Bioconjugate Chemistry

Supporting Information

Aldehyde tag-mediated site-specific ADC generation enables the exploration of structure-activity relationships at the conjugate level

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Contents

Thermofluorescence

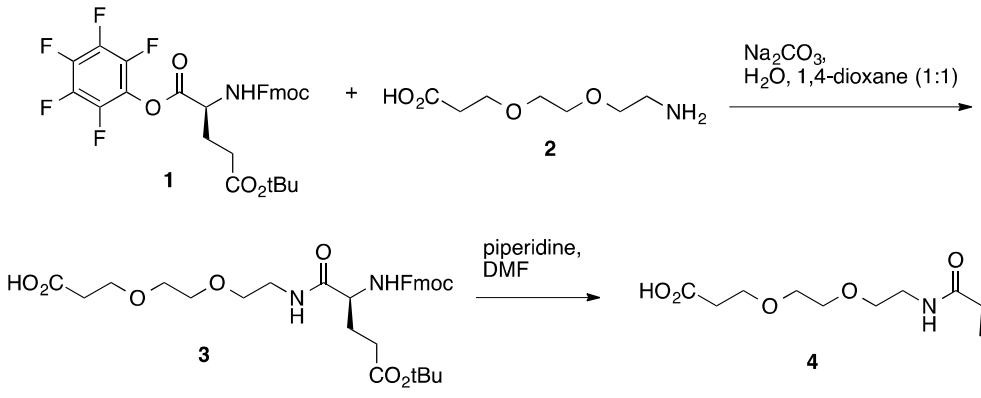
FcRN

FcRn (Sino Biologicals, #CT009-H08H) was biotinylated using NHS-LC-Biotin (Pierce, #21336) according to the manufacturer's instructions. All dilutions and binding steps for the FcRn assays were done in "Kinetic Buffer": 20 mM Phosphate, 150 mM NaCl, 0.02 % Tween-20, 0.05% sodium azide, 0.1 mg/mL bovine serum albumin. The buffer was at pH 6.0 except where otherwise noted. SA Biosensors (ForteBio, #1305291) were prehydrated in 200 µL of kinetic buffer for 10 min in a black 96 well plate. The tips were then loaded into a ForteBio Octet Red biosensor and a baseline signal was established for 1 min. Then, the tips were placed in 1.5 µg/mL biotinylated FcRn, which was captured for 320 s. After two more 1 min baseline steps, IgG (100 nM) was allowed to bind for 5 min. Finally, the tips were moved to a well containing kinetic buffer at pH 7.3, and the dissociation was monitored for 5 min.

Immunogenicity

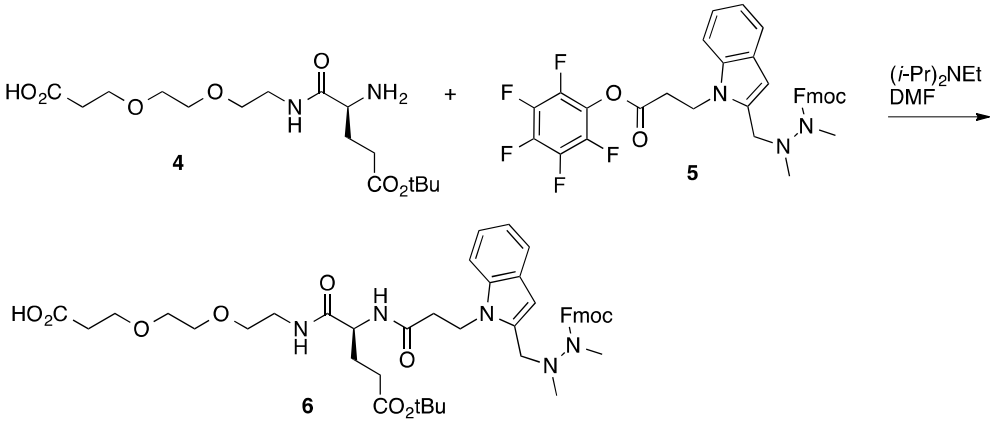
Analysis of the tagged and untagged sequences using iTope™ was performed with overlapping 9mers spanning the regions containing the tag, which were tested against each of 34 human MHC class II alleles.
Each 9mer was scored based on the potential 'fit' and interactions with the MHC class II molecules. The peptide scores calculated by the software lie between 0 and 1. Peptides that produced a high mean binding score (>0.55 in the iTope™ scoring function) were highlighted. If >=50% of the MHC class II binding peptides (i.e. 17 out of 34 alleles) had a high binding affinity (score >0.6), such peptides were defined as "promiscuous high affinity". MHC class II binding peptides binding >=50% of alleles with a score >0.55 were defined as "promiscuous moderate affinity". The sequences were also used to interrogate the TCED™ (T Cell Epitope Database) by BLAST search in order to identify any identity or high sequence homology to previously identified T cell epitopes.

