Design and characterization of homogenous antibody-drug conjugates with a drug-to-antibody ratio of one prepared using an engineered antibody and a dual-maleimide pyrrolobenzodiazepine dimer

Jason B. White[a], Ryan Fleming[a], Luke Masterson[b], Ben T. Ruddle[a], Haihong Zhong[c], Christine Fazenbaker[c], Patrick Strout[c], Kim Rosenthal[a], Molly Reed[d], Vanessa Muniz-Medina[e], Phillip Howard[b], Rakesh Dixit[d], Herren Wu[a], Mary Jane Hinrichs[d], Changshou Gao[a,*] and Nazzareno Dimasi[a,*]

[a]Antibody Discovery and Protein Engineering, MedImmune, Gaithersburg, MD, USA

[b]Spirogen Ltd, QMB Innovation Center, London, UK

[c]Oncology Research, MedImmune, Gaithersburg, MD, USA

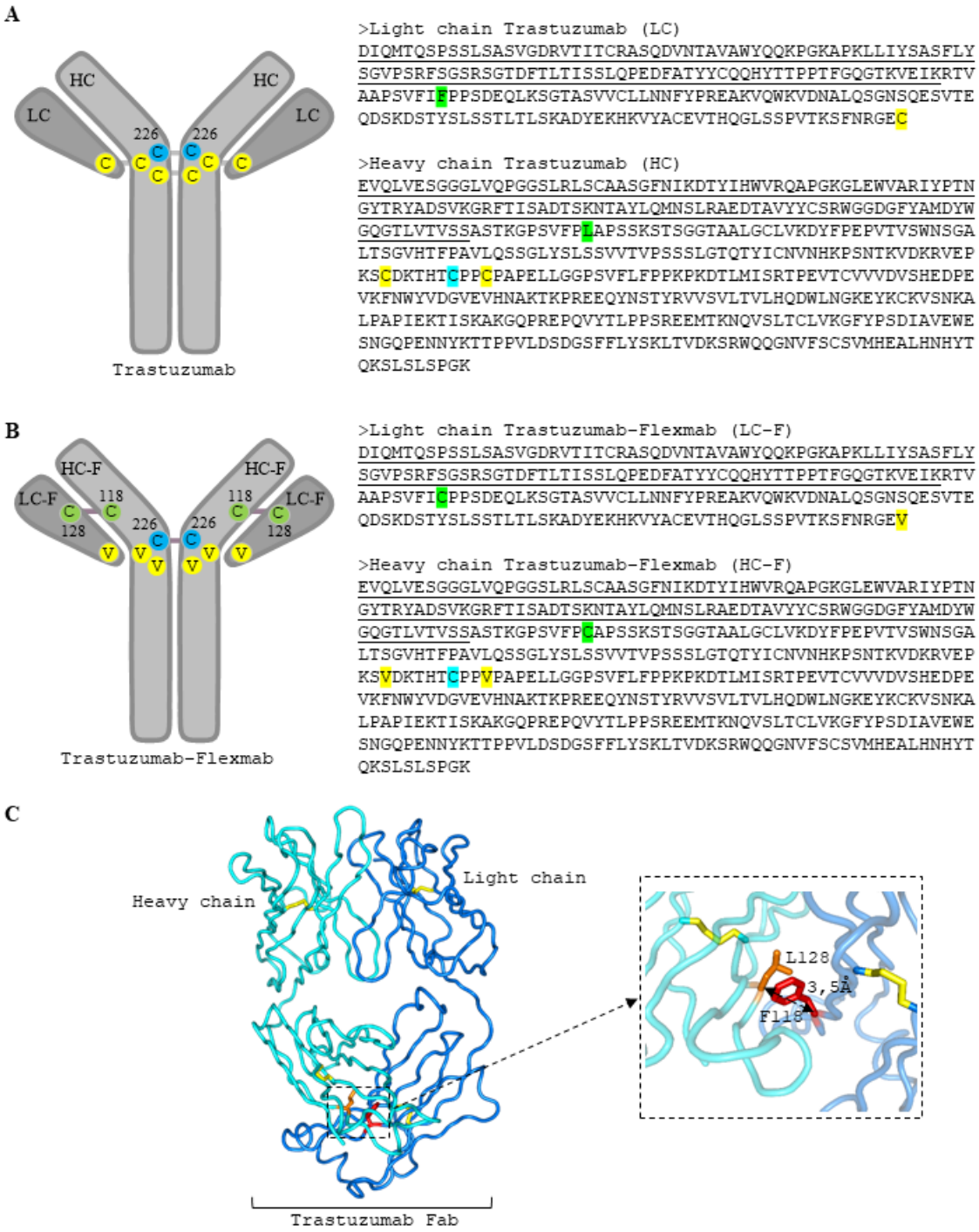
[d]Biologics Safety Assessment, MedImmune, Gaithersburg, MD, USA

[e]Biosuperiors, MedImmune, Gaithersburg, MD, USA

[*]Corresponding authors email addresses:

