Supporting Information

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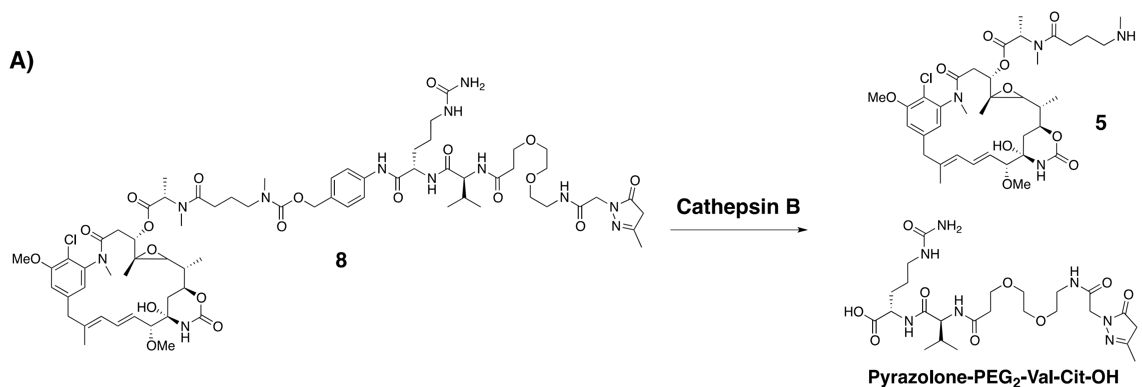


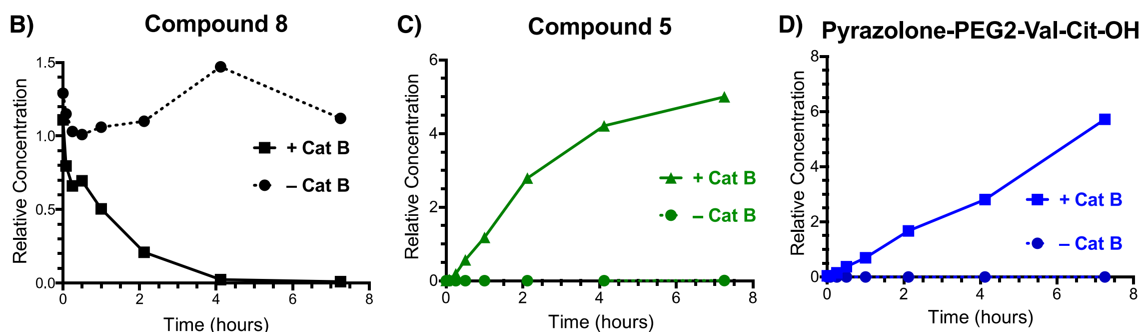
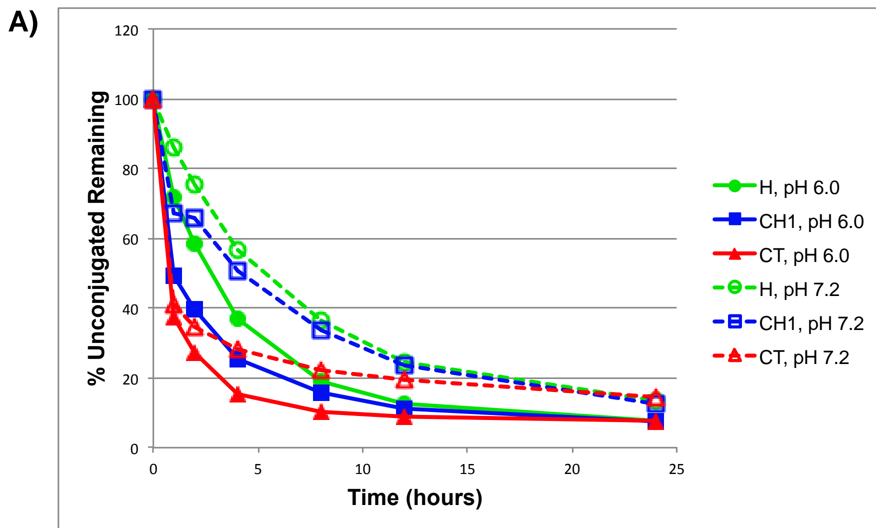


Figure S1. Subjection of compound 8 to Cathepsin B releases compound 5. In the absence of Cathepsin B, compound 5 is not detected. A) Schematic representation of the cleavage products from Cathepsin B plus compound **8**. B) Consumption of compound **8** in the presence of Cathepsin B. C and D) Generation of compound **5** (C) and Pyrazolone-PEG$_2$-Val-Cit-OH (D) in the presence of Cathepsin B.