Synthesis of HIPS-Glu-PEG2-maytansine



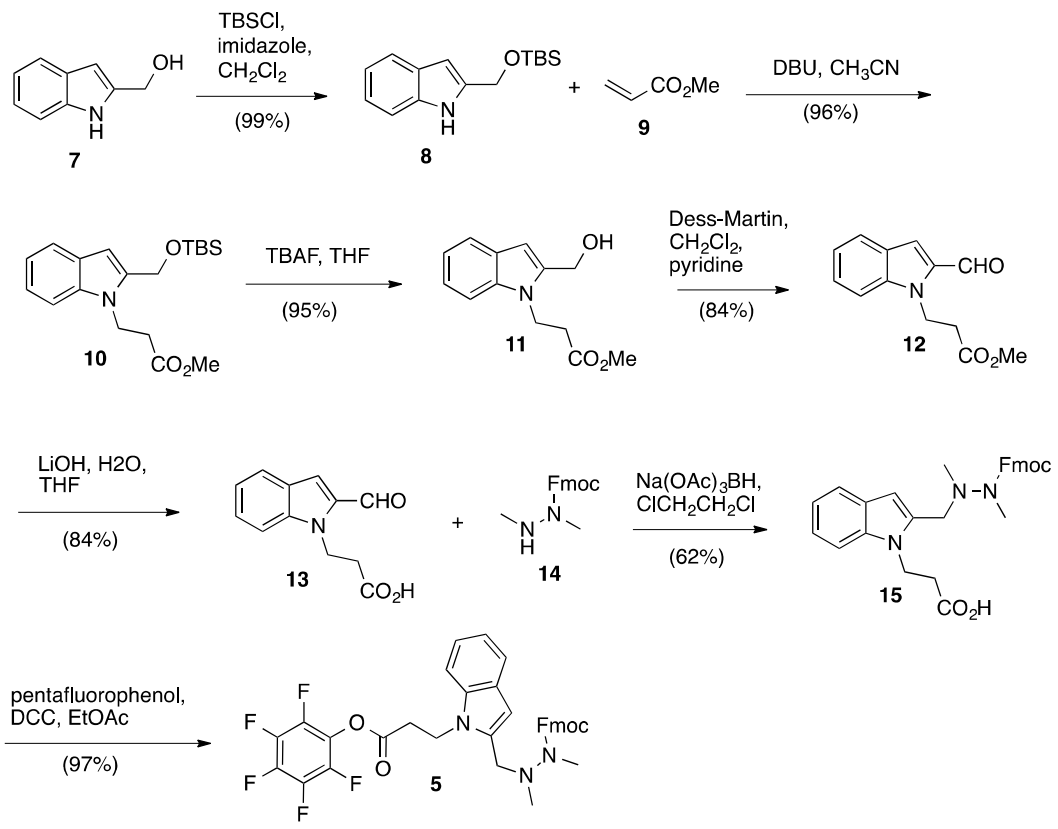
Preparation of (*S*)-5-(3-(*tert*-butoxy)-3-oxopropyl)-1-(9*H*-fluoren-9-yl)-3,6-dioxo-2,10,13-trioxa-4,7-diazahexadecan-16-oic acid (3). Amine **2** (710.3 mg, 4.0 mmol), and Na_2CO_3 (637.9 mg, 6.0 mmol), were added to a 20 mL glass scintillation vial containing a stir bar. Water (10.0 mL) was added and the solution stirred at 20 °C for 5 min. giving a clear, colorless solution. Pentafluorophenyl ester **1** (1185.7 mg, 2.0 mmol), was added to a separate 20 mL glass scintillation vial and dissolved in 10.0 mL of 1,4-dioxane. The vial was vortexed for 1 min. giving a clear, colorless solution that was added dropwise to the prepared solution above, giving a large amount of white precipitate. The reaction was stirred 20 °C for 4 h, added to 70 mL of water, acidified to pH 3 by dropwise addition of 1 M HCl, extracted with 2 x 50 mL EtOAc, and dried over Na_2SO_4. The organic fraction was filtered, evaporated, and purified by flash chromatography on C18 using a 0-100% CH_3CN-H_2O gradient as eluant. The purified product was dried under high vacuum to afford 1137.3 mg (97%) of compound **3** as a sticky, hygroscopic, white solid.

Preparation of (*S*)-7-amino-2,2-dimethyl-4,8-dioxo-3,12,15-trioxa-9-azaoctadecan-18-oic acid (4). Compound **3** (2638.7 mg, 4.513 mmol), was dissolved in a solution of piperidine (2.23 mL, 22.57 mmol) in DMF (8.92 mL) (20% v/v piperidine) and stirred at 20 °C for 1 h. A large amount of white precipitate formed. The reaction was filtered, giving a clear, pale yellow solution. The solution was evaporated and purified by flash chromatography on C18 using a 0-100% CH_3CN-H_2O gradient as eluant. The isolated product was dried under high vacuum to give 813.1 mg (50%) of compound **4** as a clear, viscous oil.