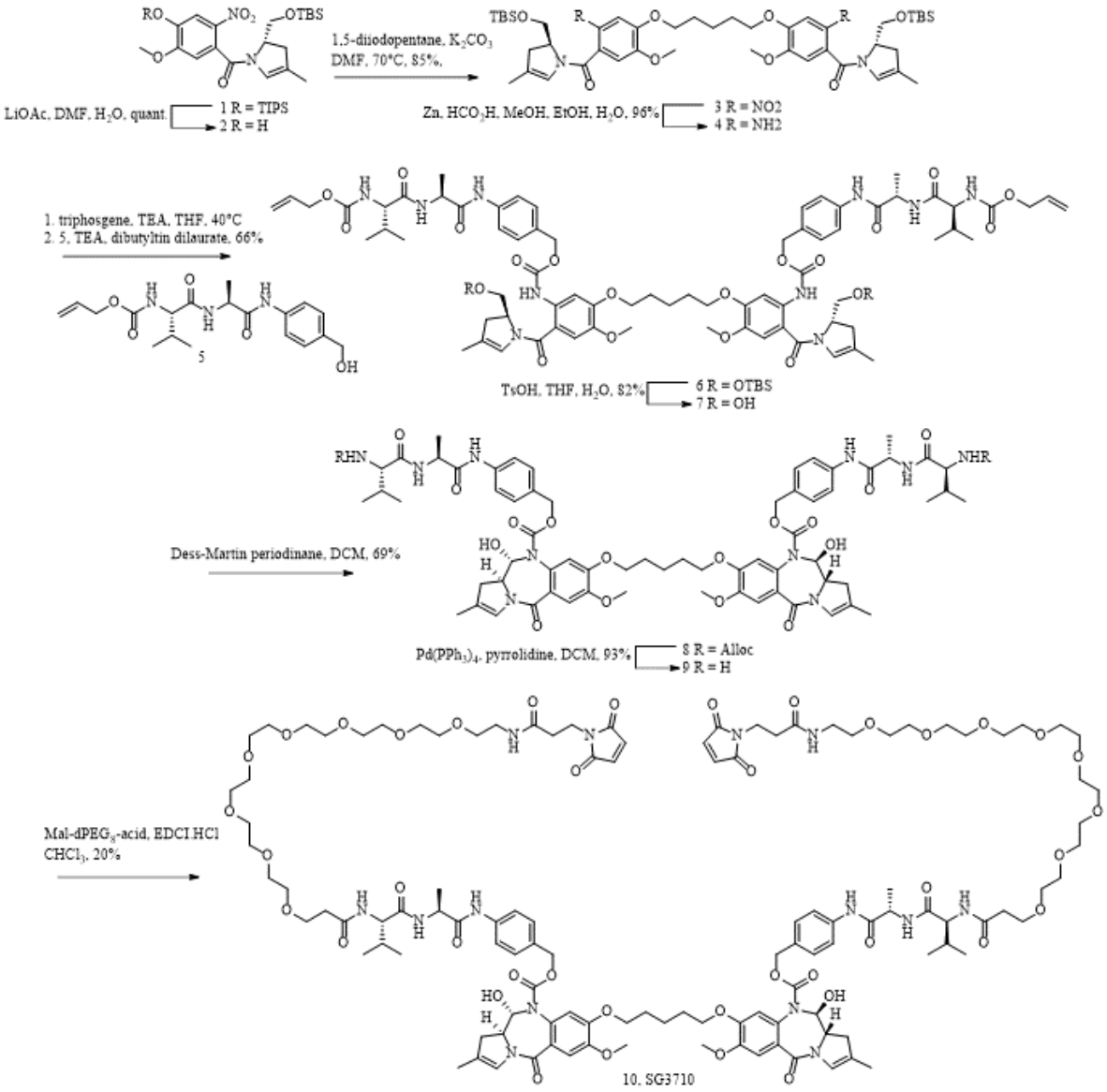
dimasin@medimmune.com

gaoc@medimune.com

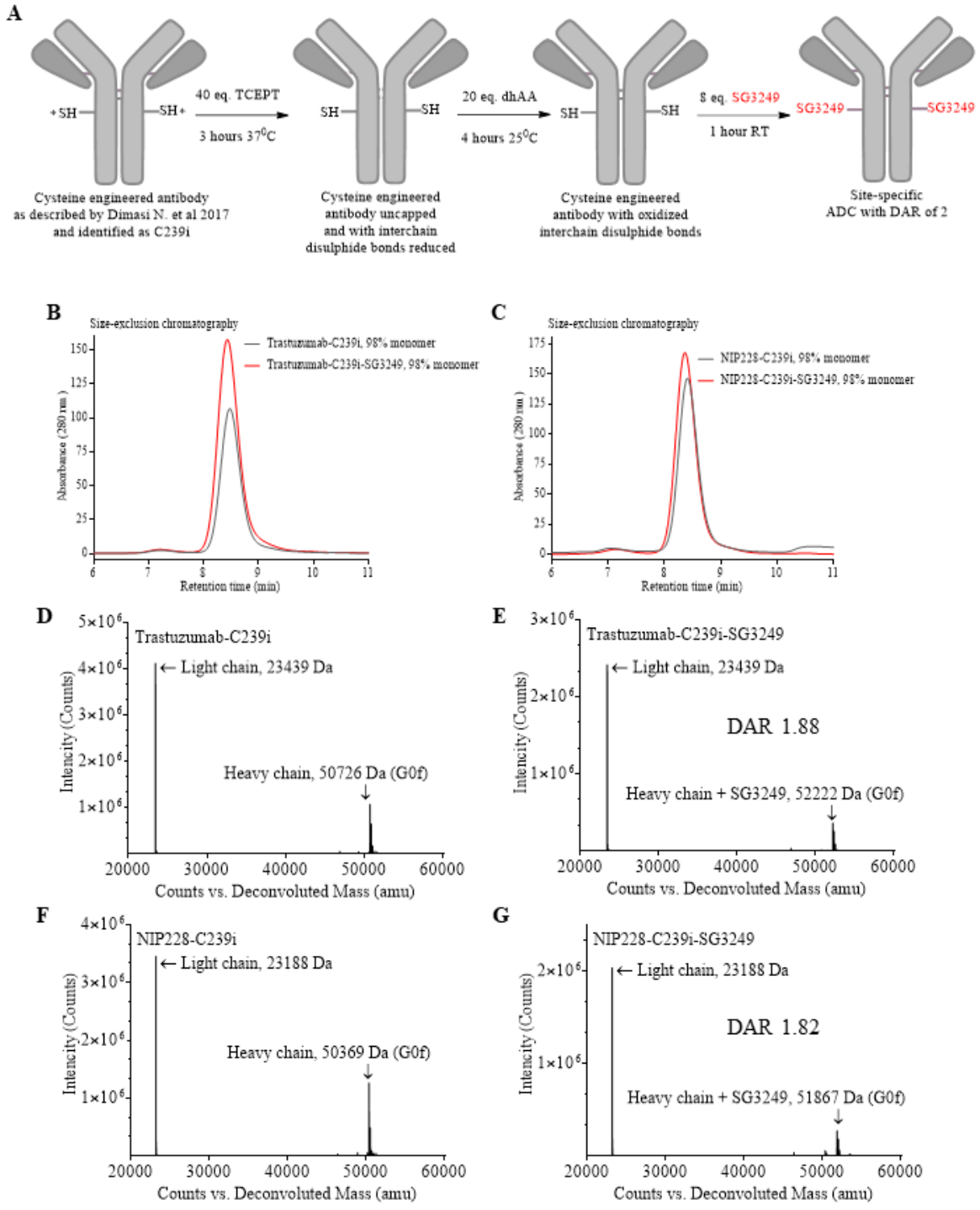


Supplemental Figure 1. Cartoon illustrations, sequences of anti-HER2 trastuzumab and trastuzumab-Flexmab antibodies, and structure of the Fab domain of trastuzumab showing the two residues mutated to cysteine. (A) Canonical interchain disulfide bridges (in yellow and blue) of trastuzumab between the heavy chains (HCs) and light chains (LCs). The amino acid sequence of the HCs and LCs of trastuzumab are shown on the right. The V-domains are underlined. The cysteines that form the interchain disulfide bridges are highlighted in yellow and

