased from Bachem Americas Inc. Torrance, CA.

H_2N-PEG8-OMe was purchased from ChemPep Inc., Wellington, FL.

tert-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate was purchased from A1 BioChem Labs, Wilmington, NC.

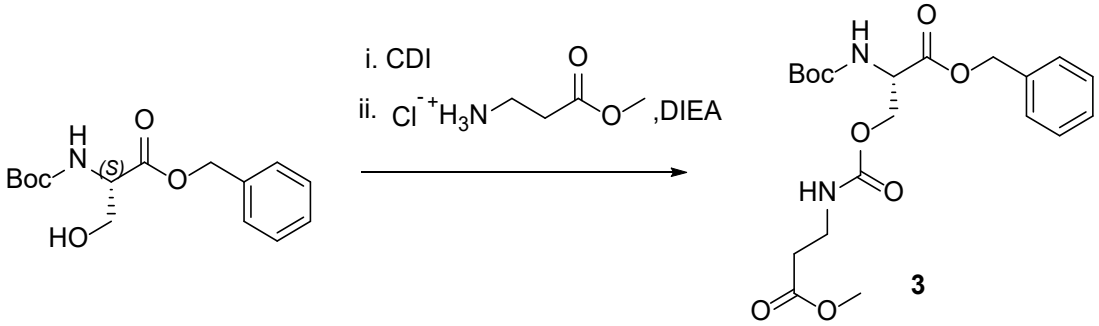
Synthesis of Dolasynthen Construct A

Synthesis of (tert-butoxycarbonyl)glycylglycylglycylglycine, compound **2**



Potassium carbonate (2.245 g, 16.25 mmol) was dissolved in dioxane/water (2:1, 120 mL) at room temperature, then 2-(2-(2-(2-aminoacetamido)acetamido)acetamido)acetic acid (compound **1**, 2 g, 8.12 mmol) was added in one portion and the mixture was stirred until the reagents dissolved. Then di-tert-butyl dicarbonate (3.55 g, 16.25 mmol) in 1,4-dioxane (5 ml) was added dropwise and the reaction mixture was stirred at room temperature for 25 hours at which point LC-MS analysis indicated the reaction was complete. The crude mixture was neutralized to ~pH 7 with 1 M HCl, then adjusted to pH 2 with citric acid (0.9 equivalents), then concentrated by rotary evaporation and purified by RP C18 column CombiFlash chromatography to give compound **2** (2.57 g, 91% yield). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 8.06-8.20 ppm (m, 2H, 2NH), 8.01 (bs, 1H, NH), 6.98 (bs, 1H, NH), 3.73 (s, 6H, 3CH_2), 3.5-3.6 (d, 2H, CH_2), 1.37 (s, 9H, *t*-Bu). ESI MS: $C_{13}H_{22}N_4O_7$ $[M+H]^+$ 347.16 found 347.10.

Synthesis of Boc-Ser(O-β-Ala-OMe)-OBenzyl, compound **3**



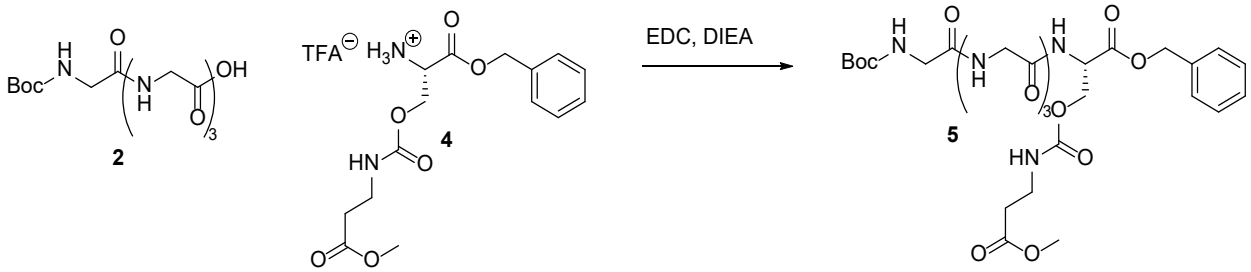
CDI **(**0.659 g, 4.06 mmol) was added in one portion to (S)-benzyl 2-((tert-butoxycarbonyl)amino)-3-hydroxypropanoate (1 g, 3.39 mmol) in THF (5 mL) at 0 °C. The reaction mixture was stirred at room temperature for 1 hour at which point LC-MS analysis indicated the reaction was complete. Then 3-methoxy-3-oxopropan-1-ammonium chloride (1.182 g, 8.47 mmol) and DIEA (1.774 ml, 10.16 mmol) in DMF (3 mL) was added and the stirring continued at room temperature for 3.5 hours at which point LC-MS indicated the reaction was complete. The crude mixture was concentrated, neutralized, purified by RP C18 column CombiFlash chromatography to give Boc-Ser(O-β-Ala-OMe)-OBenzyl **3** (1.085 g, 75% yield).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.3-5.42 (bs, 1H, NH), 5.1-5.28 (m, 3H, OCH_2-Ar), 4.5-4.6 (bs, 1H, CHN), 4.25-4.38 (dd, 2H, CH_2O), 3.71 (s, 3H, OMe), 3.35-3.50 (m, CH_2N), 2.53 (t, 2H, CH_2CO), 1.45 (s, 9H, *t*-Bu). ESI MS: $C_{20}H_{28}N_2O_8$ $[M+Na]^+$ 447.17; found 447.10.

Synthesis of (S)-3,7,11-trioxo-13-phenyl-2,8,12-trioxa-6-azatridecan-10-aminium trifluoroacetate, compound **4**

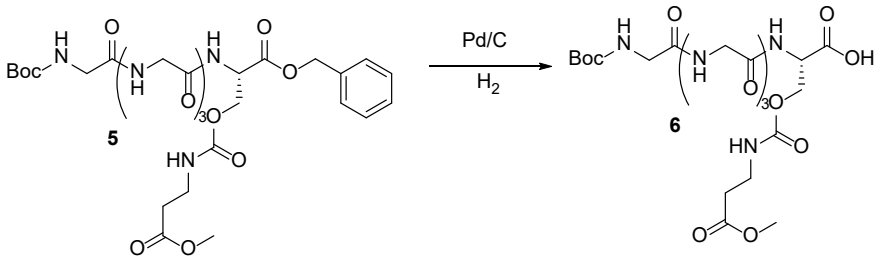
Boc-Ser(β-Ala-OMe)-OBenzyl (1.085 g, 2.56 mmol) was dissolved in DCM (3.3 mL) containing TFA (1.1 mL) at 0 °C for 1 hour and then stirred at room temperature for 30 minutes until LC-MS indicated the reaction was complete. The TFA salt of benzyl 2-amino-3-(((3-methoxy-3-oxopropyl)carbamoyl)oxy)propanoate was concentrated by rotary evaporation and use as is for synthesis of compound **5**.

Synthesis of benzyl N-(tert-butoxycarbonyl)glycylglycylglycylglycyl-O-((3-methoxy-3-oxopropyl)carbamoyl)-L-serinate, compound **5**



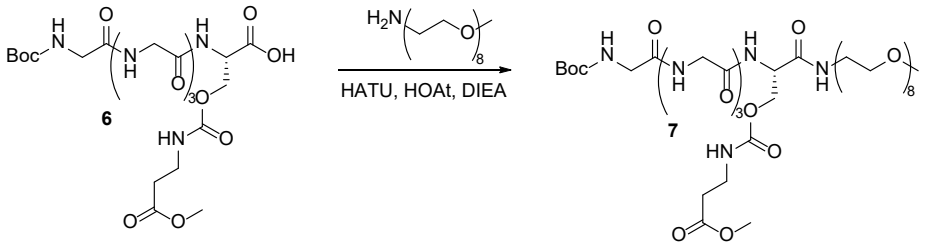
Compound **2** (805 mg, 2.324 mmol) was dissolved in DMF (17 mL), cooled to 0 ºC and NHS (401 mg, 3.49 mmol) was added followed by EDC (668 mg, 3.49 mmol) in DMF (6 mL). Separately the TFA salt of benzyl 2-amino-3-(((3-methoxy-3-oxopropyl)carbamoyl)oxy)propanoate (829 mg, 2.56 mmol) was dissolved in DMF (10 mL), stirred at 0 °C for 10 min and then DIEA (0.812 ml, 4.65 mmol), was added. This homogeneous mixture was added to the reaction mixture containing compound **2**. The resulting mixture was stirred at 0 °C for about 1 hour and then at room temperature overnight. The reaction mixture was concentrated by rotary evaporation, pH 5-7, followed by purification by RP HPLC, to give compound **5** (149 mg, 80% yield). ESI MS: $C_{28}H_{40}N_6O_{12}$ $[M+H]^+$ 653.28, found 653.20.