Preparation of (*S*)-7-(3-(2-((2-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-1,2-dimethylhydrazinyl)methyl)-1*H*-indol-1-yl)propanamido)-2,2-dimethyl-4,8-dioxo-3,12,15-trioxa-9-azaoctadecan-18-oic acid (6). Compound **4** (582.4 mg, 1.607 mmol), was added to a dried 20 mL glass scintillation vial containing a dried stir bar. Anhydrous DMF (5 mL) and (i-Pr)$_2$NEt, (0.84 mL, 4.82 mmol) were added, and the solution was stirred at 20 °C for 5 min. giving a clear, very pale yellow solution. Ester **5** (1253.7 mg, 1.930 mmol) was added in portionwise over 5 min. and the reaction was stirred at 20 °C for 2 h. The reaction mixture was purified without

additonal workup by flash chromatography on C18 using a 0-100% CH_3CN-H_2O gradient as eluant. The purified product was dried under high vacuum to afford 406.3 mg (49%) of compound **6** as a white film.



2-(((*tert*-Butyldimethylsilyl)oxy)methyl)-1*H*-indole (8): An oven-dried flask was charged with indole-2-methanol, **7**, (1.581 g, 10.74 mmol), TBSCl (1.789 g, 11.87 mmol), and imidazole (2.197 g, 32.27 mmol), and this mixture was suspended in CH_2Cl_2 (40 mL, anhydrous). After 16 h, the reaction mixture was concentrated to an orange residue. The crude mixture was taken up in Et_2O (50 mL), washed with aqueous AcOH (5% v/v, 3 x 50 mL) and brine (25 mL). The combined organic layers were dried over Na_2SO_4 and concentrated to give 2.789 g (99%) of **2** as a crystalline solid which was used without further purification. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.29 (s, 1H), 7.57 (d, J = 7.7 Hz, 1H), 7.37 (dd, J = 8.1, 0.6 Hz, 1H), 7.19 – 7.14 (m, 1H), 7.12 – 7.07 (m, 1H), 6.32 (d, J = 1.0 Hz, 1H), 4.89 (s, 2H), 0.95 (s, 9H), 0.12 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.3, 136.0, 128.6, 121.7, 120.5, 119.8, 110.9, 99.0, 59.4, 26.1, 18.5, -5.2. HRMS (ESI) calcd for $C_{15}H_{24}NOSi$ $[M+H]^+$: 262.1627; found: 262.1625.

Methyl 3-(2-(((*tert*-butyldimethylsilyl)oxy)methyl)-1*H*-indol-1-yl)propanoate (10): To a solution of indole **8** (2.789 g, 10.67 mmol) in CH_3CN (25 mL) was added methyl acrylate, **9**, (4.80 mL, 53.3 mmol) followed by 1,8-diazabicyclo[5.4.0]undec-7-ene (800 µL, 5.35 mmol), and the resulting mixture was refluxed. After 18 h, the solution was cooled and concentrated to an orange oil which was purified by silica gel chromatography (9:1 hexanes:EtOAc) to yield 3.543 g (96%) of **10** a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58 (d, J = 7.8 Hz, 1H), 7.34 (d, J = 8.2 Hz, 1H), 7.23 – 7.18 (m, 1H), 7.12 – 7.07 (m, 1H), 6.38 (s, 1H), 4.84 (s, 2H), 4.54 – 4.49 (m, 2H), 2.89 – 2.84 (m, 2H), 0.91 (s, 9H), 0.10 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.0, 138.5, 137.1, 127.7, 122.0, 121.0, 119.8, 109.3, 101.8, 58.2, 51.9, 39.5, 34.6, 26.0, 18.4, -5.2. HRMS (ESI) calcd for $C_{19}H_{30}NO_3Si$ $[M+H]^+$: 348.1995; found: 348.1996.

Methyl 3-(2-(hydroxymethyl)-1*H*-indol-1-yl)propanoate (11): To a solution of **10** (1.283 g, 3.692 mmol) in THF (20 mL) at 0 °C was added a 1.0 M solution of tetrabutylammonium fluoride in THF (3.90 mL, 3.90 mmol). After 15 minutes, the reaction mixture was diluted with Et_2O (20 mL) and washed with $NaHCO_3$ (sat. aq., 3 x 20 mL), and concentrated to a pale green oil. The oil was purified by silica gel chromatography (2:1 hexanes:EtOAc) to yield 822 mg (95%) of **11** as a white crystalline solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.60

(d, J = 7.8 Hz, 1H), 7.34 (dd, J = 8.2, 0.4 Hz, 1H), 7.27 – 7.23 (m, 1H), 7.16 – 7.11 (m, 1H), 6.44 (s, 1H), 4.77 (s, 2H), 4.49 (t, J = 7.3 Hz, 2H), 3.66 (s, 3H), 2.87 (t, J = 7.3 Hz, 2H), 2.64 (s, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.3, 138.5, 137.0, 127.6, 122.2, 121.1, 119.9, 109.3, 102.3, 57.1, 52.0, 39.1, 34.3. HRMS (ESI) calcd for $C_{13}H_{15}NNaO_3$ $[M+Na]^+$: 256.0950; found: 256.0946.