blue; the F118 and L128 residues mutated to cysteine in the LCs and HCs, respectively, are highlighted in green. (B) Single interchain disulfide bridge (in blue) and engineered interchain disulfide bridge (in green) for the anti-HER2 Flexmab between the LCs and HCs (LC-F and HC-F), respectively. The amino acid sequence with the V-domains of the LCs (LC-F) and HCs (HC-F) are shown on the right and are underlined. The valine mutations, which replace the cysteines involved in the interchain disulfide bridges, are highlighted in yellow. (C) Structure representation of the anti-HER2 trastuzumab Fab domain (Protein Data Bank entry code 1N8Z, https://www.rcsb.org/structure/1N8Z) showing the location of the L128 and F118 residues mutated to cysteines in the Flexmab. The canonical intrachain disulfide bonds in the LC and HC are shown in yellow. The right panel is a closeup view of the L128 and F118 residues showing their Cα distance, which is 3.5Å. These two residues were mutated to cysteines and demonstrated to form an intrachain disulfide bridge between the LC and HC.

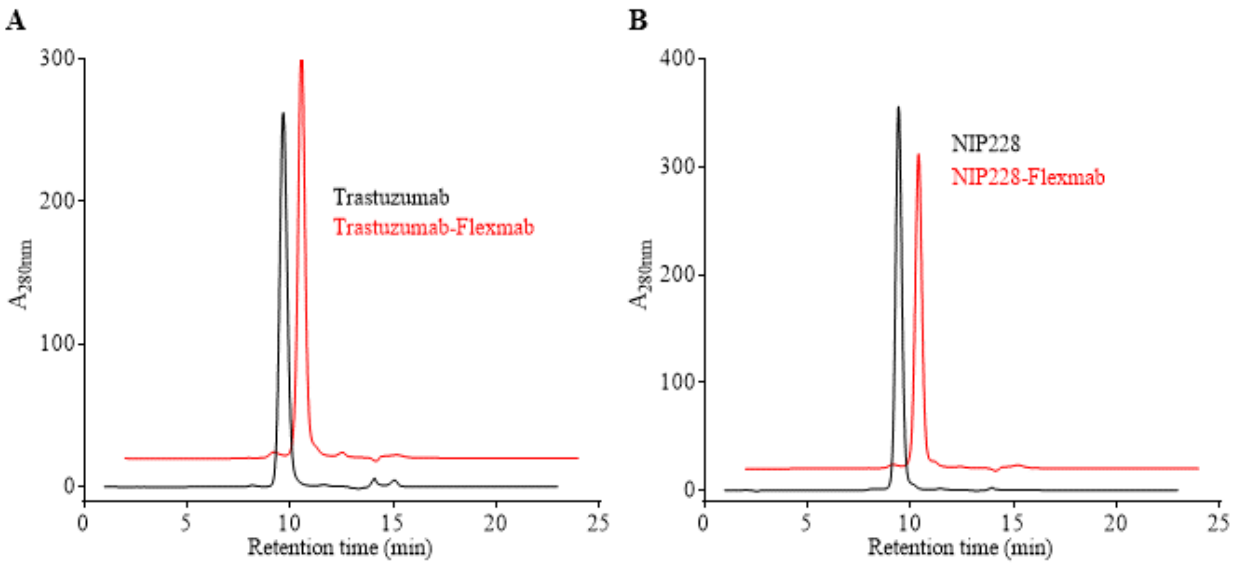


Supplemental Figure 2. Synthesis of SG3710. Detailed synthesis information is reported in the supplementary information.