Synthesis of N-(tert-butoxycarbonyl)glycylglycylglycylglycyl-O-((3-methoxy-3-oxopropyl)carbamoyl)-L-serine, compound **6**



Compound **5** (1.213 g, 1.859 mmol) was dissolved in ethanol (80 mL) with heating and stirring, then cooled to room temperature and 10% Pd/C (198 mg) was added under H_2. After 2 hours at room temperature LC-MS indicated the reaction was complete. The crude product was filtered through celite and concentrated by rotary evaporation to give compound **6** (1.103 g, ~99% yield). ESI MS: $C_{21}H_{34}N_6O_{12}$ $[M+H]^+$ 563.23, found 563.10.

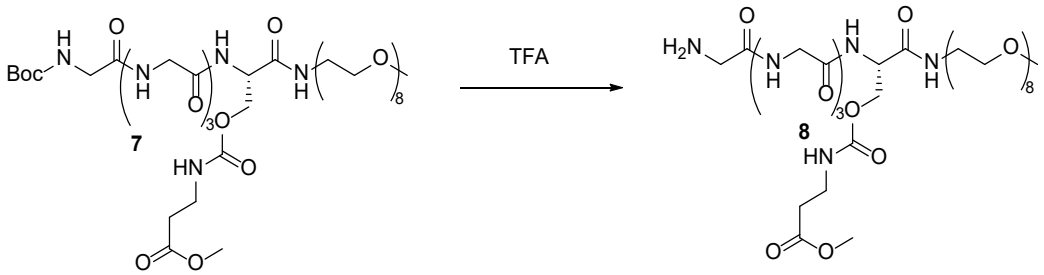
Synthesis of methyl (S)-28-(2,2-dimethyl-4,7,10,13-tetraoxo-3-oxa-5,8,11,14-tetraazahexadecan-16-amido)-27,31-dioxo-2,5,8,11,14,17,20,23,30-nonaoxa-26,32-diazapentatriacontan-35-oate, compound **7**



Compound **6** (1.05 g, 1.86 mmol) was dissolved in a mixture of toluene/DMF and concentrated by rotary evaporation multiple times to remove the residual ethanol, then H_2N-PEG_8-OMe (856 mg, 2.231 mmol) in DMF (15 mL) was added. The reaction mixture was cooled to 0 ^oC, then HOAt (380 mg, 2.79 mmol), HATU (848 mg, 2.231 mmol), DMF (5 mL) and DIEA (0.812 ml, 4.65 mmol) were added. LC-MS showed the reaction was incomplete after 5 hours. Additional HOAt (253 mg) and HATU (353 mg) were added and the mixture warmed to room temperature. After 5 hours at room temperature the reaction mixture was warmed to 35 ^oC for ~45 minutes when LC-MS showed completion of the reaction. The crude reaction mixture was concentrated by rotary evaporation and purified by RP C18 column CombiFlash chromatography using ACN/water containing AcOH (0.1%) gradient as eluant, to give compound **7** (1.26 g, 73% yield). ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.00-8.25 (m, 5H, NH), 7.20 (t, 1H, NH), 7.00 (t, 1H, NH), 4.35-4.55 (m, 1H, CH), 3.93-4.20 (m, 2H, CH_2), 3.65-3.85 (m, 6H, CH_2), 3.58 (s, 3H, OMe),

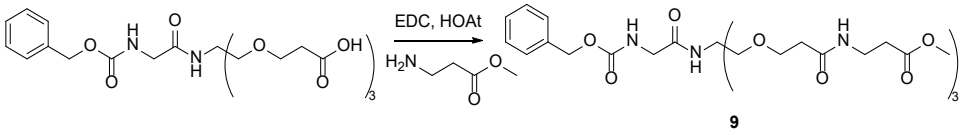
3.55-3.63 (m, 2H, CH_2), 3.50 (bs, 26H, CH_2O), 3.35-3.45 (m, 4H, CH_2), 3.23 (s, 3H, OMe), 3.12-3.25 (m, 4H, CH_2N), 2.45 (t, 2H, CH_2CO), 1.37 (s, 9H, O*t*-Bu). ESI MS: $C_{38}H_{69}N_7O_{19}$ $[M+H]^+$ 928.47, found 928.30.

Synthesis of (S)-28-(2,2-dimethyl-4,7,10,13-tetraoxo-3-oxa-5,8,11,14-tetraazahexadecan-16-amido)-27,31-dioxo-2,5,8,11,14,17,20,23,30-nonaoxa-26,32-diazapentatriacontan-35-oic acid, compound **8**



Compound **7** (2.4 g, 2.59 mmol) was dissolved in DCM (50 mL) and was cooled to 0 ºC. TFA (7 mL, 91 mmol) was added and the reaction was stirred for 1h then was allowed to reach room temperature. Solvent and excess TFA were evaporated and the residue was purified on a C18 ISCO gold catridge (3 injections) and fractions containing product were combined and evaporated and lyophilized to afford the desired compound **8** (1.448 g, 67% yield). ESI MS: $C_{33}H_{61}N_7O_{17}$ $[M+H]^+$ 828.41, found 828.36.

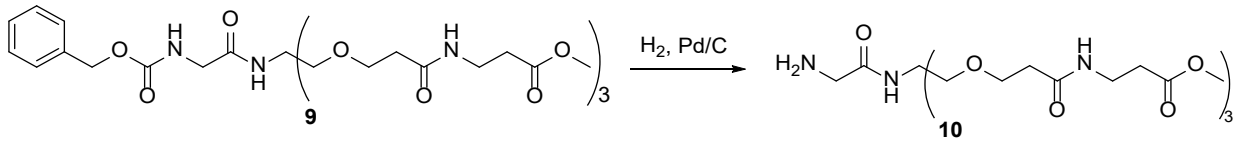
Synthesis of methyl 8,8-bis((3-((3-(l1-oxidaneyl)-3-oxopropyl)amino)-3-oxopropoxy)methyl)-3,6,13-trioxo-1-phenyl-2,10-dioxa-4,7,14-triazaheptadecan-17-oate, compound **9**



8,8-bis((2-carboxyethoxy)methyl)-3,6-dioxo-1-phenyl-2,10-dioxa-4,7-diazatridecan-13-oic acid[2] (0.5 g, 0.946 mmol) was dissolved in DCM/DMF (90:10, 25 mL), then HOAt (0.386 g, 2.84 mmol) and EDC (0.635 g, 3.31 mmol) were added and the resulting mixture was stirred at 0 ºC for 15-20 minutes. Separately β-Ala-OMe hydrochloride (0.462 g, 3.31 mmol) in DCM/DMF (9:1, 5 mL) was treated with DIEA (0.578 ml, 3.31 mmol) and then added to the reaction mixture. Additional DIEA (0.578 ml) was added and the reaction mixture was allowed to warm

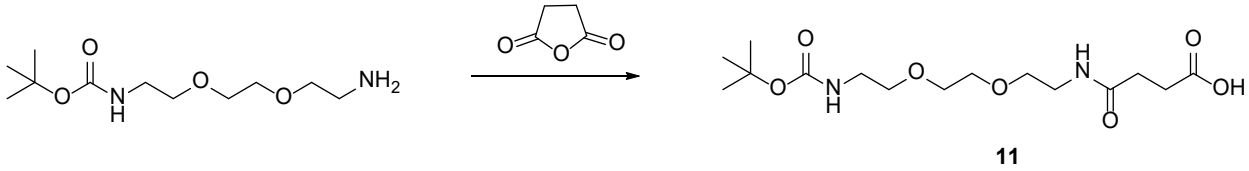
up to room temperature and stirred overnight. LC-MS indicated the reaction was nearly complete. The crude reaction mixture was diluted with DCM and washed successively with HCl (0.2 M in brine), brine and aqueous NaOH (0.2M in brine) and brine. The organic phase was dried with $MgSO_4$, filtered and concentrated to give compound **9** that was used as is. ESI MS: $C_{35}H_{53}N_5O_{15}$ $[M+H]^+$ 784.35; found 784.00.