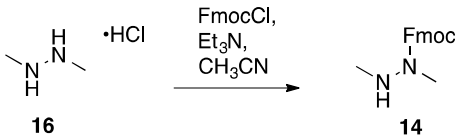
Methyl 3-(2-formyl-1*H*-indol-1-yl)propanoate (12): Dess-Martin periodinane (5.195 g, 12.25 mmol) was suspended in a mixture of CH_2Cl_2 (20 mL) and pyridine (2.70 mL, 33.5 mmol). After 5 min, the resulting white suspension was transferred to a solution of methyl 3-(2-(hydroxymethyl)-1H- indol-1-yl)propanoate (**11**; 2.611 g, 11.19 mmol) in CH_2Cl_2 (10 mL), resulting in a red-brown susupension. After 1 h, the reaction was quenched with sodium thiosulfate (10% aqueous solution, 5 mL) and $NaHCO_3$ (saturated aqueous solution, 5 mL). The aqueous layer was extracted with CH_2Cl_2 (3 x 20 mL); the combined extracts were dried over Na_2SO_4, filtered, and concentrated to a brown oil. Purification by silica gel chromatography (5-50% EtOAc in hexanes) yielded 2.165 g (84%) of **12** as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.87 (s, 1H), 7.73 (dt, J = 8.1, 1.0 Hz, 1H), 7.51 (dd, J = 8.6, 0.9 Hz, 1H), 7.45 – 7.40 (m, 1H), 7.29 (d, J = 0.9 Hz, 1H), 7.18 (ddd, J = 8.0, 6.9, 1.0 Hz, 1H), 4.84 (t, J = 7.2 Hz, 2H), 3.62 (s, 3H), 2.83 (t, J = 7.2 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 182.52, 171.75, 140.12, 135.10, 127.20, 126.39, 123.46, 121.18, 118.55, 110.62, 51.83, 40.56, 34.97. HRMS (ESI) calcd for $C_{13}H_{13}NO_3Na$ $[M+Na]^+$: 254.0793; found: 254.0786.

3-(2-Formyl-1*H*-indol-1-yl)propanoic acid (13): To a solution of indole **12** (2.369 g, 10.24 mmol) dissolved in dioxane (100 mL) was added LiOH (4 M aqueous solution, 7.68 mL, 30.73 mmol). A thick white precipitate gradually formed over the course of several hours. After 21 h, HCl (1 M aqueous solution, 30 mL) was added dropwise to give a solution with pH = 4. The solution was concentrated and the resulting pale brown oil was dissolved in EtOAc (50 mL) and washed with water (2 x 50 mL) and brine (20 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated to an orange solid. Purification by silica gel chromatography (10-50% EtOAc in hexanes with 0.1% acetic acid) yielded 1.994 g (84%) of **13** as a pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.89 (s, 1H), 7.76 (dt, J = 8.1, 0.9 Hz, 1H), 7.53 (dd, J = 8.6, 0.9 Hz, 1H), 7.48 – 7.43 (m, 1H), 7.33 (d, J = 0.8 Hz, 1H), 7.21 (ddd, J = 8.0, 6.9, 1.0 Hz, 1H), 4.85 (t, J = 7.2 Hz, 2H), 2.91 (t, J = 7.2 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 182.65, 176.96, 140.12, 135.02, 127.33, 126.42, 123.53, 121.27, 118.76, 110.55, 40.19, 34.82. HRMS (ESI) calcd for $C_{12}H_{10}NO_3$ $[M-H]^-$: 216.0666; found: 216.0665.

3-(2-((2-(((9H-Fluoren-9-yl)methoxy)carbonyl)-1,2- dimethylhydrazinyl)methyl)-1*H*-indol-1-yl)propanoic acid (15): To a solution of **7** (1.193 g, 5.492 mmol) and (9H-fluoren-9-yl)methyl 1,2-dimethylhydrazinecarboxylate, **14**, (2.147 g, 7.604 mmol) in 1,2-dichloroethane (anhydrous, 25 mL) was added sodium triacetoxyborohydride (1.273 g, 6.006 mmol). The resulting yellow suspension was stirred for 2 h and then quenched with $NaHCO_3$ (saturated aqueous solution, 10 mL), followed by addition of HCl (1 M aqueous solution) to pH 4. The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (5 x 10 mL). The pooled organic extracts were dried over Na_2SO_4, filtered, and concentrated to an orange oil. Purification by C18 silica gel chromatography (20-90% CH_3CN in water) yielded 1.656 g (62%) of **15** as a waxy pink solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, J = 7.4 Hz, 2H), 7.70 – 7.47 (br m, 3H), 7.42 – 7.16 (br m, 6H), 7.12 – 7.05 (m, 1H), 6.37 (s, 0.6H), 6.05 (s, 0.4H), 4.75 – 4.30 (br m, 4H), 4.23 (m, 1H), 4.10 (br s, 1H), 3.55 (br d, 1H), 3.11 – 2.69 (m, 5H), 2.57 (br s, 2H), 2.09 (br s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.90, 155.65, 143.81, 141.42, 136.98, 134.64, 127.75, 127.48, 127.12, 124.92, 122.00, 120.73, 120.01, 119.75, 109.19, 103.74, 67.33, 66.80, 51.39, 47.30, 39.58, 39.32, 35.23, 32.10. HRMS (ESI) calcd for $C_{29}H_{30}N_3O_4$ $[M+H]^+$: 484.2236; found: 484.2222.