A)



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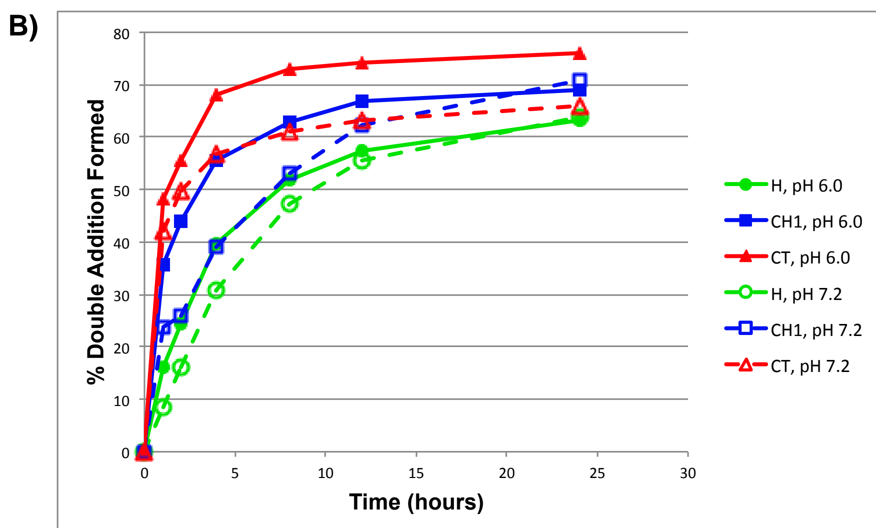


Figure S2. Relative rates of pyrazolone addition as measured by reduced reversed phase HPLC (PLRP-S). A) Monitoring the Knoevenagel Condensation at pH 6.0 and pH 7.2 over time reveals differences in the relative rates of first addition of the pyrazolone among tag sites; from fastest to slowest the sites ranked C-Term > CH-1 > Hinge. B) Similarly, monitoring the Michael Addition at pH 6.0 and pH 7.2 shows that the speed of the second addition of the pyrazolone also varies among tag sites; from fastest to slowest the sites ranked C-Term > CH-1 > Hinge.

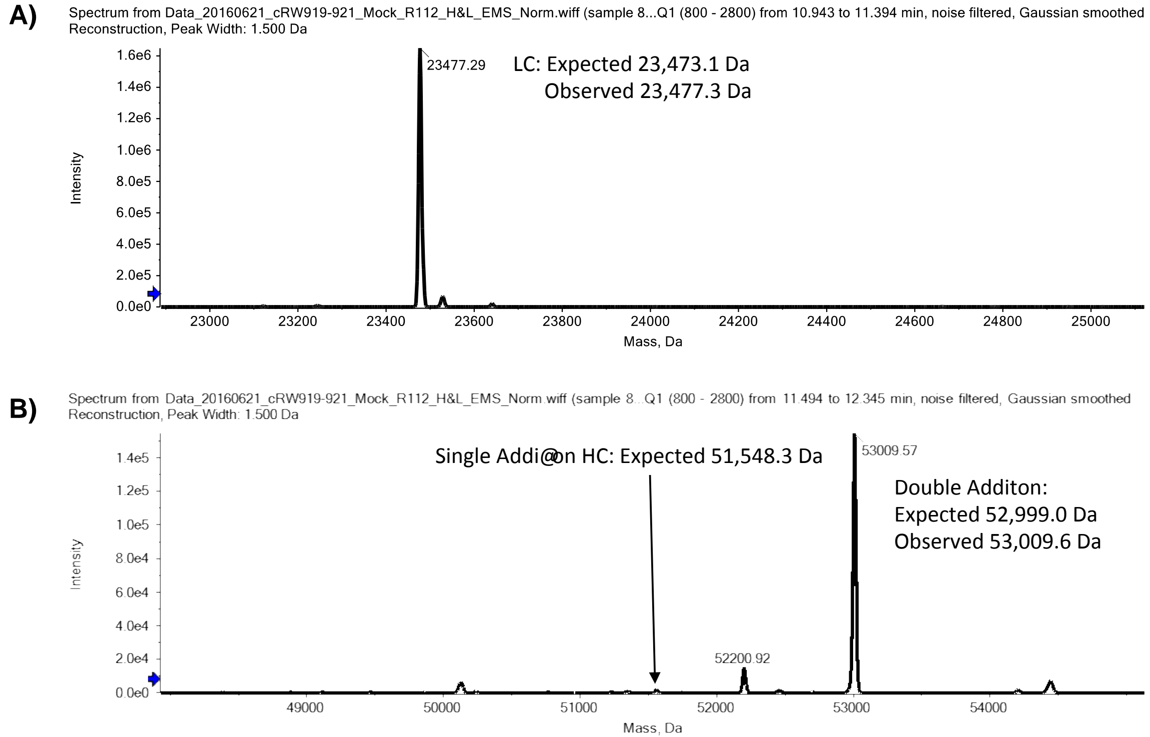


Figure S3. Representative mass spectral data highlighting double addition to the heavy chain of the antibody. A) Mass spectral data for the light chain portion of the antibody showing no modification. B) Mass spectral data for the heavy chain portion showing double addition resulting from TKM ligation of compound **8**.