Synthesis of methyl 10-((3-((3-(l1-oxidaneyl)-3-oxopropyl)amino)-3-oxopropoxy)methyl)-10-(2-aminoacetamido)-19-(l1-oxidaneyl)-5,15,19-trioxo-8,12-dioxa-4,16-diazanonadecanoate, compound **10**



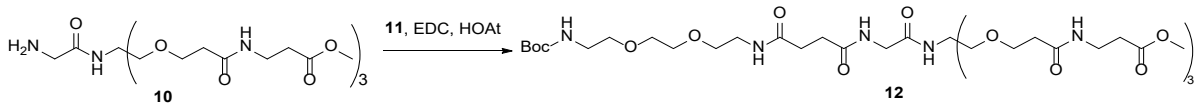
To compound **9** (735 mg, 0.938 mmol) in ethanol (50 mL) was added 10% Pd/C (100 mg, 0.094 mmol) under H_2. When LC-MS indicated the reaction was complete, the reaction mixture was filtered through Celite and concentrated to give compound **10** (0.565 g. 93% yield). ESI MS: $C_{27}H_{47}N_5O_{13}$ $[M+H]^+$ 650.32; found 650.00.

Synthesis of 2,2-dimethyl-4,15-dioxo-3,8,11-trioxa-5,14-diazaoctadecan-18-oic acid, compound **11**



To tert-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate (1 g, 4.03 mmol) in $CHCl_3$ (25 ml), at 0 °C was added dihydrofuran-2,5-dione (0.403 g, 4.03 mmol) and the reaction mixture was allowed to warm up to room temperature. After 20 hours, the reaction mixture was concentrated by rotary evaporation to give compound **11** (1.535 g). ESI MS: $C_{15}H_{28}N_2O_7$ $[M-H]^-$ 347.19; found 347.20.

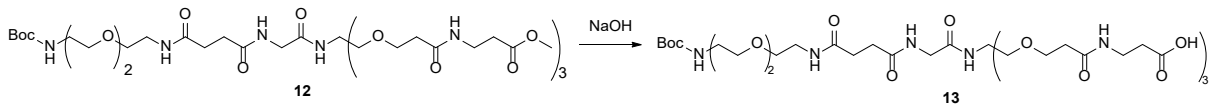
Synthesis of dimethyl 10-(2,2-dimethyl-4,15,18-trioxo-3,8,11-trioxa-5,14,19-triazahenicosan-21-amido)-10-((3-((3-methoxy-3-oxopropyl)amino)-3-oxopropoxy)methyl)-5,15-dioxo-8,12-dioxa-4,16-diazanonadecanedioate, compound **12**



10 **12**

To compound **11** (201 mg, 0.576 mmol) in DMF (4.3 mL) at 0 ºC was added HOAt (115 mg, 0.847 mmol) and EDC (162 mg, 0.847 mmol). After 15 minutes, compound **10** (220 mg, 0.339 mmol) in DMF (0.7 mL) was added followed by DIEA (0.148 ml, 0.847 mmol). The reaction mixture was allowed to warm up slowly to room temperature and stirred overnight, then concentrated by rotary evaporation and purified by RP C18 column CombiFlash chromatography using ACN/water containing AcOH (0.1%) gradient as eluant, to give compound **12** (250 mg, 75% yield). ESI MS: $C_{42}H_{73}N_7O_{19}$ $[M+H]^+$ 979.50; found 980.00.

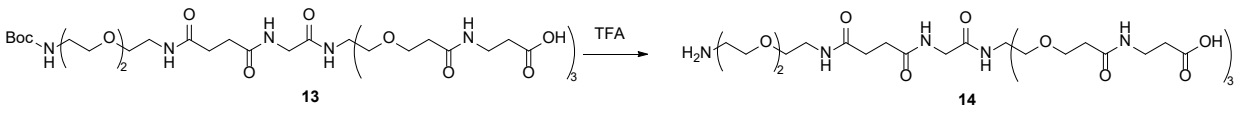
Synthesis of 10-((3-((2-carboxyethyl)amino)-3-oxopropoxy)methyl)-10-(2,2-dimethyl-4,15,18-trioxo-3,8,11-trioxa-5,14,19-triazahenicosan-21-amido)-5,15-dioxo-8,12-dioxa-4,16-diazanonadecanedioic acid, compound **13.**



12 **13**

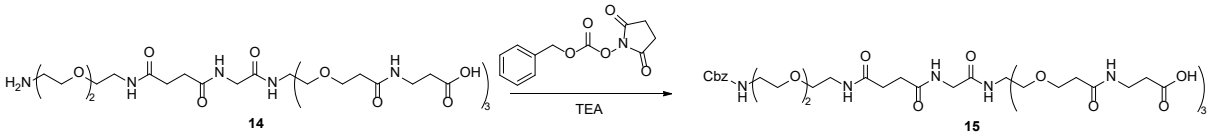
To compound **12** (250 mg, 0.255 mmol) in MeOH (4 ml) and water (0.5 mL) at 0 ºC was added NaOH (51.0 mg, 1.275 mmol in water (0.5 mL)). After 45 minutes the reaction mixture was warmed to room temperature and stirred for 3 hours at room temperature, then neutralized with aqueous HCl (1M), concentrated and purified by C18 RP chromatography to give compound **13** (207 mg, 87% yield. ESI MS: $C_{39}H_{67}N_7O_{19}$ $[M+H]^+$ 938.45; found 938.00.

Synthesis of 10-(1-amino-10,13-dioxo-3,6-dioxa-9,14-diazahexadecan-16-amido)-10-((3-((2-carboxyethyl)amino)-3-oxopropoxy)methyl)-5,15-dioxo-8,12-dioxa-4,16-diazanonadecanedioic acid, compound **14**



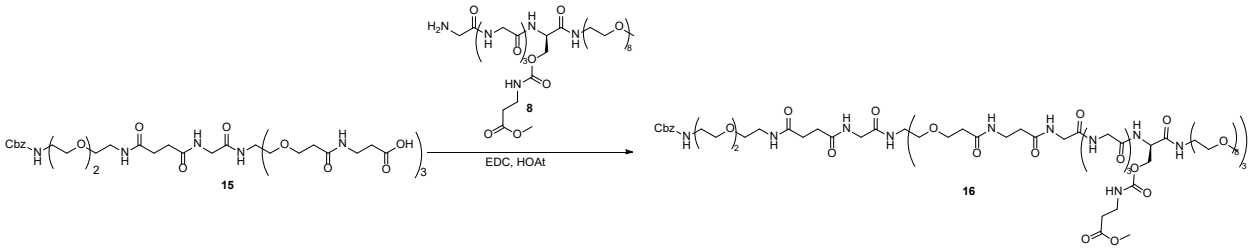
Compound **13** (950 mg, 1.013 mmol) was disolved in of DCM (9.5 mL) and the reaction mixture was cooled to 0 ºC and of TFA (5.7 mL, 75 mmol) was then added and allowed to warm up to room temperature. After 1 hour at room temperature, excess TFA and the solvent were evaporated and the residue was dried under high vaccum overnight. The crude oil was dissolved in 2.5 mL of water and loaded on C18 RP CombiFlash column. The fractions containing product were combined, were concentrated and were lyophilized to give compound **14** (500 mg, 59% yield). ESI MS: $C_{34}H_{59}N_7O_{17}$ $[M+H]^+$ 838.40; found 838.42.