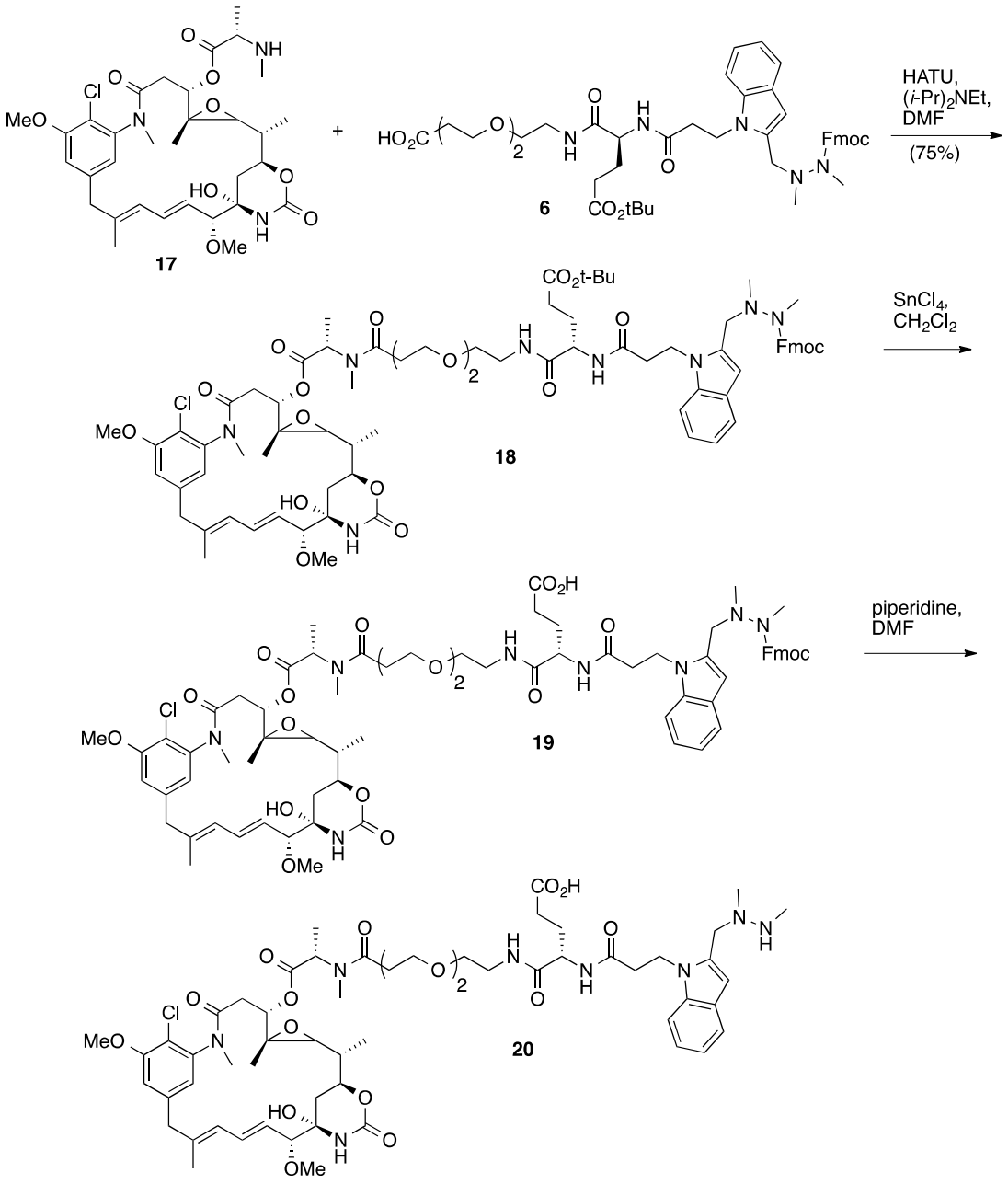
(9*H*-Fluoren-9-yl)methyl 1,2-dimethyl-2-((1-(3-oxo-3-(perfluorophenoxy)propyl)-1*H*-indol-2-yl)methyl)hydrazine-1-carboxylate (5). Compound **15** (5.006 g, 10.4 mmol), was added to a dried 100 mL 2-neck round bottom flask containing a dried stir bar. Anhydrous EtOAc, 40 mL, was added by syringe and the solution stirred at 20 °C for 5 min. giving a clear, pale, yellow-green solution. The solution was cooled to 0 °C in an ice water bath and pentafluorophenol (2098.8 mg, 11.4 mmol), in 3 mL of anhydrous EtOAc, was added dropwise. The solution was stirred at 0 °C for 5 min. DCC (2348.0 mg, 11.4 mmol), in 7 mL of anhydrous EtOAc, was added dropwise, slowly by syringe. The solution was stirred at 0 °C for 5 min, then removed from