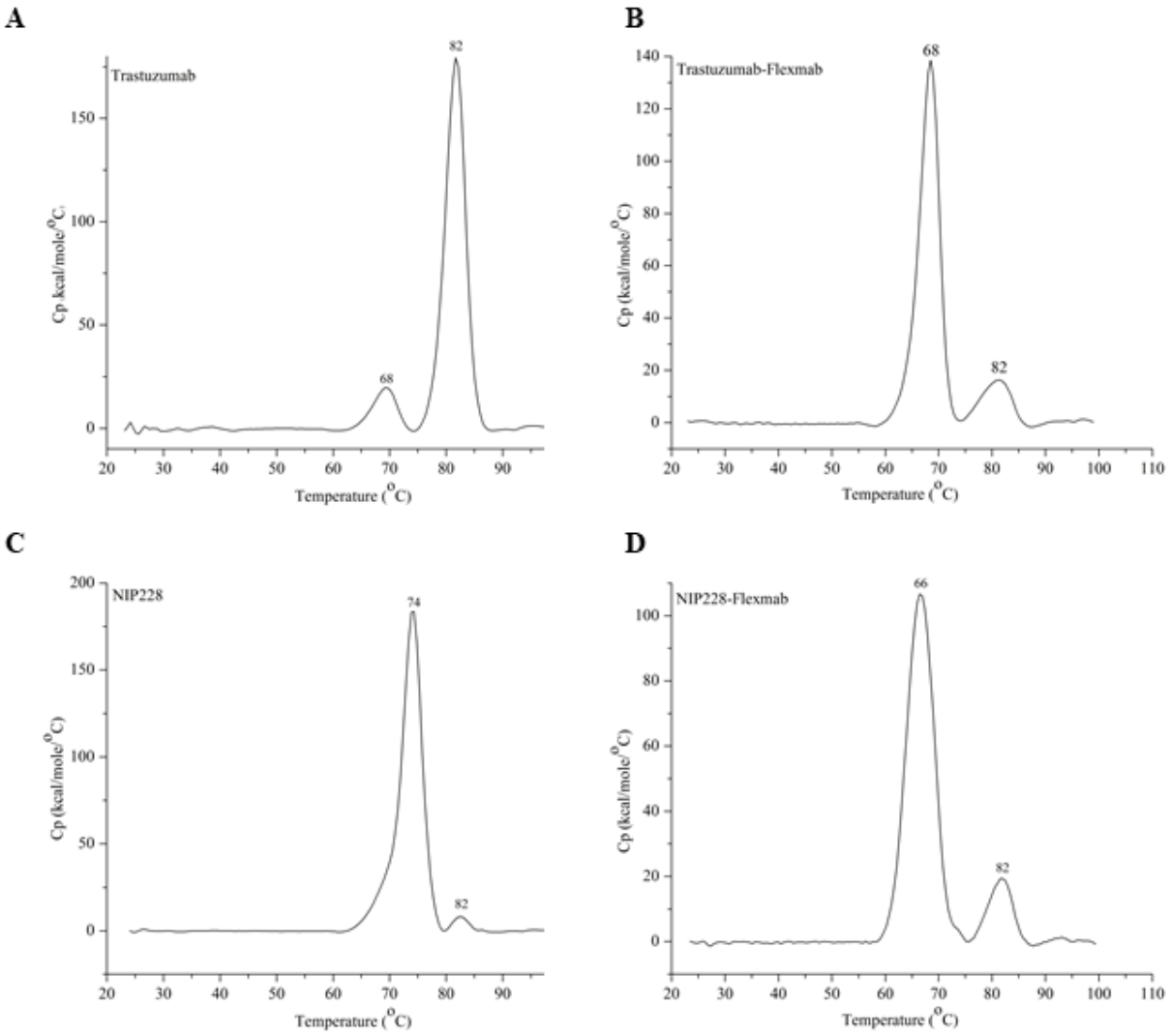


Supplemental Figure 3. Preparation and characterization of trastuzumab and NIP228 site-specific ADCs with DAR of two using SG3249. (A) Site-specific conjugation process as

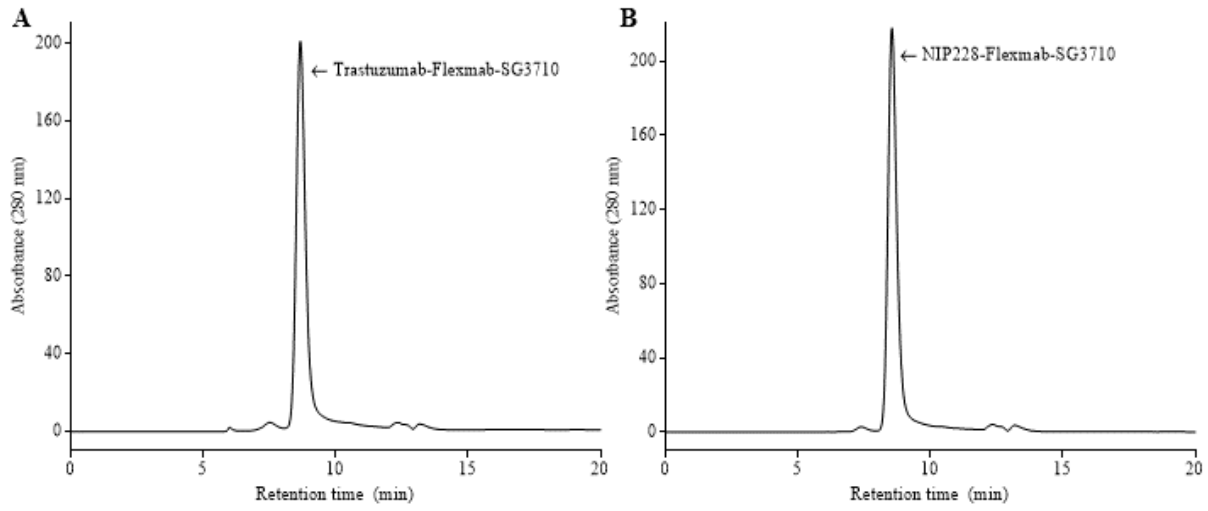
described by Dimasi N. et al. 2017 [doi:10.1021/acs.molpharmaceut.6b00995], used to prepare trastuzumab and NIP228 site-specific ADCs with a DAR of two. The symbols * signify that the inserted cysteines are capped with another cysteine. (B) SEC-HPLC of non-conjugated (grey) and conjugated (red) trastuzumab-C239i-SG3249. (C) SEC-HPLC of non-conjugated (grey) and conjugated (red) NIP228-C239i-SG3249. rLCMS of (D) trastuzumab-C239i; (E) trastuzumab-C239i-SG3249; (F) NIP228-C239i; (G) and NIP228-C239i-SG3249. For the heavy chains, the peaks with the highest ion intensity correspond to the G0f glycoform. The rLCMS data show that conjugation is on the heavy chains with no conjugation detectable on the light chains. The molecular weight of trastuzumab-C239i-SG3249 heavy-chain G0f is 52222 Da, whereas the molecular weight of non-conjugated heavy-chain is 50726 Da. The difference in mass between conjugated and non-conjugated heavy-chains is 1497 Da, which corresponds to the molecular weight of SG3249 (Figure 1 manuscript). The molecular weight of NIP228-C239i-SG3249 heavy-chain G0f is 51867 Da, whereas the molecular weight of the non-conjugated-heavy chain is 50369 Da. The difference in mass between conjugated versus non-conjugated heavy-chains is 1497.21 Da, which corresponds to the molecular weight of SG3249 (Figure 1 manuscript). By determining the ratio of the conjugated versus non-conjugated peak height intensity of the G0f glycoform DARs of 1.88 and 1.82 were determined for trastuzumab-C239i-SG3249 and NIP228-C239i-SG3249, respectively. The light-chains molecular weights are 23439 Da and 23188 Da for trastuzumab-C239i and NIP228-C239i, respectively.