Synthesis of 10-((3-((2-carboxyethyl)amino)-3-oxopropoxy)methyl)-5,15-dioxo-10-(3,14,17-trioxo-1-phenyl-2,7,10-trioxa-4,13,18-triazaicosan-20-amido)-8,12-dioxa-4,16-diazanonadecanedioic acid, compound **15**



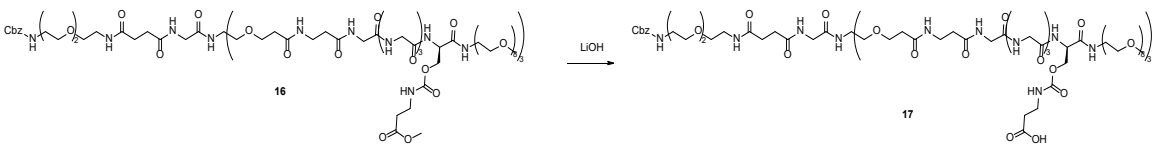
Compound **14** (500 mg, 0.597 mmol) was dissolved in DMF (4.5 mL) and TEA (166 mL, 1.2 mmol) was added followed by addition of benzyl (2,5-dioxopyrrolidin-1-yl) carbonate (164 mg, 0.656 mmol) as a solution in anhydrous 1,4-dioxane (1 mL). The reaction is monitored by LC-MS and was complete in 2.5 hours. The solvents and excess TEA were evaporated to dryness. The remaining crude product was diluted with water (2.5 mL) and was loaded on C18 CombiFlash column for purification, using gradient ACN/water +0.1 %AcOH buffer. Fractions containing pure product were combined, concentrated and lyophilized to yield compound **15** (430 mg,74% yield). ESI MS: $C_{42}H_{65}N_7O_{19}$ $[M+H]^+$ 972.43; found 972.44.

Synthesis of methyl (28R,74R)-74-((2,5,8,11,14,17,20,23-octaoxapentacosan-25-yl)carbamoyl)-28-methyl-51-((R)-28-methyl-27,30,33,36,39,42,46-heptaoxo-2,5,8,11,14,17,20,23,49-nonaoxa-26,29,32,35,38,41,45-heptaazapentacontan-50-yl)-27,30,33,36,39,42,46,56,60,63,66,69,72,77-tetradecaoxo-51-(3,14,17-trioxo-1-phenyl-2,7,10-trioxa-4,13,18-triazaicosan-20-amido)-2,5,8,11,14,17,20,23,49,53,76-undecaoxa-26,29,32,35,38,41,45,57,61,64,67,70,73,78-tetradecaazahenoctacontan-81-oate, compound **16**



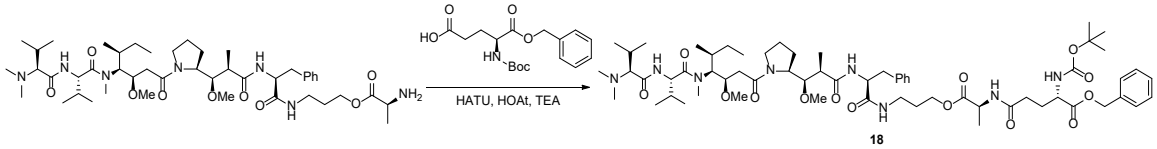
To an ice cold solution of compound **15** (210 mg, 0.216 mmol) in water (3 ml) was added HOAt (88 mg, 0.648 mmol) in NMP (400 mL) followed by the addition of EDC (124 mg, 0.648 mmol) and reaction mixture was stirred for 15 min at 0 ºC. A solution of compound **8** (627 mg, 0.756 mmol) was prepared by dissolution in water (3 ml) was added and the pH of the reaction mixture was adjusted to ~ 6.3 with 1N $NaHCO_3$ and the rection mixture was allowed to reach room temperature. After 1 h the reaction mixture was cooled and added HOAt (88 mg, 0.648 mmol) in NMP (400 mL) followed the addition of EDC (124 mg, 0.648 mmol) and pH was adjusted the pH 6 and let warm to room temperature overnight. MS analysis indicated the reaction was not complete, and compound **8** (179 mg, 252 mmol) and EDC (41 mg, 216 mmol) were added. After 2 h the reaction was complete and was purified on C18, 150 g ISCO Gold cartridge with a an ACN/water gradient buffered with 0.1% AcOH. The fractions containing the desired product were lyophilized to a white amorphous solid compound **16** (539 mg, 73% yield). ESI MS: $C_{141}H_{242}N_{28}O_{67}$ $[M+2H]^{2+}/2$ = 1700.82; found 1701.23.

Synthesis of (28R,74R)-74-((2,5,8,11,14,17,20,23-octaoxapentacosan-25-yl)carbamoyl)-51-(2,2-dimethyl-4,15,18-trioxo-3,8,11-trioxa-5,14,19-triazahenicosan-21-amido)-28-methyl-51-((R)-28-methyl-27,30,33,36,39,42,46-heptaoxo-2,5,8,11,14,17,20,23,49-nonaoxa-26,29,32,35,38,41,45-heptaazapentacontan-50-yl)-27,30,33,36,39,42,46,56,60,63,66,69,72,77-tetradecaoxo-2,5,8,11,14,17,20,23,49,53,76-undecaoxa-26,29,32,35,38,41,45,57,61,64,67,70,73,78-tetradecaazahenoctacontan-81-oic acid, compound **17**



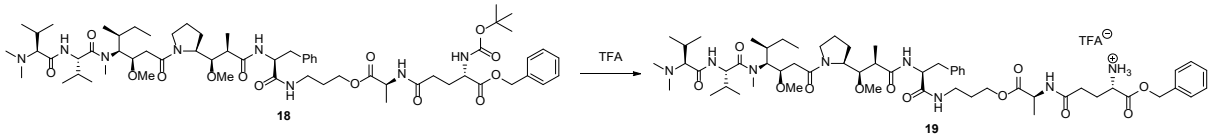
To an ice-cold solution of compound **16** (231 mg, 0.068 mmol) was added lithium hydroxide (11.4 mg, 4 eq.) dissolved in 400 mL water and was stirred cold for 2h 45 min. 1 N HCl (272 mL) was added and was injected directly onto a 150 g C18 Gold cartridge for CombiFlash purification, mobile phase ACN/water buffered with 0.1% AcOH. The desired fractions lyophilized to a white amorphous solid of compound **17** (195 mg, 85% yield). ESI MS: $C_{138}H_{236}N_{28}O_{67}$ $[M+2H]^{2+}/2$ = 1679.5; found 1680.16.