the bath and warmed to 20 °C. The reaction was stirred for 2 h, cooled to 0 °C, and filtered to give a clear, pale, yellow-green solution. The solution was diluted with 50 mL of EtOAc, and washed with 2 x 25 mL H_2O, 1 x 25 mL 5 M NaCl, and dried over Na_2SO_4. The solution was filtered, evaporated, and dried under high vacuum, giving 6552.5 mg (97%) of **5** as a greenish-white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 780 (d, J = 7.2 Hz, 2H), 7.58 (m, 3H), 7.45-7.22 (m, 6H), 7.14 (dd(appt. t), J = 7.4 Hz, 1H), 6.42 & 6.10 (2 br s, 1H), 4.74 (dd(appt. t), J = 5.4 Hz, 2H), 3.65-3.18 (br, 3H), 3.08 & 2.65 (2 br s, 3H), 2.88 (s, 3H).



16 **14**

(9*H*-Fluoren-9-yl)methyl 1,2-dimethylhydrazine-1-carboxylate (14). MeNHNHMe•2HCl, **16**, (5.0 g, 37.6 mmol) was dissolved in MeCN (80 mL). Et_3N (22 mL, 158 mmol) was added and the precipitate that formed was removed by filtration. To the remaining solution of MeNHNHMe, a solution of FmocCl (0.49 g, 18.9 mmol, 0.5 eq) was added dropwise over 2.5 h at -20 °C. The reaction mixture was then diluted with EtOAc, washed with H_2O, brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by flash chromatography on silica (hexanes/EtOAc = 3:2) to give 3.6 g (34%) of **14**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75-7.37 (m, 8 H), 4.48 (br s, 2H), 4.27 (t, J = 6.0 Hz, 1H), 3.05 (s, 3H), 2.55 (br s, 3H).



Maytansinol 3-(2*S*,15*R*)-19-(2-((2-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-1,2-dimethylhydrazinyl)methyl)-1*H*-indol-1-yl)-15-(2-(*t*-butoxycarbonyl)ethyl)-2,3-dimethyl-4,14,17-trioxo-7,10-dioxa-3,13,16-triazanonadecanoate (18). A solution of maytansinol 3-(*S*)-□-*N*-methylaminopropionate **(17)**[1] (0.426 g, 0.655 mmol), carboxylic acid **6** (0.597 g, 0.721 mmol), and (*i*-Pr)$_2$NEt (0.35 mL, 2.00 mmol) in 3.0 mL of DMF was stirred at room temperature as HATU (0.277 g, 0.729 mmol) was added. The reaction mixture was stirred for 2.5 h and concentrated by rotary evaporation. The product was isolated by flash chromatography on silica gel using a 0-10% MeOH-CH_2Cl_2 gradient. Product-containing fractions were combined, concentrated, and re-subjected to flash chromatography on C18 using a 0-100% CH_3CN-H_2O gradient to yield 0.721 g (75%) of maytansinoid **18** as a white solid. MS (ESI) calcd for $C_{75}H_{95}ClN_8O_{17}$ $[M+Na]^+$: 1458.7; found: 1481.8.

Maytansinol 3-(2*S*,15*R*)-19-(2-((2-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-1,2-dimethylhydrazinyl)methyl)-1*H*-indol-1-yl)-15-(2-(carboxy)ethyl)-2,3-dimethyl-4,14,17-trioxo-7,10-dioxa-3,13,16-triazanonadecanoate

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[1]1. Widdison, W. C. *et al.* Semisynthetic Maytansine Analogues for the Targeted Treatment of Cancer. *J. Med. Chem.* **49**, 4392–4408 (2006).

(19). A solution of maytansinoid **18** (110.5 mg, 0.08 mmol) in 1.0 mL of anhydrous CH_2Cl_2 was stirred at 0 °C as a 1.0 M solution of $SnCl_4$ in CH_2Cl_2 (0.378 mL, 0.378 mmol) was added dropwise. A yellow precipitate formed. The reaction mixture was purified, without additonal workup, by flash chromatography on C18 using a 0-100% CH_3CN-H_20 gradient as eluant to afford 65.6 mg (62%) of maytansinoid **19** as a white film. MS (ESI) calcd for $C_{73}H_{91}ClN_8O_{18}$ $[M-H]^-$: 1401.6 found 1401.1.

Maytansinol 3-(2*S*,15*R*)-19-(2-(2-(1,2-dimethylhydrazinyl)methyl)-1*H*-indol-1-yl)-15-(2-(carboxy)ethyl)-2,3-dimethyl-4,14,17-trioxo-7,10-dioxa-3,13,16-triazanonadecanoate (20). A solution of piperidine (90.7 □L, 0.92 mmol) in 453.6 mL of DMA was stirred at room temperature as maytansinoid **19** (64.5 mg, 0.05 mmol) was added. The reaction mixture was stirred for 20 min. The reaction mixture was purified, without additonal workup, by flash chromatography on C18 using a 0-100% CH_3CN-H_20 gradient as eluant to afford 49.1 mg (90%) maytansinoid **20** as a white film. MS (ESI) calcd for $C_{58}H_{82}ClN_8O_{16}$ $[M+H]^+$: 1181.6 found 1181.3.

