SUPPLEMENTARY INFORMATION

Site-Specific Lysine Arylation as an Alternative Bioconjugation Strategy for Chemically Programmed Antibodies and Antibody-Drug Conjugates

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SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Synthesis of MS-PODA and β-lactam hapten derivatives of folate and LLP2A

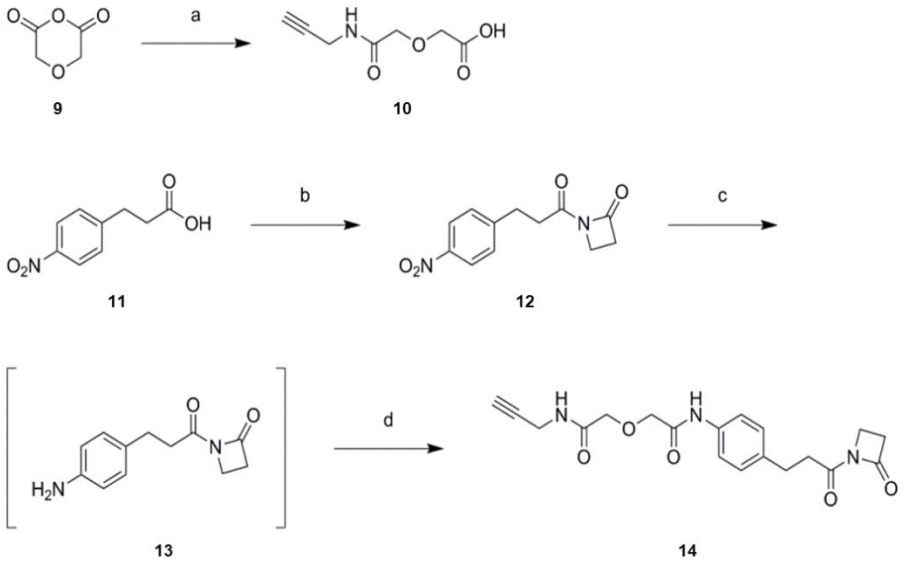
General methods

All experiments involving moisture-sensitive compounds were conducted under anhydrous conditions (positive argon pressure) using standard syringe, cannula, and septa apparatus. Commercial reagents were purchased from Sigma-Aldrich, TCI America, Acros, Chem-Impex, Ambeed, and Novabiochem. All solvents were purchased in anhydrous form (Sigma-Aldrich) and used without further drying. HPLC-grade hexanes, ethyl acetate (EtOAc), dichloromethane (DCM), and methanol were used in chromatography. Silica gel column chromatography employed a Teledyne CombiFlash Rf 200i instrument with either hexane/EtOAc or DCM/methanol gradients. NMR spectra were recorded using a Varian Inova 400 MHz instrument. Coupling constants are reported in Hertz (Hz), and peak shifts are reported in δ (ppm) relative to $CDCl_3$ (^1H 7.26 ppm, ^{13}C 77.16 ppm). Low-resolution mass spectra (ESI) were measured with an Agilent 1200 series LC/MSD-SL system. High resolution mass spectra (HRMS) were obtained by positive ion, ESI analysis on a Thermo Fisher Scientific LTQ Orbitrap XL mass spectrometer with HPLC sample introduction using a short narrow-bore C18 reversed-phase (RP) column with acetonitrile (MeCN)-H_2O gradients. Preparative HPLC purification was performed using a Waters 2545 binary pump (MeCN/H_2O gradient) with a Phenomenex Gemini-C_{18} (5 µm, 250 x 21 mm) preparative column and UV detection at 210 nm. Semi-preparative HPLC purification was performed using an Agilent 1200 series quaternary pump (MeCN/H_2O gradient) with a Phenomenex Kinetix-C_{18} (5 µm, 250 x 10 mm) semi-preparative column, 3 mL/min flow rate, and UV detection at 210 nm. Analytical HPLC analyses of purified peptides were performed using an Agilent 1200 series quaternary pump (MeCN/H_2O gradient) with a Phenomenex Gemini-C_{18} (5 µm, 250 x 4 mm) analytical column, 1 mL/min flow rate, and UV detection at 210 nm.