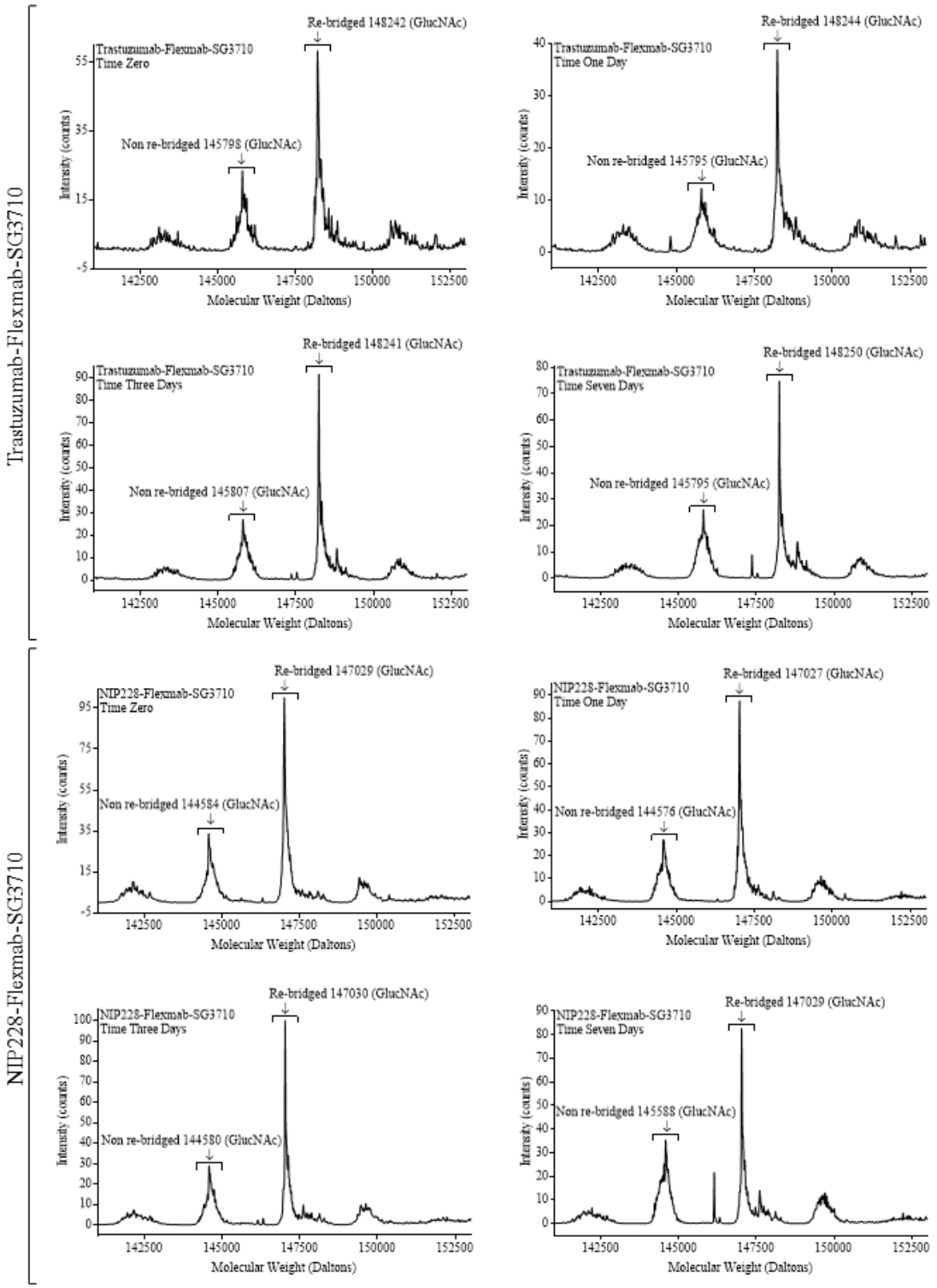
Supplemental Figure 4. SEC-HPLC, absorbance 280 nm, after protein A purification of antibodies (black) and Flexmabs (red). (A) SEC-HPLC results for trastuzumab and trastuzumab-Flexmab. (B) SEC-HPLC results for NIP228 and NIP22-Flaxmab. Monomeric content for antibodies and Flexmabs after protein A purification was 98%.



Supplemental Figure 5. Thermostability of antibodies and Flexmabs using DSC. Transition temperatures ($T_{M,}$ °C) for trastuzumab (A), trastuzumab-Flexmab (B), NIP228 (C), and NIP228-Flexmab (D).

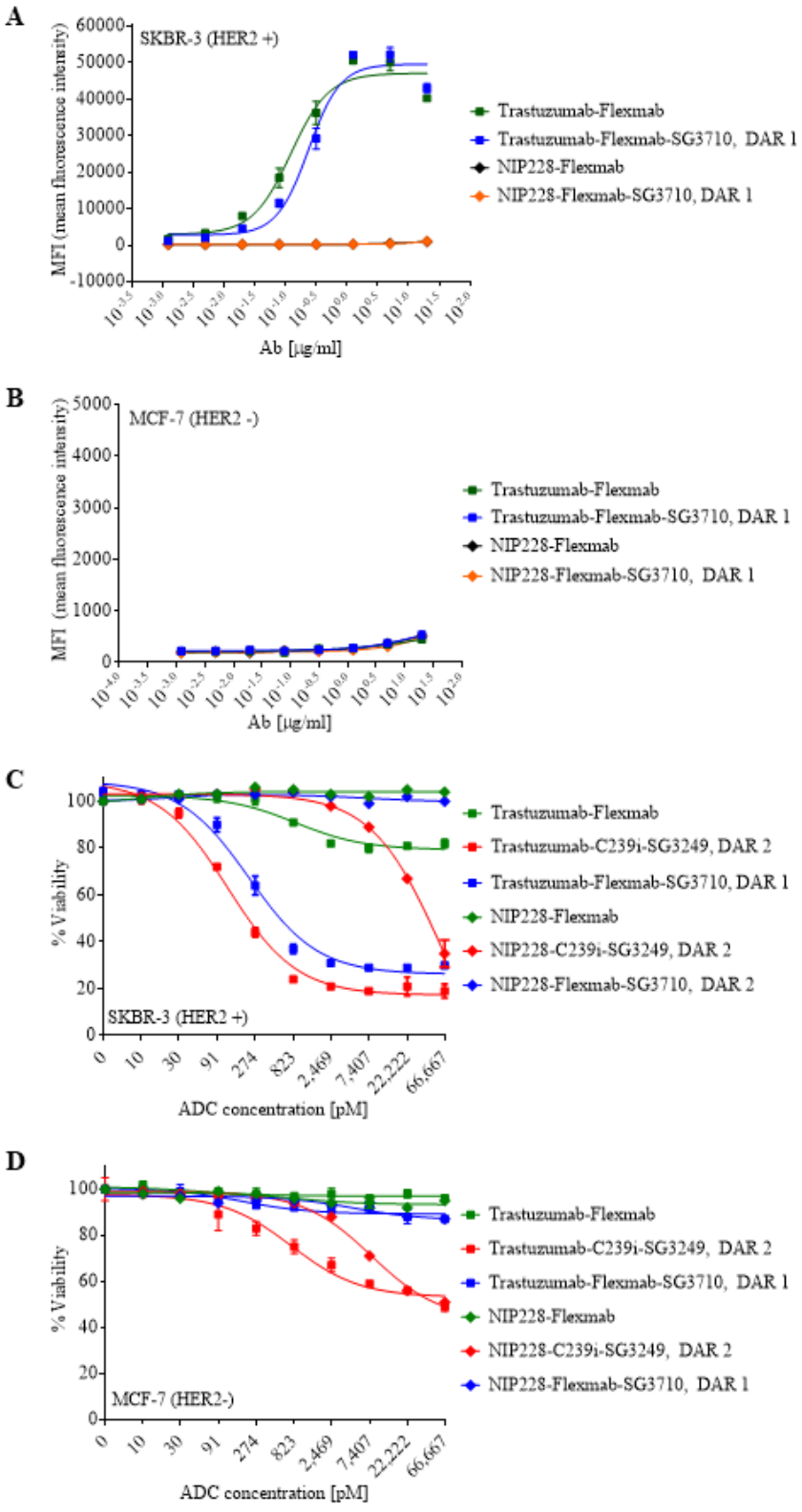


Supplemental Figure 6. SEC-HPLC, absorbance 280 nm, of the site-specific re-bridged ADCs. (A) SEC-HPLC results for rastuzumab-Flexmab-SG3710. (B) SEC-HPLC results for NIP228-Flexmab-SG3710. Monomeric content for both site-specific re-bridged ADCs was 98%.