Figure S1.

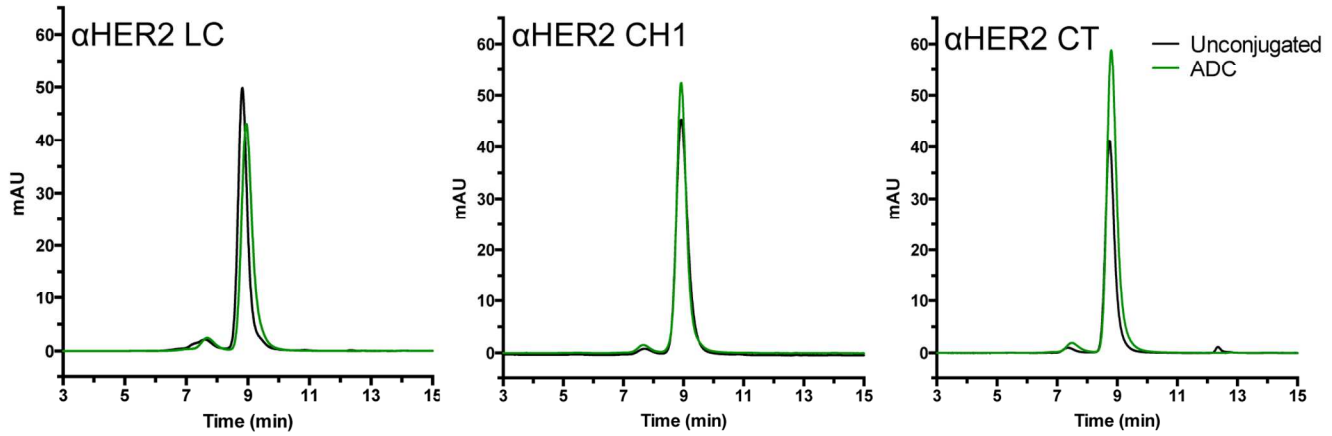

Figure S1. Size-exclusion chromatographic analysis reveals minimal aggregation in preparations of αHER2 ADCs bearing the aldehyde tag at various locations. Unconjugated (black) and HIPS-Glu-PEG2-maytansine conjugated ADCs tagged at the indicated locations were analyzed by SEC. Total aggregate was ≤5% in all cases.

Figure S2.

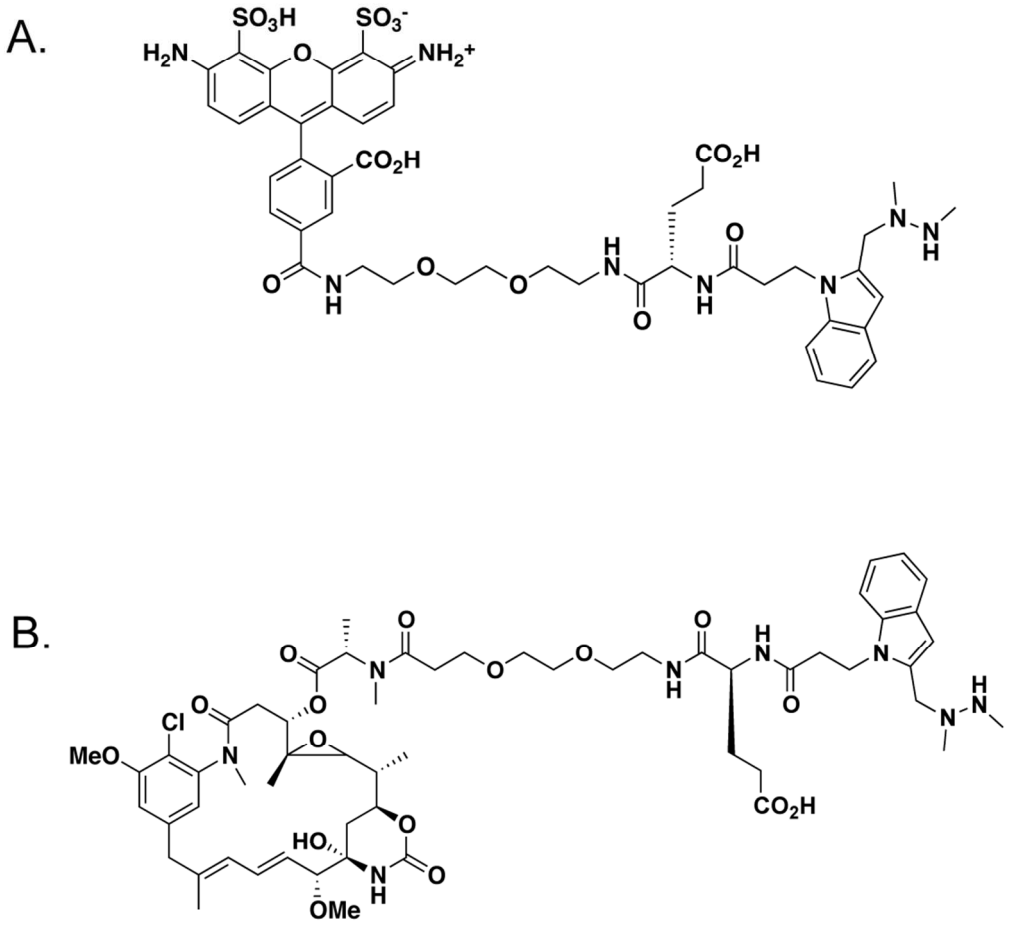

Figure S2. A comparison of the HIPS-Glu-PEG2-AF488 and HIPS-Glu-PEG2-maytansine structures shows that they have different chemical bonds at the point of payload attachment. (A) Alexa Fluor 488 is attached to the PEG2 moiety via an aryl amide bond. (B) Maytansine is attached to the PEG2 moiety via an ester bond.