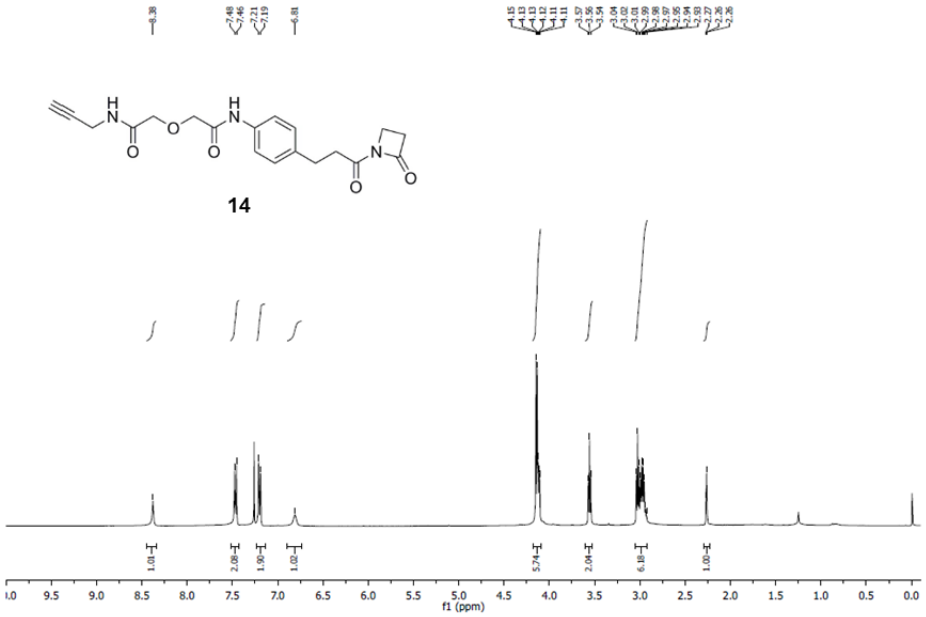
Synthesis of 2-(2-oxo-2-((4-(3-oxo-3-(2-oxoazetidin-1-yl)propyl)phenyl)amino) ethoxy)-*N*-(prop-2-yn-1-yl)acetamide (14)

The synthesis of β-lactam-hapten-alkyne **14** was achieved by coupling compounds **10** and **13** (**Scheme S1**). Compound **10** was prepared by treatment of diglycolic anhydride **9** with propargylamine. Compound **12** was synthesized according to literature procedures[1], hydrogenated to the corresponding aniline-containing **13** and then subsequently used for coupling with **10** to afford **14**. In detail, to a solution of 1-(3-(4-nitrophenyl)propanoyl)azetidin-2-one (**12**) (100 mg, 0.40 mmol) in EtOAc (15 mL) was added Pd/C (10% (w/w), 43 mg, 0.040 mmol). The mixture was degassed *in vacuo* and then the vessel was charged with H_2. After stirring at RT for 6 h, the mixture was filtered through Celite and the filtrate was concentrated *in vacuo*. To a solution of the resulting crude **13** in dimethylformamide (DMF; 15 mL) was added hydroxybenzotriazole hydrate (HOBt·H_2O; 80 mg, 0.52 mmol), **10** (76 mg, 0.44 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl; 85 mg, 0.44 mmol) and the mixture was stirred at RT for 2.5 h. DMF was removed by evaporation, H_2O was added,