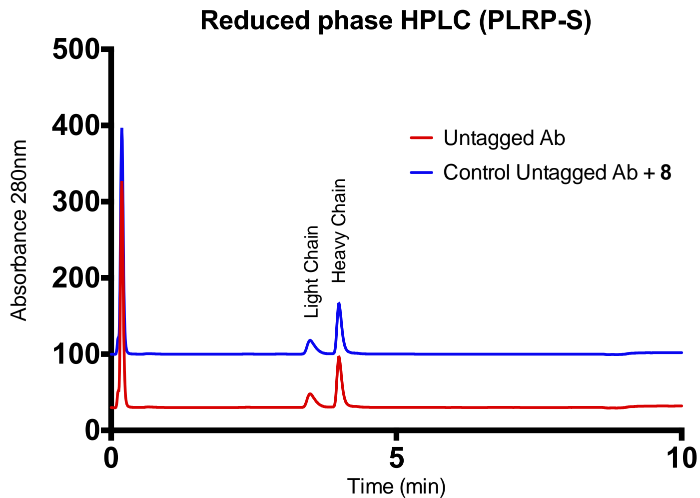
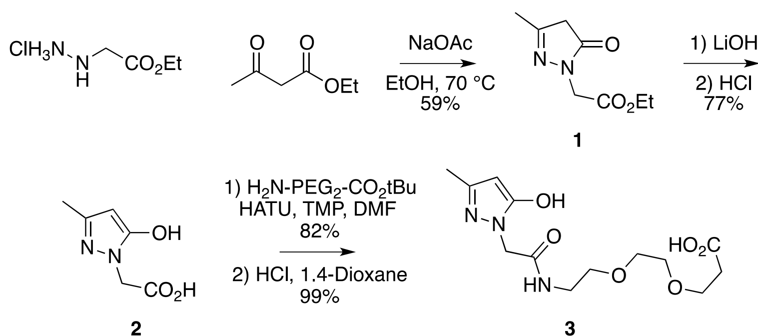


Figure S4. Control experiment showing no reaction of compound 8 to an untagged antibody. When an untagged (wild-type) antibody bearing no aldehyde group was subjected to the general bioconjugation conditions with compound **8**, no addition of compound **8** was observed by reversed phase HPLC (PLRP-S).

General.

Synthetic reagents were purchased from Sigma-Aldrich, Acros, and TCI and were used without purification unless noted otherwise. Compound **6** was purchased from Levena Biopharma. All reactions were carried out in flame-dried glassware under N_2 unless otherwise noted. Anhydrous solvents were obtained from commercial sources in sealed bottles. In all cases, solvent was removed by reduced pressure with a Buchi Rotovapor R-114 equipped with a Buchi V-700 vacuum pump. Column chromatography was performed with a Biotage Isolera Prime chromatograph. HPLC analyses were conducted on an Agilent 1100 Series Analytical HPLC equipped with a Model G1322A Degasser, Model G1311A Quarternary Pump, Model G1329A Autosampler, Model G1314 Variable Wavelength Detector, Agilent Poroshell 120 SB C18, 4.6 mm x 50 mm column at room temperature using a 10-100% gradient of water and acetonitrile containing 0.1% formic acid. HPLCs were monitored at 254 nm. Emeryville Pharmaceutical Services acquired NMR spectra on a Bruker 400 MHz spectrometer. The NMR data are reported as follows: chemical shift in ppm from an internal tetramethylsilane standard on the δ scale, multiplicity (br = broad, app = apparent, s = singlet, d = doublet, t = triplet, q = quartet, and m = multiplet), coupling constants (Hz), and integration.



Scheme S1. Synthesis of compound 3.

Synthesis of ethyl 2-(3-methyl-5-oxo-4,5-dihydro-1*H*-pyrazol-1-yl)acetate (1).

A 200 mL round bottom flask was charged with 2.63 g (17.1 mmol) of ethyl hydrazinoacetate hydrochloride, sodium acetate (1.4 g, 17.1 mmol) and 50 mL of ethanol. The solution was stirred at room temperature for 5 min. Ethyl acetoacetate 2.18 mL (17.1 mmol) was added and the solution was stirred at 70 °C for 18 h. The

heterogeneous solution was then cooled to room temperature and filtered. The filtrate was washed with 20 mL of cold ethanol thrice. The solution was collected, concentrated by rotary evaporation and then purified by reversed phase flash chromatography using acetonitrile:water [5 column volumes (CV)] of water followed by a gradient of water to 50% acetonitrile:water over 15 CV to afford 1.857 g of the desired product in 59% yield. H NMR (400MHz, $CDCl_3$) δ 4.43 (s, 2H), 4.24 (q, J = 7.2 Hz, 2H), 3.28 (s, 2H), 2.14 (s, 3H), 1.30 (t, J = 7.2 Hz, 3H). LRMS calcd for $C_8H_{12}N_2O_3$ $[M+H]^+$: 185.0; found 185.0.