Figure S3.

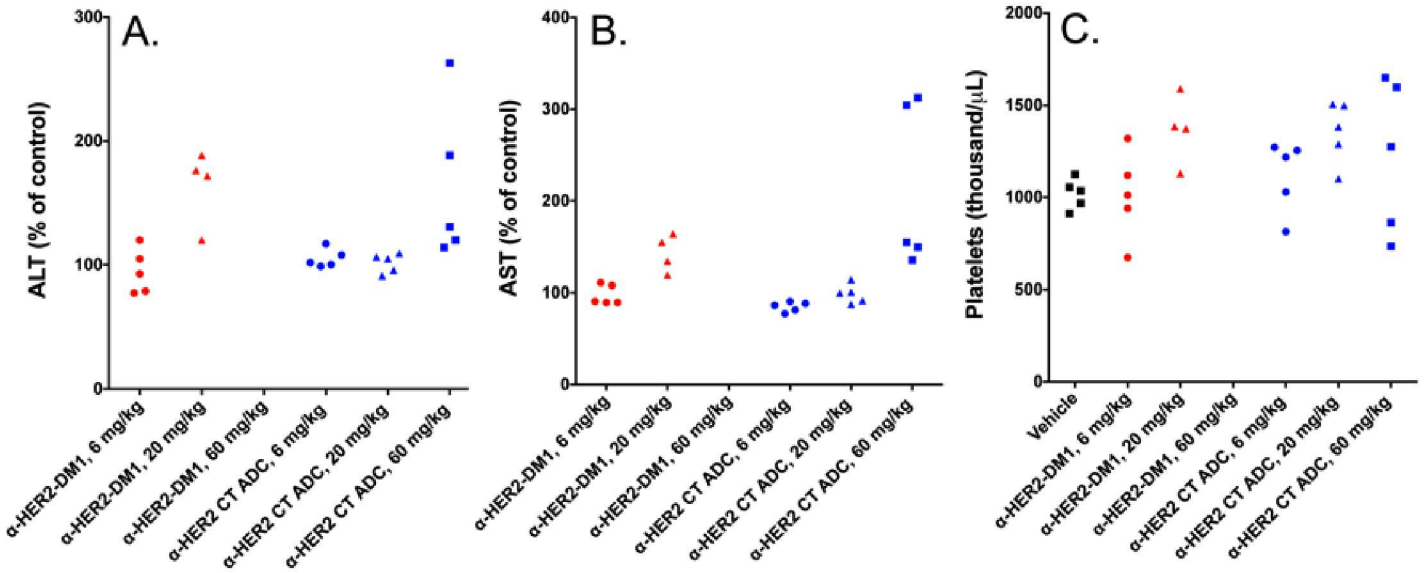

Figure S3. The toxicity indicators that were observed at day 5 have mostly resolved by day 12. Alanine aminotransferase (A), aspartate aminotransferase (B), and platelet counts (C) were assessed at day 12 post-dose. Note the differences in the *y*-axes of these graphs as compared to those in Figure 8. The increased platelet levels at all treatment groups reflect a compensatory rebound to return to homeostasis after depletion.

Table S1. Aldehyde tag insertion and ADC production does not impact thermal stability as measured by thermofluorescence.

	Untagged αHER2	αHER2 CH1	αHER2 CT	αHER2 LC	αHER2 CT HIPS-Glu-PEG2-May
Reading 1*	67	68	67	68	67
Reading 2	68	68	68	68	67
Reading 3	68	68	68	68	66
Average	**67.7**	**68.0**	**67.7**	**68.0**	**66.7**
Standard deviation	0.6	0.0	0.6	0.0	0.6

* Numbers indicate the first observed thermal transition in °C.

Table S2. The LC, CH1, and CT aldehyde tags do not induce immune responses in T-cells from donors representing the world MHC class II allotypes.

Sample	% Response*
Wild-type αHER2	4
αHER2 CH1 unconjugated.	8
αHER2 CT unconjugated.	10
αHER2 LC unconjugated.	2
αHER2 CH1 ADC	4
αHER2 CT ADC	6
αHER2 LC ADC	8
Control 1, Humanized A33**	22
Control 2, KLH***	74

* The % response summarizes the results of the T-cell proliferation assay.

** A relatively immunogenic antibody for which benchmark clinical immunogenicity data are available.

*** A broadly recognized immunostimulatory protein.