Synthesis of , benzyl N2-(tert-butoxycarbonyl)-N5-((S)-1-(3-((S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-((S)-2-(dimethylamino)-3-methylbutanamido)-N,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanamido)propoxy)-1-oxopropan-2-yl)-L-glutaminate, compound **18**



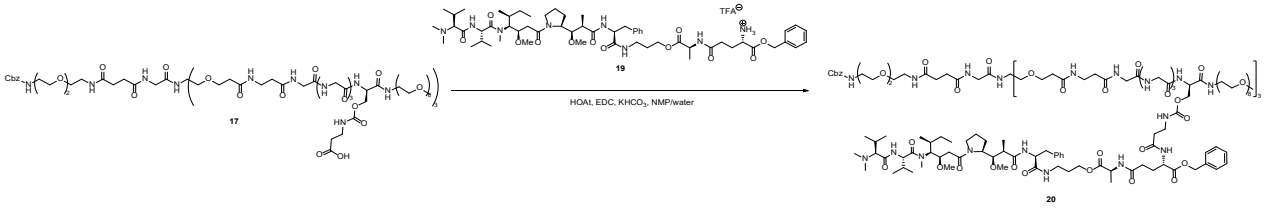
Auristatin F-HPA-Ala-NH_2 TFA-salt[1] (1.000 g, 0.933 mmol) was dissolved in DMF/DCM (8.5 mL) and Boc-L-Glu-OBn (0.304 g, 0.901 mmol) was added. Reaction temperature was lowered to 0° C and HOAt (0.061 g, 0.451 mmol), TEA (0.251 ml, 1.802 mmol) and HATU (0.343 g, 0.901 mmol) were added and stirred for 1.5h. Reaction mixture was allowed to warm to room temperature. The reaction progress was monitored via LC-MS. After 24 h at room temperature the reaction mixture was diluted with EtOAc (135 mL), was washed with water, then twice with saturated aqueous NH_4Cl and with 0.35 M $KHCO_3$,dried over anhydrous $MgSO_4$ and filtered. Following evaporation of solvent the solid was dried overnight under the high vacuum to afford compound **18**, 0.95 g (88%).ESI MS: $C_{63}H_{100}N_8O_{14}$ $[M+H]^+$ 1193.74 found 1193.82.

Synthesis of , benzyl N2-(tert-butoxycarbonyl)-N5-((S)-1-(3-((S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-((S)-2-(dimethylamino)-3-methylbutanamido)-N,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanamido)propoxy)-1-oxopropan-2-yl)-L-glutaminate, compound **19**



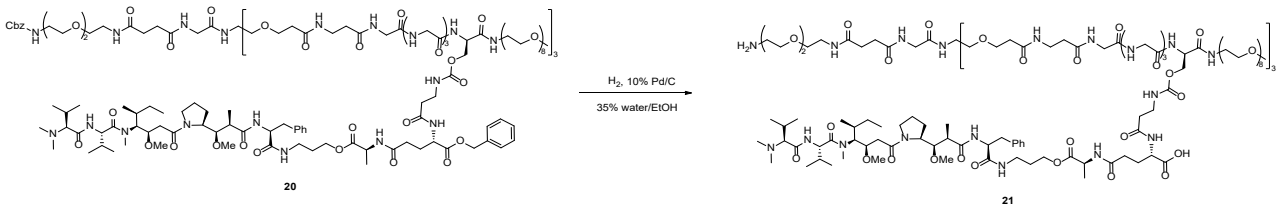
Compound **18** (1.98 g, 1.659 mmol) was dissolved in DCM (17 mL), was cooled to 0° C and TFA (3 mL, 36 mmol) was added. After 2 h stirring, the reaction mixture was warmed up to room temperature. The crude reaction mixture was concentrated, and the product was kept under the high vacuum for 24 hours then lyophilized to yield Auristatin F-HPA-Ala-i-Glu(OBn)-NH_2 TFA-salt (1.98 g, 99%). ESI MS: $C_{58}H_{92}N_8O_{12}$ $[M+H]^+$ 1093.69 found 1093.75.

Synthesis of compound **20**



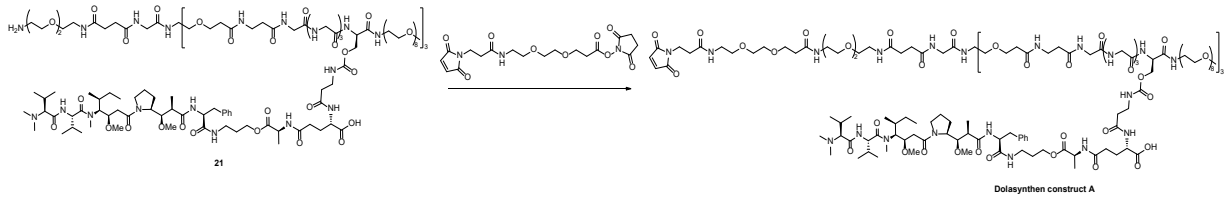
Compound **17** (450 mg, 0.134 mmol) was dissolved in 9 mL of water, chilled to 0 ºC and treated with HOAt (91 mg, 0.670 mmol) dissolved in NMP (1.5 mL) and EDC (128 mg, 0.670 mmol) at 0 ºC, stirring continued at 0 ºC for 15 min. A solution in a mixture of water (0.65 mL) and NMP (0.9 mL) of compound **19** (566 mg, 0.469 mmol), was neutralized with aqueous $KHCO_3$ (1M). The solution of compound **19** was added to the reaction mixture, was stirred at 0 ºC, for 1.5 h, and was allowed to warm up to room temperature over 30 min. Additional 2.5 eq. of EDC were added to the reaction mixture at 0 ºC and stirring was continued for 2.5 h then allowed to warm to room temperature. Additional compound **19** was added in two aliquots (first 50 mg in 0.35 mL and second 70 mg in 0.6 mL 2.5/1 v/v water-NMP) and 5 eq HOAt and 5 eq EDC after each aliquot of compound **19** while keeping the temperature at 0 ºC and pH =5.5-7. The resulting crude product was purified on C18 RP CombiFlash column, +0.1% AcOH buffer to afford compound **20** (619 mg) after lyophilization. ESI MS $C_{312}H_{506}N_{52}O_{100}$ calcd $[M+H]^+/3$ 2194.33 found 2196.37 (+3)

Synthesis of compound **21**



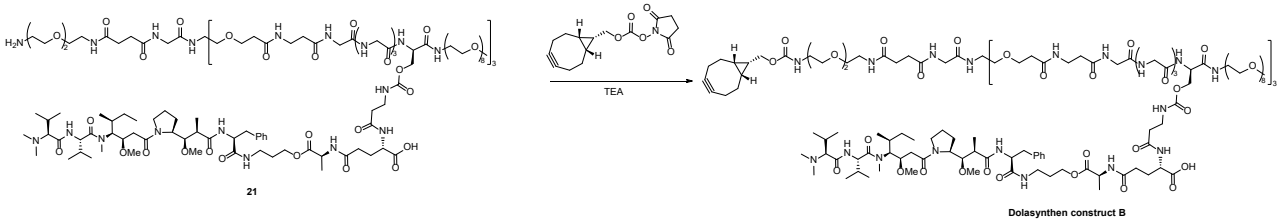
Compound **20** (678 mg, 0.103 mmol) was dissolved in ethanol (7 mL) and water (1.7 ml) and 10 mol-% Pd/C (32 mg) was added to the reaction mixture, and the reaction was stirred under an atmosphere of H_2 for 8 h. The crude material was filtered through polyethylene frit filter, then concentrated. The crude material was redissolved in 1:1 ACN/EtOH and filtered via a plug of celite. The filtrate was collected and concentrated and lyophilized to afford compound **21** (632 mg, 97%). ESI MS $C_{283}H_{482}N_{52}O_{98}$ calcd $[M+H]^+/3$ 2059.48; found 2061.260 (+3).

Synthesis of Dolasynthen Construct A



To an ice-cold solution of compound **21** (246 mg, 0.04 mmol)) in DCM (10 ml) and DMF (1 mL) was added 2,5-dioxopyrrolidin-1-yl 3-(2-(2-(3-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)propanamido)ethoxy)ethoxy)propanoate ester (76 mg, 0.179 mmol) and DIEA (0.063 mL, 0.358 mmol). The pH was adjusted to ~8-9 by addition of DIEA. Reaction mixture was stirred for 1 h then AcOH (10 µL, 0.165 mmol) was added to adjust the pH ~6-7 while stirring and cooling for 1 h and then allowed to reach rt and stirred for 1 h. DCM was evaporated and the residue was diluted with 1 ml of DMF, and purified by preparative HPLC, 2 injections ~1ml each with a gradient of ACN/water buffered with 0.1% AcOH. The fractions containing the product were concentrated and lyophilized to a white amorphous solid **Construct A** (149 mg, 57%). ESI MS: $C_{297}H_{500}N_{54}O_{104}$ $[M+3H]^{3+}/3$ 2163.52; found 2164.79.