Synthesis of 2-(3-methyl-5-oxo-4,5-dihydro-1*H*-pyrazol-1-yl)acetic acid (2).

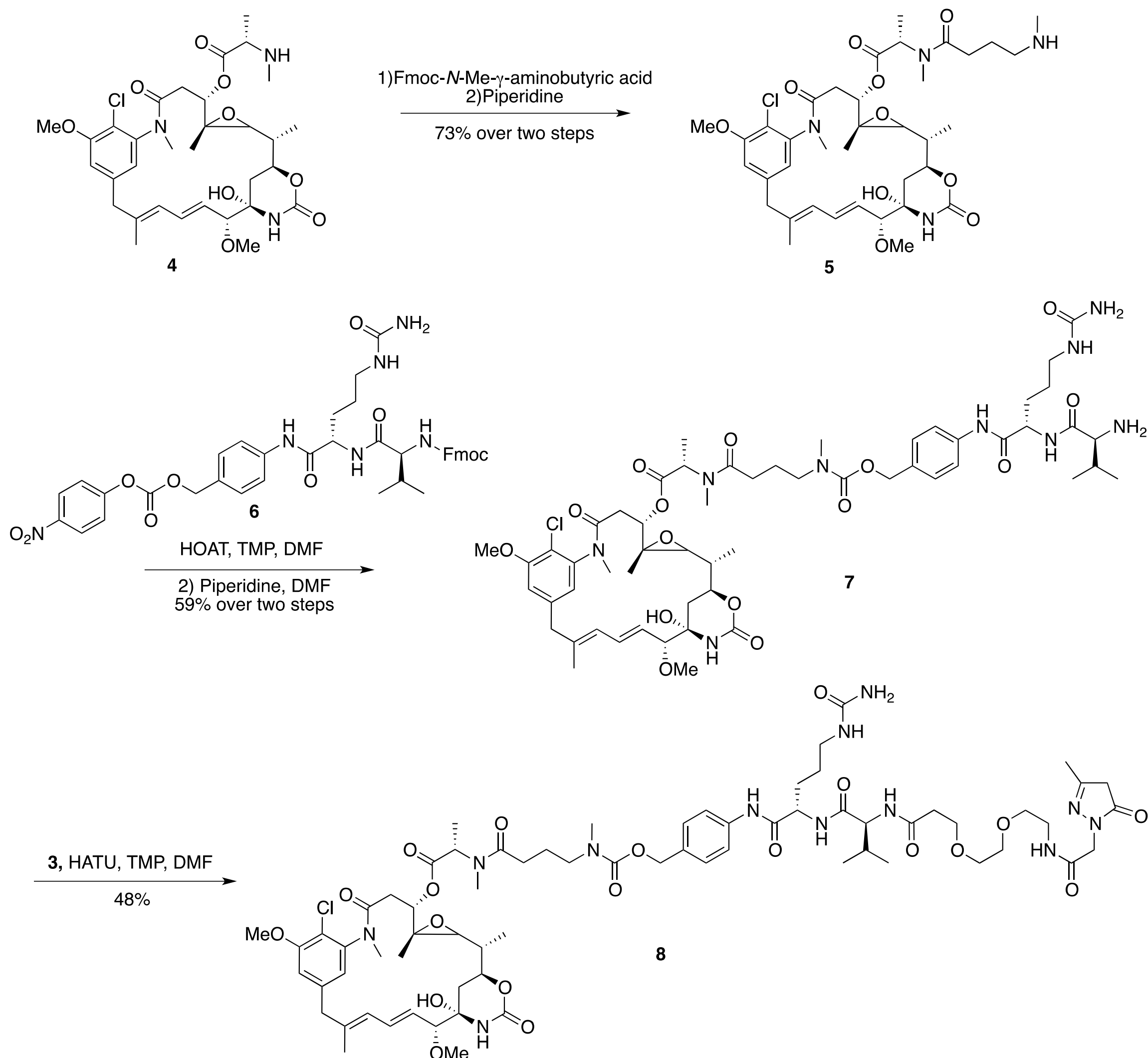
A 20 mL scintillation vial was charged with 428.4 mg (2.33 mmol) of compound **1** and 2 mL of water. To this solution was added 2 mL of a 2.3 M aqueous solution of lithium hydroxide and stirred for 18 h. The reaction was cooled to 0 °C and 1.165 mL of 4 M HCl (aq) dropwise. The crude reaction was concentrated by rotary evaporation and purified by reversed phase chromatography using just water as the eluting solvent to yield 570.3 mg of the product. The compound was repurified by reversed phase flash chromatography using water as the eluting solvent to afford 279.5 mg of the desired product in 77% yield. ^1H NMR (400MHz, DMSO) δ 5.65 (s, 1H), 4.78 (s, 2H), 2.22 (s, 3H). ^{13}C NMR (125MHz, DMSO) δ 167.98, 159.96, 146.51, 90.10, 46.64, 12.07. LRMS calcd for $C_6H_8N_2O_3$ $[M+H]^+$: 157.0; found 157.0.