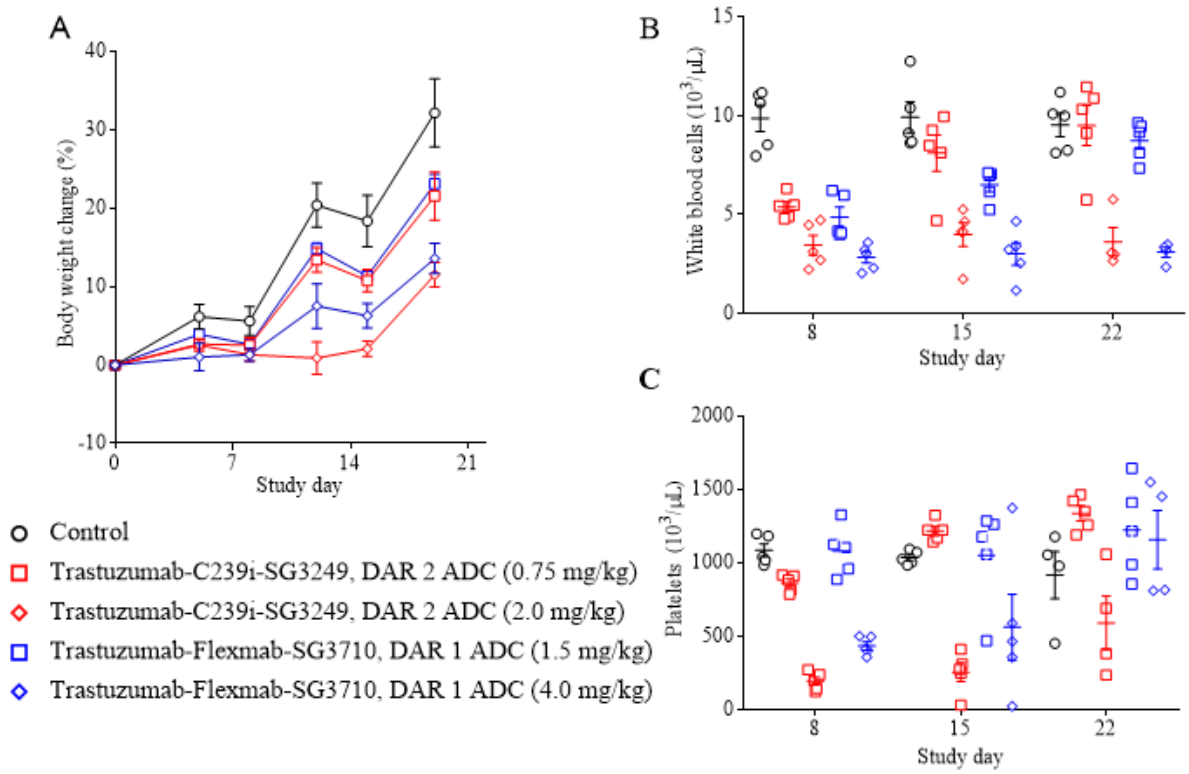
Supplemental Figure 7. Mouse serum stability of re-bridged ADCs determined by affinity capture non-reduced liquid LCMS. Trastuzumab-Flexmab-SG3710 and NIP228-Flexmab-

SG3710 were incubated in mouse serum at 37°C for one, three and seven days, and the loss of SG3710, determined by affinity capture non-reduced LCMS, was compared to that at the start of the incubation. The four top panels show trastuzumab-Flexmab-SG3710 at baseline (time zero), after one day, three days and seven days, left to right and top to bottom, respectively. The four bottom panels show NIP228-Flexmab-SG3710 at baseline (time zero), after one day, three days and seven days, respectively, left to right. The data show that the re-bridged peak intensity of the ADCs remained unchanged throughout the incubation time, which indicated that the re-bridged ADCs are highly stable in mouse serum. To facilitate interpretation of the data, the two ADCs were deglycosylated before the mass spectrometry analysis.



Supplemental Figure 8. Cell-based affinity and cytotoxicity of non-conjugated and ADCs prepared using SG3710 (DAR 1) and SG3249 (DAR 2). Fluorescence-activated cell sorting

binding after incubation of serial dilutions (20 µg/mL to 1 ng/mL) of Flexmabs and Flexmabs-ADCs on SKBR-3 (A) and MCF-7 cells (B), respectively. Cytotoxicity assay results after incubation of serial dilutions (30 µg/mL to 1.5 ng/mL) of Flexmabs and Flexmabs-ADCs on SKBR-3 (C) and MCF-7 cells (D), respectively. SKBR-3 are HER2 positive, and MCF-7 are HER2 negative.



Supplemental Figure 9. Tolerability results of single-dose injections of trastuzumab-Flexmab-SG3710 DAR 1 ADC and trastuzumab-Flexmab-C239i-SG3249 DAR 2 ADC in male Sprague-Dawley rats (n=5/group). Changes in body weights after dosing (A), as well as changes in peripheral white blood cells (B), and platelet (C) counts after administration of the ADCs at indicated doses.

General synthesis information for SG3710

Flash chromatography was performed using silica gel under pressure. Fractions were checked for purity using thin-layer chromatography (TLC) with Merck Kieselgel 60 F254 silica gel, and a fluorescent indicator on aluminum plates. Visualization of TLC was achieved with UV light or iodine vapor unless otherwise stated. Extraction and chromatography solvents were bought from VWR and used without further purification. All fine chemicals were purchased from Sigma-Aldrich, unless otherwise stated. Pegylated reagents were obtained from Quanta BioDesign or from Thermo Fisher Scientific.

^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR) spectra were obtained on a Bruker Avance® 400 MHz spectrometer. Coupling constants are quoted in hertz (Hz). Chemical shifts are recorded in parts per million (ppm) downfield from tetramethylsilane. Spin multiplicities are described as s (singlet), bs (broad singlet), d (doublet), t (triplet), and m (multiplet).