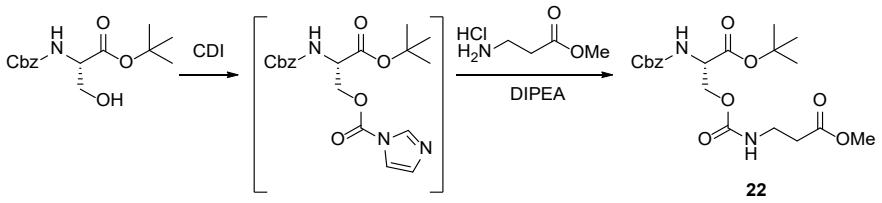
Synthesis of Dolasynthen Construct B



Compound **21** (238 mg, 0.039 mmol) was dissolved in DCM (5 mL) and DMF (0.85 mL) under argon. ((1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (2,5-dioxopyrrolidin-1-yl) carbonate (39.3 mg, 0.135 mmol) was added to the reaction mixture, followed by TEA (26.8 µL, 0.193 mmol), while cooling to 0 ºC. The reaction was stirred at 0 ºC for 1 h and was allowed to warm to room temperature and was stirred for 3 h. The reaction mixture was concentrated, then AcOH (18.74 µL, 0.327 mmol) was added and the reaction mixture was loaded on C18 RP CombiFlash column (30g), gradient 10-45% ACN/water. The fractions containing the desired product were pooled, were concentrated under vacuum, and were lyophilized to afford **Construct B** (142 mg, yield: 58%). ESI MS $C_{294}H_{494}N_{52}O_{100}$ calcd $[M+3H]^{3+}/3$ 2118.84 found 2120.29 (3+)

Synthesis of Dolasynthen Construct C

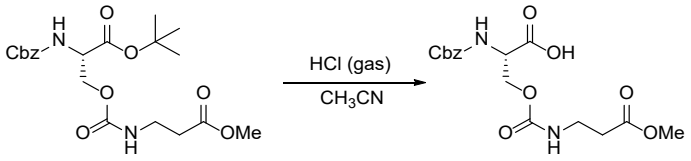
Synthesis of methyl (S)-5-(tert-butoxycarbonyl)-3,8-dioxo-1-phenyl-2,7-dioxa-4,9-diazadodecan-12-oate, compound **22**



To a solution of tert-butyl ((benzyloxy)carbonyl)-L-serinate (10 g, 33.88 mmol, 1 equiv.) in THF (40 mL, 4 vol.) was added CDI (6.6 g, 40.65mmol, 1.2 equiv.) at room temperature under N_2. The resulting mixture was stirred at room temperature for 3h. After SM was disappeared, a solution of DIPEA (13.13 g, 101.64 mmol, 3 equiv.) and methyl 3-aminopropanoate hydrochloride (5.67 g, 40.65 mmol, 1.2 equiv.) in DMF (24 mL, 2.4 vol.) was added dropwise and the resulting mixture was stirred at room temperature for 3h. The mixture was concentrated in vacuum, washed with aq. HCl (1N) and extracted with ethyl acetate. The organic phase was washed with brine and concentrated under vacuum. The residue was recrystallized from 20% EA/n-heptane to give the compound **22** (10.37g, Yield: 73%).

^{1}H NMR (400 MHz, DMSO): δ 7.66-7.63 (s, 1H), 7.36-7.26 (m, 6H), 5.05 (s, 2H), 4.20 (s, 2H), 4.07 (s, 1H), 3.58 (s, 3H), 3.18-3.21 (m, 2H), 2.47-2.44 (m, 2H), 1.39-1.23 (m, 9H); ESI MS $C_{20}H_{28}N_2O_8$ calcd $[M+Na]^+$ 447.20 found 447.20.

Synthesis of N-((benzyloxy)carbonyl)-O-((3-methoxy-3-oxopropyl)carbamoyl)-L-serine, compound **23**

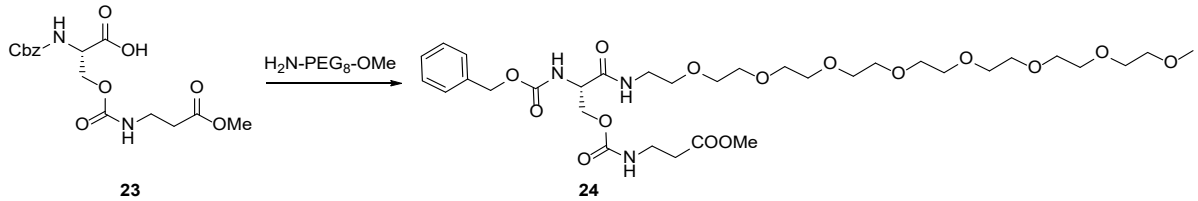


22 **23**

To a solution of compound **22** (50 g, 117.87 mmol, 1 equiv.) in ACN (590 mL) was bubbled HCl_{gas} at room temperature for 2h. The resulting solution was concentrated, pH was adjusted to 7 and was extracted with EA. The aqueous layer was separated, acidified with 1N HCl and extracted with EA. The organic phase was washed with brine, dried over anhydrous $MgSO_4$, was filtered, and was evaporated under reduced pressure to give compound **23** (42.63 g, Yield: 98%).

^{1}H NMR (400 MHz, DMSO): δ 12.9 (s, 1H), 7.64 (s, 1H), 7.41-7.28 (m, 6H), 5.05 (s, 2H), 4.32 (s, 2H), 4.09-4.06 (m, 2H), 3.59 (s, 3H), 3.24-3.19 (m, 2H), 2.52-2.50 (m, 2H); ESI MS $C_{16}H_{20}N_2O_8$ calcd $[M+Na]^+$ 391.20 found 391.10.

Synthesis of methyl (S)-28-(((benzyloxy)carbonyl)amino)-27,31-dioxo-2,5,8,11,14,17,20,23,30-nonaoxa-26,32-diazapentatriacontan-35-oate, compound **25**



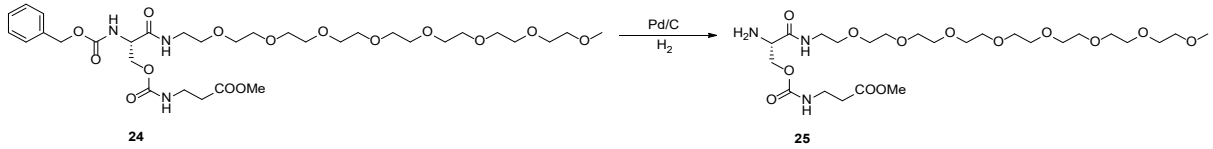
23 **24**

To a solution of compound **23** (11.3 g, 30.6 mmol, 1.2 equiv.) and H_2N-PEG_8-OMe (9.8 g, 25.5 mmol, 1.0 eq.) in DCM (250 mL) were added EDC.HCl (8.8 g, 46 mmol, 1.8 equiv.) and DMAP (3.1 g, 25.5 mmol, 1.0 equiv.) at room temperature under N_2. The resulting mixture was stirred at

room temperature for 3h. The reaction mixture was washed with aqueous HCl (1 M, 100 mL) twice. The organic layer was separated, dried over anhydrous $MgSO_4$, filtered and evaporated under reduced pressure. The resulting residue was purified by normal phase column chromatography (5%, MeOH/DCM) to give compound **24** (14.9 g, Yield:80%).

^{1}H NMR (400 MHz, DMSO): δ 8.06 (s, 1H), 7.48-7.44 (m, 1H), 7.36-7.30 (m, 5H), 7.22 (s, 1H), 5.03 (s, 2H), 4.28-4.10 (m, 2H), 4.01-3.96 (m, 1H), 3.58 (s, 3H), 3.50-3.19 (m, 37H), 2.46-2.43 (m, 2H); ESI MS $C_{33}H_{55}N_3O_{15}$ calcd $[M+H]^+$ 734.40 found 734.40.