Preparation of 3-(2-(2-(2-(3-methyl-5-oxo-4,5-dihydro-1H-pyrazol-1-yl)acetamido)ethoxy)ethoxy)propanoic acid (3).

To a dried 20 mL glass scintillation vial containing a dried pea stir bar was added acid **2** (937.4 mg, 4.9 mmol), HATU (1903.0 mg, 5.0 mmol), 2,4,6-trimethylpyridine (605.9 mg, 660.7 μL, 5.0 mmol), and anhydrous DMF (13 mL). The clear, colorless solution was stirred at room temperature for 15 min. In a separate vial, *tert*-butyl 3-(2-(2-aminoethoxy)ethoxy)propanoate (1172.7 mg, 5.0 mmol), 2,4,6-trimethylpyridine (1211.8 mg, 1321.5 μL, 10.0 mmol), and anhydrous DMF (3 mL) were combined and added dropwise, slowly, to the stirring solution. The reaction was allowed to stir at room temperature for 2 h, then purified by reversed phase flash chromatography using a gradient of 0-100% acetonitrile:water, giving the *tert*-butyl 3-(2-(2-(2-(5-hydroxy-3-methyl-1*H*-pyrazol-1-yl)acetamido)ethoxy)ethoxy)propanoate as a brownish colored viscous mass (1517.3 mg, 82% yield). ^1H NMR (400MHz, DMSO) δ 10.85 (bs, 1H), 7.81 (bs, 1H), 5.12 (bs, 1H), 4.34 (bs, 2H), 3.59 (t, J = 6.2 Hz, 2H), 3.49 (s, 4H), 3.39 (t, J =

5.8 Hz, 2H), 3.21 (app q, *J* = 5.9 Hz, 2H), 2.42 (t, *J* = 6.4 Hz, 2H), 2.00 (s, 3H), 1.40 (s, 9H). LRMS calcd for $C_{17}H_{30}N_3O_6$ $[M+H]^+$: 372.2; found 372.1.

To a dried 20 mL glass scintillation vial containing a dried stir bar was added *tert*-butyl 3-(2-(2-(2-(5-hydroxy-3-methyl-1*H*-pyrazol-1-yl)acetamido)ethoxy)ethoxy)propanoate (722.6 mg, 1.9 mmol). A solution of HCl (4.0 M in 1,4-dioxane, 4.9 mL) was added by syringe and the solution stirred at room temperature for 12 h. The solution was concentrated by rotary evaporation and purified by reversed phase flash chromatorgraphy using a gradient of 0-100% acetonitrile:water, giving compound **3** as a pale yellow solid (HCl salt)(684.5 mg, 99% yield). ^{1}H NMR (400MHz, DMSO) δ 8.23 (bs, 1H), 5.58 (s, 1H), 4.57 (s, 2H), 3.61 (t, *J* = 6.4 Hz, 2H), 3.51 (s, 4H), 3.43 (t, *J* = 5.6 Hz, 2H), 3.26 (app q, *J* = 5.6 Hz, 2H), 2.45 (t, *J* = 6.4 Hz, 2H), 2.19 (s, 3H). ^{13}C NMR (125MHz, DMSO) δ 173.09, 165.19, 156.92, 146.23, 89.98, 69.99, 69.98, 69.26, 66.69, 47.57, 35.22, 12.13, 1.63. LRMS calcd for $C_{13}H_{22}N_3O_6$ $[M+H]^+$: 316.2; found 316.3.