The analytical liquid chromatography mass spectroscopy (LCMS) conditions (for reaction monitoring and purity determination) were as follows. Positive mode electrospray mass spectrometry was performed using a Shimadzu Nexera®/Prominence® LCMS-2020. Mobile phases used were solvent A (H2O with 0.1% formic acid) and solvent B (CH3CN with 0.1% formic acid). Gradient for routine 3-minute run: Initial composition 5% B held over 25 seconds, then increased from 5% B to 100% B over a 1 minute 35 seconds' period. The composition was held for 50 seconds at 100% B, then returned to 5% B in 5 seconds and held there for 5 seconds. The total duration of the gradient run was 3.0 minutes. Gradient for 15-minute run: Initial composition 5% B held over 1.25 minutes, then increased from 5% B to 100% B over an 8.75 minute period. The composition was held for 2.5 minutes at 100% B, then returned to 5% B in 30 seconds and held there for 2 minutes. The total duration of the gradient run was 15.0 minutes. Flow rate was

0.8 mL/minute (for 3-minute run) and 0.5 mL/minute (for 15-minute run). Detection was at 254 nm. Columns: Waters Acquity UPLC® BEH Shield RP18 1.7µm 2.1 x 50 mm at 50 °C fitted with Waters Acquity UPLC® BEH Shield RP18 VanGuard Pre-column, 130A, 1.7µm, 2.1 mm x 5 mm (routine 3-minute run); and Waters Acquity UPLC CSH C18, 1.7µ, 2.1 x 100mm fitted with Waters Acquity UPLC® BEH Shield RP18 VanGuard Pre-column, 130A, 1.7µm, 2.1 mm x 5 mm (15 minute run).

The preparative HPLC conditions were as follows: Reverse-phase ultra-fast high-performance liquid chromatography (UFLC) was carried out on a Shimazdzu Prominence® machine using a Phenomenex® Gemini NX 5µ C18 column (at 50 °C) 150 x 21.2 mm. Eluents used were solvent A (H2O with 0.05% formic acid) and solvent B (CH3CN with 0.05% formic acid). All UFLC experiments were performed with gradient conditions: Initial composition 13% B, the composition was then increased to 100% B over a total of 17 minutes at a gradient suitable to effect the desired separation, then held for 1 minute at 100% B, then returned to 13% B in 0.1 minute and held there for 1.9 minutes. The total duration of the gradient run was 20.0 minutes. Flow rate was 20.0 mL/minute and detection was at 254 and 280 nm.

(*S)-(2-(((tert-butyldimethylsilyl)oxy)methyl)-4-methyl-2,3-dihydro-1H-pyrrol-1-yl)(4-hydroxy-5-methoxy-2-nitrophenyl)methanone (2)*

Lithium acetate dihydrate (3.52 g, 34.5 mmol, 1.0 eq.) was added to a stirred solution of TIPS ether (1) (19.96 g, 34.5 mmol, 1.0 eq.) in DMF/H2O (300 mL/4 mL). The resultant red solution was stirred at room temperature for 3.5h. The reaction mixture was diluted with EtOAc (600 mL) and washed with 1M citric acid solution (2 x 250 mL), H2O (2 x 250 mL), saturated brine (300

mL) and dried (MgSO4). The solvent was evaporated under reduced pressure to afford the product as a yellow solid (14.57 g, 100%). The product was used without further purification. Analytical Data: LC/MS, RT 1.74 min; MS (ES+) m/z (relative intensity) 423 ([M + H]+., 100); 445 ([M + Na])+., 75).

((Pentane-1,5-diylbis(oxy))bis(5-methoxy-2-nitro-4,1-phenylene))bis(((S)-2-(((tert-butyldimethylsilyl)oxy)methyl)-4-methyl-2,3-dihydro-1H-pyrrol-1-yl)methanone) (3)

Potassium carbonate (5.03 g, 36.44 mmol, 1.1 eq.) was added to a stirred solution of phenol (2) (14 g, 33.13 mmol, 1.0 eq.) and 1,5 diiodopentane (21.46 g, 9.86 mL, 66.26 mmol, 2.0 eq.) in DMF (250 mL). The solution was heated at 70°C for 3.5h. The solution was poured into a mixture of ice/water (800 mL) and extracted with EtOAc (4 x 500 mL). The combined extracts were washed with H2O (2 x 250 mL), saturated brine (400 mL), dried (MgSO4) and evaporated under reduced pressure to give a brown oil. Purification by flash column chromatography [n-heptane/EtOAc 40% to 80% in 10% increments] gave the product as a yellow foam (12.7 g, 85%). Analytical Data: LC/MS, RT 2.16 min; MS (ES+) m/z (relative intensity) 913 ([M + H]+., 100); 935 ([M + Na])+., 100).

((Pentane-1,5-diylbis(oxy))bis(2-amino-5-methoxy-4,1-phenylene))bis(((S)-2-(((tert-butyldimethylsilyl)oxy)methyl)-4-methyl-2,3-dihydro-1H-pyrrol-1-yl)methanone) (4)

Zinc dust (19.9 g, 304 mmol, 40 eq.) was treated with 1M HCl (100 mL) and stirred for 10 minutes at room temperature. The mixture was then sonicated for 10 minutes and the activated Zinc collected by vacuum filtration then washed with 1M HCl (50 mL), H2O (to pH 6 to 7), MeOH

and dried in vacuo on the filter pad. The activated zinc was added to a vigorously stirred solution of the bis nitro compound (3) (6.94 g, 7.6 mmol, 1.0 eq.) in EtOH/H2O/EtOAc (60 mL/4 mL/60 mL) at room temperature. The reaction mixture was treated drop-wise with a solution of 5% v/v HCO2H in MeOH (76 mL). A colour change from green to metallic grey and an exotherm to 42 °C were observed. Once the exotherm had subsided to 30°C LC/MS indicated that the reaction was not complete. A further portion of 5% v/v HCO2H in MeOH (20 mL) was added and a further exotherm was observed (34°C) The reaction mixture was allowed to cool to room temperature at which point analysis by LC/MS revealed complete conversion to desired product. The mixture was filtered through celite® and the pad washed with EtOAc. The filtrate was washed with saturated aqueous NaHCO3 (2 x 300 mL), water (300 mL), saturated brine (300 mL), dried (MgSO4), filtered and evaporated in vacuo to provide the bis-aniline as a yellow foam (6.22g, 96%). The product was used without further purification. Analytical Data: LC/MS, RT 2.12 min; MS (ES+) m/z (relative intensity) 853 ([M + H]+., 15).