Synthesis of methyl (S)-28-amino-27,31-dioxo-2,5,8,11,14,17,20,23,30-nonaoxa-26,32-diazapentatriacontan-35-oate, Compound **25**

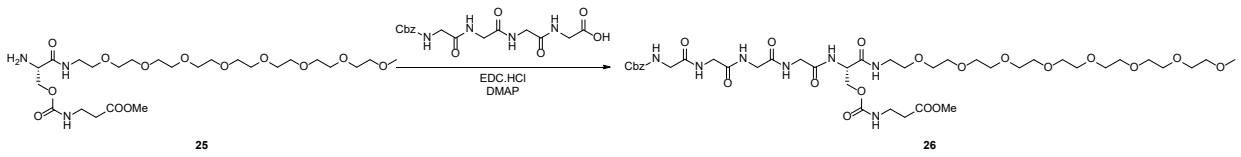


24 25

To a solution of compound **24** (24 g, 32.7 mmol) in MeOH (100 mL) was added Pd/C (2.4 g, 10%, wt%) and H_2 gas was bubbled. The mixture was stirring at room temperature for 4h. Pd/C was filtered via a plug of diatomaceous earth and the filtrate was concentrated under reduced pressure to give compound **25** (18.8 g, Yield: 96%).

^{1}H NMR (400 MHz, DMSO): δ 8.05-7.95 (m, 1H), 7.25-7.20 (m, 1H), 4.10-3.95 (m, 2H), 3.70-3.68 (s, 3H), 3.65-3.30 (m, 35H), 3.30-3.10 (s, 3H), 2.58-2.40 (m, 2H), 1.80-1.60(m, 2H); ESI MS $C_{25}H_{49}N_3O_{13}$ calcd $[M+H]^+$ 600.33 found 600.37.

Synthesis of methyl (S)-27,31-dioxo-28-(3,6,9,12-tetraoxo-1-phenyl-2-oxa-4,7,10,13-tetraazapentadecan-15-amido)-2,5,8,11,14,17,20,23,30-nonaoxa-26,32-diazapentatriacontan-35-oate, compound **26**

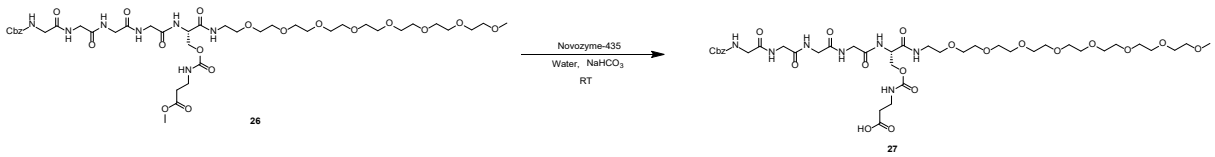


25 26

To a solution of compound **25** (12 g, 20 mmol) in DMF (100 mL) were added ((benzyloxy)carbonyl)glycylglycylglycylglycine (8 g, 21 mmol, 1.05 equiv.), EDC.HCl (6.1 g, 31.6 mmol, 1.6 equiv.) and DMAP (2.6 g, 21 mmol, 1.0 equiv.). The resulting mixture was stirred at 75°C for 2.5h under N_2. The reaction mixture was diluted with DCM (2 L) and washed with aq. HCl (1M, 200 mL) three times. The organic layer was separated, dried over anhydrous $MgSO_4$, filtered, and evaporated under reduced pressure to give compound **26** (15.3 g, Yield:80%).

^{1}H NMR (400 MHz, DMSO): δ 8.19-8.01 (m, 5H), 7.50-7.47 (m, 1H), 7.36-7.30 (m, 6H), 7.20-7.18 (m, 1H), 5.03 (s, 2H), 4.50-4.44 (m, 1H), 4.14-4.00 (m, 2H), 3.76-3.66 (m, 10H), 3.58 (s, 3H), 3.50-3.19 (m, 37H), 2.50-2.45 (4H); ESI MS $C_{41}H_{67}N_7O_{19}$ calcd $[M+H]^+$ 962.50 found 962.40.

Synthesis of (S)-27,31-dioxo-28-(3,6,9,12-tetraoxo-1-phenyl-2-oxa-4,7,10,13-tetraazapentadecan-15-amido)-2,5,8,11,14,17,20,23,30-nonaoxa-26,32-diazapentatriacontan-35-oic acid, compound **27**

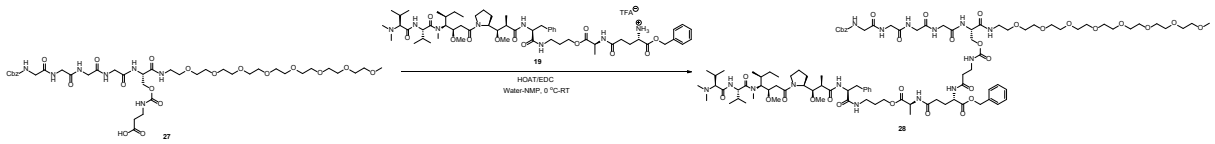


26 27

To a solution of compound **26** (2 g, 2.079 mmol) in 20 mL of water on was added Novozyme-435 (4 g, 2equiv w/w) followed by aqueous $NaHCO_3$ (1M, 2.079 mmol). The resulting mixture was stirred at room temperature for 3h. The reaction mixture was filtered through diatomaceous earth. The cake was washed with water (24 mL). The combined filtrates were then lyophilized to give the desired compound **27** as a sodium salt as off white solid. (1.81 g, Yield: 92%). ESI MS $C_{40}H_{65}N_7O_{19}$ calcd [M+H]+ 948.43 found 948.44.

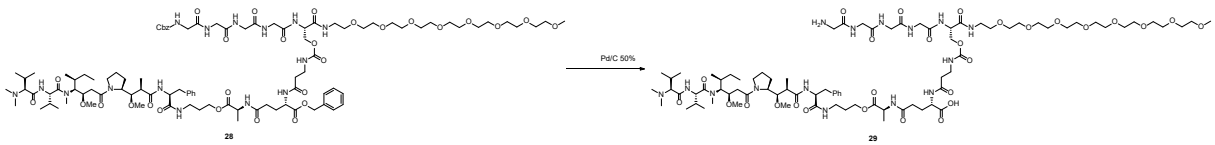
Synthesis of benzyl N2-((S)-28-(2-(2-(2-(2-(((benzyloxy)carbonyl)amino)acetamido)acetamido)acetamido)acetamido)-27,31-dioxo-2,5,8,11,14,17,20,23,30-nonaoxa-26,32-diazapentatriacontan-35-oyl)-N5-((S)-1-(3-((S)-2-

((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-((S)-2-(dimethylamino)-3-methylbutanamido)-N,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanamido)propoxy)-1-oxopropan-2-yl)-L-glutaminate, compound **28**



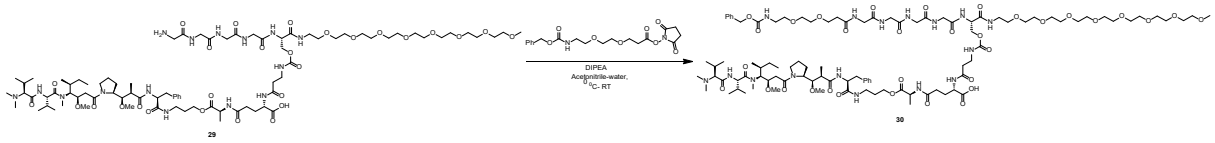
A solution of compound **27** (0.8g, 0.844 mmol) dissolved in water (16 mL), and cooled to 0 ºC and then was added dropwise a solution of HOAt (0.138 g, 1.013 mmol) in NMP (2.5 mL). Compound **19** (1.121 g, 0.928 mmol) was dissolved in water (1.5 mL) and NMP (3 mL) and neutralized with aqueous $KHCO_3$ (1M, ~2 mL), and added to the reaction mixture via syringe (pH ~ 7.04). EDC HCl (0.582 g, 3.04 mmol) was added to the reaction mixture in three portions at 1 h interval (~200 mg each) and stirring continued for 44 h. The crude mixture was purified by reverse phase C18 column chromatography using 0-30% B (A: 0.1 % acetic acid in water: B: 0.1 % acetic acid in Acetonitrile). The pure fractions were combined, and the solvent were evaporated to 10 mL under reduced pressure and lyophilized to afford compound **28** as a white solid. (1.17 g, Yield: 68%). ESI MS $C_{98}H_{155}N_{15}O_{30}$ calcd $[M+H]^+$ 2022.31 found 2023.69.