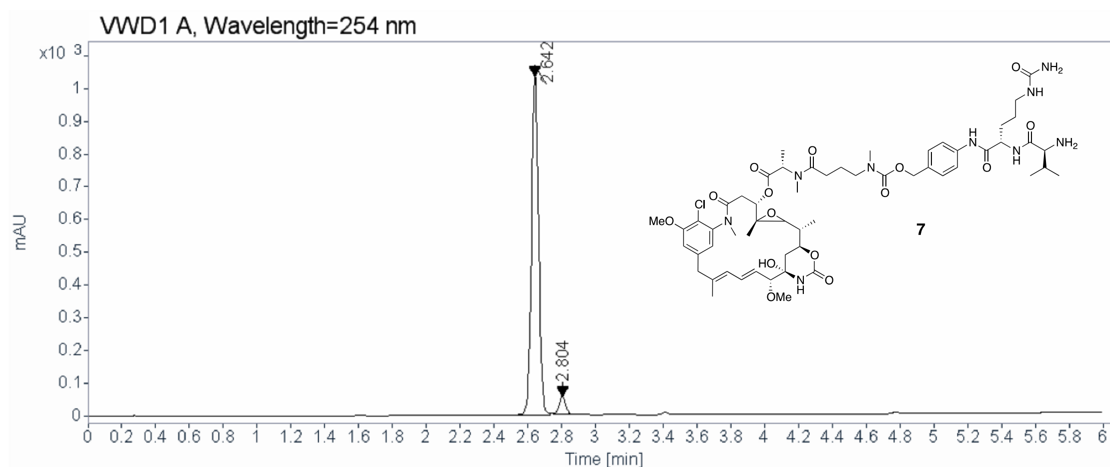


Scheme S2. Synthesis of compound 8.

Synthesis of compound 5. To a solution of compound **4** (0.95 g, 1.46 mmol)[1] was added Fmoc-*N*-methyl-γ-aminobutyric acid (0.59 g, 1.75 mmol), HATU (0.66 g, 1.47 mmol), and DIPEA (0.76 mL, 4.38 mmol). The reaction was stirred for 1.5 h and purified by silica gel flash chormatorgraphy using a 1-15% MeOH:DCM gradient. Upon concentration, the purified material was immediately diluted with 2 mL of DMF and 0.4 mL of piperidine was added. The reaction was stirred for 15 min and purified by reversed phase flash chromatography using a gradient of 0-100% of acetonitrile:water with 0.1% formic acid in the eluent. This afforded 843 mg of desired product in 78% yield over two steps. ^{1}H NMR (400MHz, DMSO) δ 8.36 (s, 1H), 7.20 (s, 1H), 6.91 (s, 1H), 6.63-6.52 (m, 3H), 5.56 (dd, *J* = 14, 9.2 Hz, 1H), 5.33 (q, *J* = 6.8 Hz, 1H), 4.52 (dd,

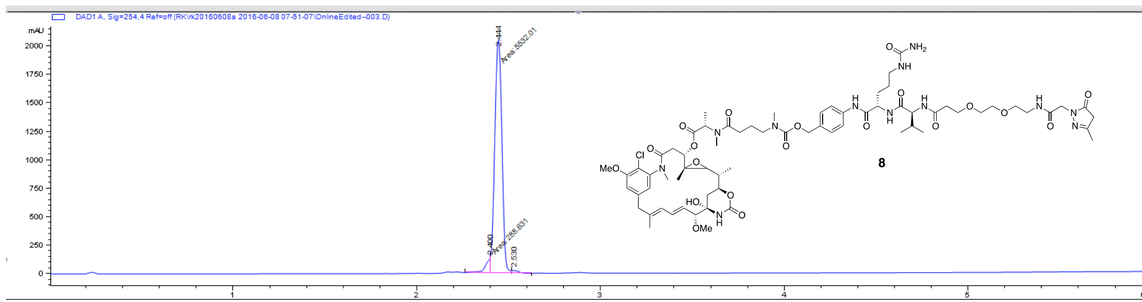
J = 12.4, 2.8 Hz, 1H), 4.08 (t, J = 10.4 Hz, 1H), 3.94 (s, 3H), 3.49 (d, J = 9.2 Hz, 1H), 3.44 (d, J = 12.4 Hz, 1H), 3.25-3.20 (m, 5H), 3.10 (s, 3H), 2.79 (d, J = 9.6 Hz, 1H), 2.73-2.64 (m, 5H), 2.56-2.53 (m, 1H), 2.48 (m, 1H), 2.31 (s, 3H), 2.29-2.21 (m, 1H), 2.03 (dd, J = 14, 2.4 Hz, 1H), 1.85-1.76 (m, 1H), 1.69-1.63 (m, 1H), 1.60 (s, 3H), 1.50-1.41 (m, 2H), 1.24 (d, J = 12.8 Hz, 1H), 1.17 (d, J = 6.8 Hz, 3H), 1.12 (d, J = 6.4 Hz, 3H), 0.78 (s, 3H). ^{13}C NMR (125MHz, DMSO) δ 155.73, 151.69, 141.84, 141.66, 138.78, 133.04, 128.97, 125.64, 122.10, 117.65, 114.38, 88.65, 80.45, 78.08, 73.62, 67.22, 67.21, 60.50, 57.04, 56.58, 51.95, 48.99, 45.91, 45.89, 45.87, 38.19, 36.82, 35.62, 33.67, 32.41, 30.60, 30.15, 22.65, 15.48, 14.89, 13.54, 11.81. LRMS calcd for $C_{37}H_{54}ClN_4O_{10}$ $[M+H]^+$: 749.4; found 748.9.