Bis(4-((S)-2-((S)-2-(((allyloxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl) ((pentane-1,5-diylbis(oxy))bis(6-((S)-2-(((tert-butyldimethylsilyl)oxy)methyl)-4-methyl-2,3-dihydro-1H-pyrrole-1-carbonyl)-4-methoxy-3,1-phenylene))dicarbamate (6)

Triethylamine (0.171 g, 235 µL, 1.69 mmol, 4.4 eq.) was added via syringe to a stirred solution of bis aniline (4) (0.33 g, 0.38 mmol, 1.0 eq.) and triphosgene (0.082 g, 0.28 mmol, 0.72 eq.) in dry THF under an argon atmosphere. The resultant suspension was heated to 40°C and after 5 min sampled in MeOH for LC/MS as the bis methyl carbamate (MS (ES+) m/z (relative intensity) 969 ([M + H]+., 80); 992 ([M + Na])+., 100). Dibutyltin dilaurate (0.024 g, 23 µL, 38 µmol, 0.1 eq.) then solid linker (5) (0.319 g, 0.85 mmol, 2.2 eq.) and trimethylamine (0.085 g, 118

µL, 0.85 mmol, 2.2 eq.) were added and the mixture heated at 40°C with stirring under an argon atmosphere for 5h. The reaction mixture was allowed to cool, filtered and the THF evaporated under reduced pressure. The residue was purified by flash column chromatography [CHCl3/MeOH 0%, 1%, 1.5%, 2%, gradient elution] to give the product as a yellow foam (0.42 g, 66%). Analytical Data: LC/MS, RT 2.16 min; MS (ES+) m/z (relative intensity) 1660 ([M + H]+., 60); 1682 ([M + Na])+., 65).

Bis(4-((S)-2-((S)-2-(((allyloxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl) ((pentane-1,5-diylbis(oxy))bis(6-((S)-2-(hydroxymethyl)-4-methyl-2,3-dihydro-1H-pyrrole-1-carbonyl)-4-methoxy-3,1-phenylene))dicarbamate (7)

p-Toluenesulfonic acid (0.296 g, 1.7 mmol, 2.2 eq.) was added to a stirred solution of bis-tert-butyldimethylsilyl ether (6) (1.26 g, 0.76 mmol, 1.0 eq.) in 10% v/v H2O in THF. The solution was stirred at room temperature for 18h. The reaction mixture was diluted with EtOAc (100 mL) and washed with saturated NaHCO3 solution (2 x 100 mL), H2O (100 mL), saturated brine (100 mL), dried (MgSO4) and evaporated under reduced pressure. The residue was purified by flash column chromatography [CHCl3/MeOH 0% to 5% in 1% increments] to give the product as a white foam (0.896 g, 92%). Analytical Data: LC/MS, RT 1.61 min; MS (ES+) m/z (relative intensity) 1432 ([M + H]+., 5); 1454 ([M + Na])+., 5).

Bis(4-((S)-2-((S)-2-(((allyloxy)carbonyl)amino)-3-methylbutanamido)propanamido)benzyl) 8,8'-(pentane-1,5-diylbis(oxy))(11S,11aS,11'S,11a'S)-bis(11-hydroxy-7-methoxy-2-methyl-5-oxo-11,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepine-10(5H)-carboxylate) (8)

Dess-Martin periodinane (0.24 g, 0.57 mmol, 2.0 eq.) was added to a stirred solution of bis-alcohol (7) in dry DCM (20 mL). The resultant white suspension was stirred at room temperature for 24h. The reaction mixture was diluted with DCM (100 mL) and extracted with saturated NaHCO3 solution (2 x 100 mL), water (100 mL), saturated brine (100 mL), dried (MgSO4) and evaporated under reduced pressure. Purification by flash column chromatography [CHCl3/MeOH 0% to 3% in 0.5% increments] gave the product as a white foam (0.28 g, 69%). Analytical Data: LC/MS, RT 1.58 min; MS (ES+) m/z (relative intensity) 1428 ([M + H]+., 20); 1450 ([M + Na])+., 30).

Bis(4-((S)-2-((S)-2-amino-3-methylbutanamido)propanamido)benzyl) 8,8'-(pentane-1,5-diylbis(oxy))(11S,11aS,11'S,11a'S)-bis(11-hydroxy-7-methoxy-2-methyl-5-oxo-11,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepine-10(5H)-carboxylate) (9)

Pd(PPh3)4 (8 mg,7 µmol, 0.04 eq.) was added to a stirred solution of bis-alloc derivative (8) (0.25 g, 0.176 mmol 1.0 eq.) and pyrrolidine (31 mg, 36 µL 0.44 mmol, 2.5 eq.) in dry DCM (10 mL). The solution was stirred at room temperature for 2h. The reaction mixture was partitioned between saturated NH4Cl solution (50 mL) and DCM (50 mL). The DCM was separated and washed with saturated brine (100 mL), dried (MgSO4) and evaporated under reduced pressure. The solid residue was triturated/sonicated with Et2O (3 x 15 mL) and dried under vacuum to give the product as a white solid (0.207 g, 93%). The product was used without further purification. Analytical Data: LC/MS, RT 1.06 min; MS (ES+) m/z (relative intensity) 630 ([M + 2H]+., 100).

Bis(4-((2S,5S)-37-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-5-isopropyl-2-methyl-4,7,35-trioxo-10,13,16,19,22,25,28,31-octaoxa-3,6,34-triazaheptatriacontanamido)benzyl) 8,8'-(pentane-1,5-

diylbis(oxy))(11S,11aS,11'S,11a'S)-bis(11-hydroxy-7-methoxy-2-methyl-5-oxo-11,11a-dihydro-1H-pyrrolo[2,1-c][1,4]benzodiazepine-10(5H)-carboxylate) (10) SG3710

EDCI.HCl (56 mg, 0.29 mmol, 3 eq.) was added to a stirred solution of bis-amine (9) (0.123 g, 98 µmol, 1.0 eq.) and MaldPEG®OH (0.128 g, 0.22 mmol, 2.2 eq.) in CHCl3 (15 mL). The reaction mixture was stirred at room temperature for 30 min then diluted with CHCl3 (50 mL) washed with H2O (100 mL), saturated brine (100 mL), dried (MgSO4) and evaporated under reduced pressure. Purification by preparative HPLC followed by lyophilisation gave the product as a white foam (0.047 g, 20%). Analytical Data: LC/MS, RT 6.61 min; MS (ES+) m/z (relative intensity) 1205 ([M + 2H]+., 55).