Synthesis of N2-((S)-28-(2-(2-(2-(2-(3-(2-(2-aminoethoxy)ethoxy)propanamido)acetamido)acetamido)acetamido)acetamido)-27,31-dioxo-2,5,8,11,14,17,20,23,30-nonaoxa-26,32-diazapentatriacontan-35-oyl)-N5-((S)-1-(3-((S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-((S)-2-(dimethylamino)-3-methylbutanamido)-N,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanamido)propoxy)-1-oxopropan-2-yl)-L-glutamine, compound **29**



Compound **28** (1.174 g, 0.580 mmol) was dissolved in 22 mL of 50% water-ethanol mixture and palladium (0.111g) for 50 % wet (220 mg) was added. After 20 h, the reaction mixture was filtered through diatomaceous earth (60 mL, Chemrus, prewashed with 10 mL of water) followed by 0.2-µm filter. The solvent was removed under reduced pressure to a final volume of 15 mL and was lyophilized to afford the desired compound **29** as a white solid. (1.015 g, Yield: 93 %). ESI MS $C_{83}H_{143}N_{15}O_{28}$ calcd $[M+H]^+$ 1798.02 found 901.32 $[M+2H]^+/2$.

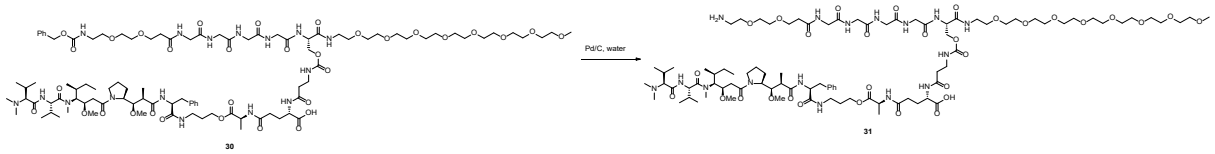
Synthesis of N5-((S)-1-(3-((S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-((S)-2-(dimethylamino)-3-methylbutanamido)-N,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-methylpropanamido)-3-phenylpropanamido)propoxy)-1-oxopropan-2-yl)-N2-((S)-27,31-dioxo-28-(2-(2-(2-(2-(3-oxo-1-phenyl-2,7,10-trioxa-4-azatridecan-13-amido)acetamido)acetamido)acetamido)acetamido)-2,5,8,11,14,17,20,23,30-nonaoxa-26,32-diazapentatriacontan-35-oyl)-L-glutamine, compound **30**



To a solution of compound **29** (200 mg, 0.111 mmol) in 1 mL of (1:1) ACN -water was added 2,5-dioxopyrrolidin-1-yl 3-oxo-1-phenyl-2,7,10-trioxa-4-azatridecan-13-oate (68.1 mg, 0.167 mmol) in ACN (0.5 mL) followed by DIEA (19.84 µL, 0.111 mmol). After 2h, the reaction mixture was subjected to RP-HPLC purification using 30 % buffer B (0.1 % acetic acid in ACN in buffer A (0.1 % acetic acid in water). The pure fractions were combined and then lyophilized to afford compound **30** (100 mg, Yield: 43 %). ESI MS $C_{98}H_{162}N_{16}O_{33}$ calcd $[M+H]^+$ 2091.72 found 2092.72.

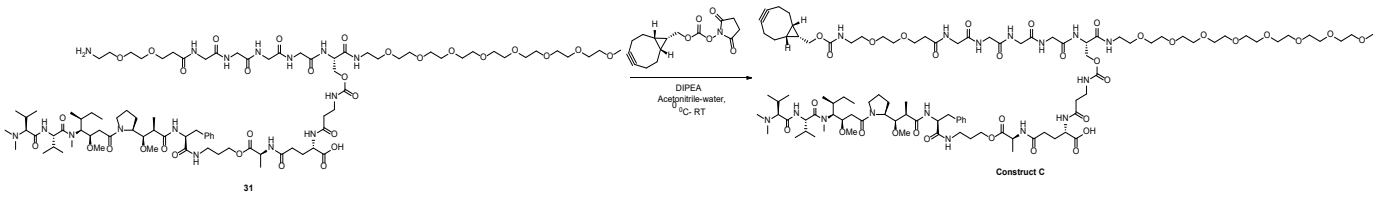
Synthesis of N2-((S)-28-(2-(2-(2-(2-(3-(2-(2-aminoethoxy)ethoxy)propanamido)acetamido)acetamido)acetamido)acetamido)-27,31-dioxo-2,5,8,11,14,17,20,23,30-nonaoxa-26,32-diazapentatriacontan-35-oyl)-N5-((S)-1-(3-((S)-2-((2R,3R)-3-((S)-1-((3R,4S,5S)-4-((S)-2-((S)-2-(dimethylamino)-3-methylbutanamido)-N,3-dimethylbutanamido)-3-methoxy-5-methylheptanoyl)pyrrolidin-2-yl)-3-methoxy-2-

methylpropanamido)-3-phenylpropanamido)propoxy)-1-oxopropan-2-yl)-L-glutamine, compound **31**



Compound **30** (1.03 g, 0.492 mmol) was dissolved in 20 mL of 50% water-ethanol mixture and added palladium (0.1 g)/C 50 % wet (200 mg). After 21 h, the reaction mixture was filtered through diatomaceous earth (60 mL, pre-washed with 10 mL of water) followed by filtration through 0.2 µm filter. The crude was purified by RP-HPLC using 30 % buffer B (0.1 % acetic acid in ACN in buffer A (0.1 % acetic acid in water). The fractions containing pure products were combined and the solvent was evaporated under reduced pressure and was lyophilized to afford the desired compound **31** as white solid. (600 mg, Yield: 62%). ESI MS $C_{90}H_{156}N_{16}O_{31}$ calcd $[M+H]^+$ 1958.11 found 1958.98.

Synthesis of Construct C



To a cold (ice bath) solution of compound **31** (600 mg, 0.297 mmol) in 5 mL of ACN-water (1:1) was added DIEA (212 µl, 1.189 mmol) at 0°C. To this solution, ((1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (2,5-dioxopyrrolidin-1-yl) carbonate (173 mg, 0.595 mmol) dissolved ACN (1.2 mL). After 15 min the ice bath was removed and stirring was continued at RT for 2h. The reaction mixture was purified using Combiflash C18, RP-HPLC using 0-30 % mobile phase B (0.1 Acetic acid in acetonitrile) in mobile phase A (0.1 % acetic acid in water). The fractions containing pure product were combined, and the solvent was evaporated under reduced pressure and the solution was lyophilized to afford the desired compound **Construct C** as white solid. (480 mg, Yield: 76%). (Purity: 97%). ESI MS $C_{101}H_{168}N_{16}O_{33}$ calcd [M+H]+ 2134.22 found 2134.76.

References

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(2) Sliedregt, L. A. J. M.; Rensen, P. C. N.; Rump, E. T.; van Santbrink, P. J.; Bijsterbosch, M. K.; Valentijn, A. R. P. M.; van der Marel, G. A.; van Boom, J. H.; van Berkel, T. J. C.; Biessen, E. A. L. Design and Synthesis of Novel Amphiphilic Dendritic Galactosides for Selective Targeting of Liposomes to the Hepatic Asialoglycoprotein Receptor. *J Med Chem* **1999**, *42* (4), 609–618. https://doi.org/10.1021/jm981078h.