Synthesis of compound 7. To a solution of compound **5** (45.8 mg, 0.058 mmol) in 300 μL of DMF was added 44.1 mg (0.058 mmol) of (9*H*-fluoren-9-yl)methyl ((*S*)-3-methyl-1-(((*S*)-1-((4-((((4-nitrophenoxy)carbonyl)oxy)methyl)phenyl)amino)-1-oxo-5-ureidopentan-2-yl)amino)-1-oxobutan-2-yl)carbamate (compound **6**), HOAT (7.9 mg, 0.058 mmol), and 2,4,6-trimethylpyridine (15.3 μL, 0.115 mmol). The reaction was stirred for 18 h after which 100 μl of piperdine was added to the reaction. The reaction was stirred for 30 min and then purified by reversed phase flash chromatography using a gradient of 0-100% acetonitrile:water, giving 39.5 mg of the compound **7** in 59% yield over two steps in 95% purity. LRMS calcd for $C_{56}H_{81}ClN_9O_{15}$ $[M+H]^+$: 1154.6; found 1154.2.



Synthesis of compound 8. To a stirred solution of compound **3** (10.2 mg, 0.029 mmol) in 200 μL of DMF was added compound **7** (33.4 mg, 0.029 mmol), HATU (13.4 mg, 0.035 mmol), 2,4,6-trimethylpyridine (7.9 μL, 0.058 mmol). The reaction was stirred for 5

hours, then purified by reversed phase flash chromatography using a gradient of 0-100% acetonitrile:water, giving the 20.2 mg of the compound **7** in 48% yield and in 94% purity. LRMS calcd for $C_{69}H_{100}ClN_{12}O_{20}$ $[M+H]^+$: 1451.7; found 1451.2.



General Bioconjugation Conditions.

The fGly-tagged antibody was conjugated to compound **8** at 15 mg/mL and 8 drug:antibody equivalents for 16 h at 37 °C in 50 mM sodium citrate, 50 mM NaCl pH 7.2 in the presence of 5% DMA and 0.085% Triton X-100. Free drug was removed using tangential flow filtration (TFF). Then, the ADCs were run on a preparative hydrophobic interaction chromatography column (HIC; GE Healthcare 28-4110-05; mobile phase A: 1.0 M ammonium sulfate/25 mM sodium phosphate, pH 6.8, and mobile phase B: 25% isopropanol, 18.75 mM sodium phosphate, pH 6.8). An isocratic gradient of 33% B was used to elute unconjugated material, followed by a linear gradient of 41-95% B to separate the differentially conjugated species. The differentially conjugated species were combined to yield a final drug-antibody ratio (DAR) of >3.5. The combined material was then buffer exchanged into 20 mM NaCitrate/50 mM NaCl, pH 5.5. To determine the DAR of the final product, ADCs were examined by reversed-phase chromatography of DTT-reduced samples (Agilent PLRP-S #PL1912-1802; mobile phase A: 0.1% trifluoroacetic acid (TFA) and mobile phase B: 0.1% trifluoroacetic acid in CH_3CN). To monitor aggregation, samples were analyzed using size exclusion chromatography (SEC; Tosoh #08541) with a mobile phase of 300 mM NaCl, 25 mM sodium phosphate pH 6.8. Final products contained less than 5% aggregate.

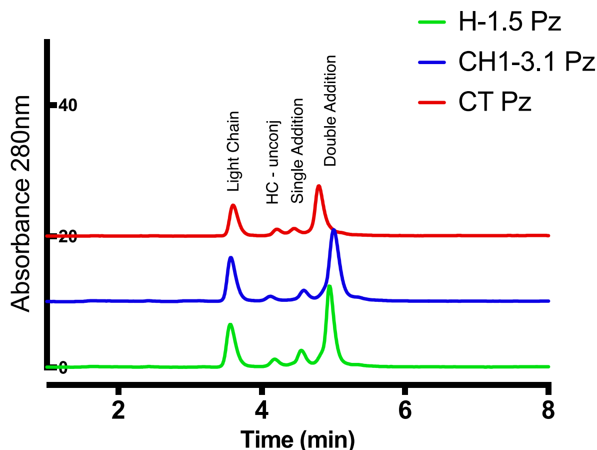


Figure S5. Reduced phase HPLC (PLRP-S) traces of CH1 (blue), CT (red), and Hinge (green) showing reaction of compound 8 with aldehyde tagged antibodies. Retention times of conjugates vary with respect to payload placement on the heavy chain of the protein sequence.

In vitro cytotoxicity assay

The antigen-positive suspension cell line is maintained in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Seradigm) and Glutamax (Invitrogen) in a 37 °C incubator with 5% CO_2. Cells are passaged regularly to ensure log-phase growth. On the day of the assay, 5000 cells are added per well into 96-well plates in 100 μL normal growth medium and returned to the incubator for 4-5 h to equilibrate to plating conditions. Cells are then treated with 20 μL of serially diluted drug/linkers or antibody-drug conjugates at 6x final desired concentration. After 5 d of incubation, cell viability is measured using CellTiter-Glo reagent (Promega) following manufacturer's recommendation. Luminescence is read on SpectraMax M5 plate reader. GraphPad Prism software is used for data analysis, including calculation of an IC_{50} from luminescence values normalized to controls present on each plate.

Xenograft studies

Female ICR SCID mice were inoculated subcutaneously with 5 x 10^7 tumor cells in 50% Matrigel. Dosing was initiated when the tumors reached an average of 261 mm^3. Animals (8 mice/group) were given an intravenous dose (10 mg/kg ADC or vehicle alone) every four days for a total of four doses. The animals were monitored twice

weekly for body weight and tumor size. Tumor volume was calculated using the formula:

$$\text{Tumor volume (mm}^3) = \frac{w^2\ x\ l}{2}$$

Tumor growth inhibition (TGI%) was calculated on day 15 when the negative control group reached the predetermined endpoint tumor volume (2,000 mm^3). TGI (%) was defined using the following formula:

TGI (%) = ($TV_{control}$ group - $TV_{treated}$ group)/$TV_{control}$ x 100

Time to endpoint (TTE) of individual tumors was defined as the time (in days) required for the tumor to reach to the endpoint volume. Tumor Growth Delay (TGD) for each treatment group was defined as median $TTE_{treated}$ - median $TTE_{control}$.

Pharmacokinetic (PK) study design

Male Sprague-Dawley rats (3 per group) were dosed intravenously with a single 3 mg/kg bolus of ADC. Plasma was collected at 1 h, 8 h and 24 h, and 2, 4, 6, 8, 10, 14, and 21 days post-dose. Plasma samples were stored at -80 °C until use.

PK sample analysis

The concentrations of total antibody, and total ADC were quantified by ELISA. For total antibody, conjugates were captured with an anti-human IgG-specific antibody and detected with an HRP-conjugated anti-human Fc-specific antibody. For total ADC, conjugates were captured with an anti-human Fab-specific antibody and detected with a mouse anti-maytansine primary antibody, followed by an HRP-conjugated anti-mouse IgG-subclass 1-specific secondary antibody. Bound secondary antibody was detected using Ultra TMB One-Step ELISA substrate (Thermo Fisher). After quenching the reaction with sulfuric acid, signals were read by taking the absorbance at 450 nm on a Molecular Devices Spectra Max M5 plate reader equipped with SoftMax Pro software. Data were analyzed using GraphPad Prism and Microsoft Excel software.

Cathepsin B Assay

Cathepsin B (Cat B) (purchased from R&D systems) is divided into 1 µL aliquots and kept at -80°C until use. On the day of assay, freshly prepared 300 mM DTT is diluted 1:10 in 50 mM MES, pH 5.0 to make "activation buffer". 4 µL activation buffer is added to 1 µL Cat B, and is incubated at room temperature for 15 min. Then, 95 µL 25 mM MES pH 5.0 (assay buffer) is added to the activated Cat B. 100 µL of a no enzyme blank solution is prepared by diluting a 1 mg/mL BSA solution to 2.2 µg/mL in activation buffer diluted 1:20 with assay buffer. 100 µL of substrate is prepared from 1 mM DMSO stock solution by diluting 1:200 into 30% ACN in water. All solutions are prewarmed to 37 °C, and then mixed and kept at 37 °C for the duration of the assay. 25 µL aliquots are sampled at the following time points: 0, 0.5, 1, 1.5, 2, 3, 5, and 7 hours. The aliquots are immediately quenched with 25 µL ice cold 0.5 µM Tolbutamide in ACN, and are kept on ice until analysis. Analysis was done by LC/MS/MS using an Agilent Series 1100 HPLC in-line with an AB Sciex 4000 QTRAP mass spectrometer. Separation was done on a 2.1 x 50 mm biphenyl column. Multiple-reaction monitoring was performed targeting the intact linker-payload, the toxin payload alone, and the cleaved linker alone. Analyte counts at each time point were divided by the Tolbutamide internal standard counts, and reported as relative concentration.

References

(1) Albers, A. E.; Garofalo, A. W.; Drake, P. M.; Kudirka, R.; de Hart, G. W.; Barfield, R. M.; Baker, J.; Banas, S.; Rabuka, D. European Journal of Medicinal Chemistry. *European Journal of Medicinal Chemistry* **2014**, 1–7.