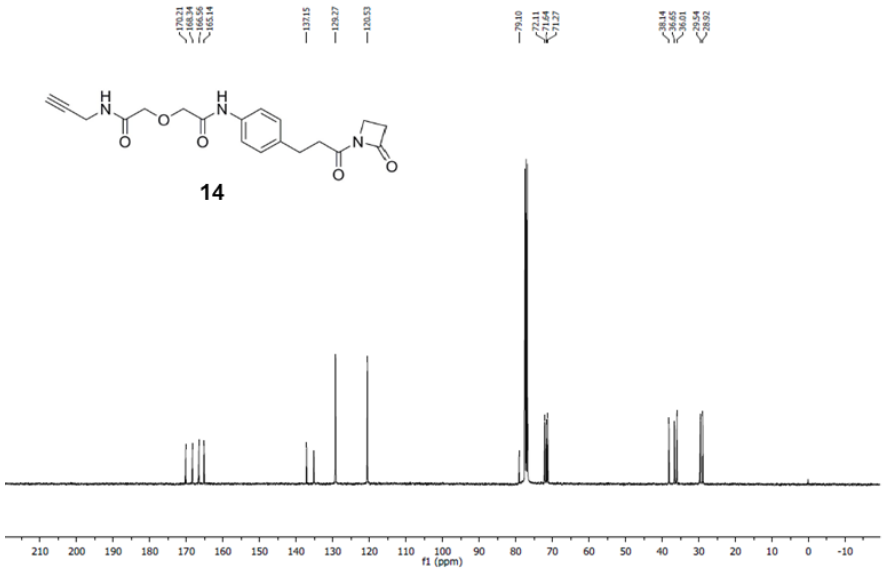
and the mixture was extracted 3 times with chloroform ($CHCl_3$). The organic layer was washed with brine, dried (Na_2SO_4) and the crude material was purified by silica gel CombiFlash chromatography (hexane/EtOAc gradient, 0-100% over 30 min) to afford **14** as an off-white powder (89 mg, 59%, 2 steps from **12**).^{1}H NMR (400 MHz, chloroform-d) δ 8.38 (s, 1H), 7.47 (d, J = 8.4 Hz, 2H), 7.20 (d, J = 8.4 Hz, 2H), 6.81 (s, 1H), 4.15 – 4.11 (m, 6H), 3.56 (t, J = 5.3 Hz, 2H), 3.04 – 2.93 (m, 6H), 2.26 (t, J = 2.5 Hz, 1H). ^{13}C NMR (101 MHz, chloroform-d) δ 170.21, 168.34, 166.56, 165.14, 137.15, 135.19, 129.27, 120.53, 79.10, 72.11, 71.64, 71.27, 38.14, 36.65, 36.01, 29.54, 28.92. HR-MS (ESI+) calculated for $C_{19}H_{22}N_3O_5$: 372.1554 $[M+H]^+$; found: 372.1552.



Scheme S1. Synthesis of compound **14**. (a) Propargylamine, THF, RT, 1 d, 43%; (b) thionyl chloride, 0°C to 60°C, 1.5 h then azetidin-2-one, *n*-BuLi, THF, -78°C to RT, overnight, 24%; (c) Pd/C, H_2, EtOAc, RT, 6 h; (d) **10**, EDC·HCl, HOBt·H_2O, DMF, 2.5 h, 59% (2 steps from **12**).



Spectrum 1. ^{1}H-NMR of compound **14.**



Spectrum 2. ^{13}C-NMR of compound **14.**

General procedure for solid-phase peptide synthesis (SPPS)

SPPS resin was pre-swollen in *N*-methyl-2-pyrrolidone (NMP) with shaking (20 min). Sieber Amide resin (Novabiochem, 0.71 mmol/g) was utilized for certain peptides and the loading procedure is described where applicable. On-resin fluorenylmethyloxycarbonyl (Fmoc) deprotection was achieved using 20% (v/v) piperidine in DMF with shaking (10 min). Fmoc-protected amino acids (2.0 – 4.0 eq based on resin) were dissolved in NMP and pre-activated by the addition of HATU (0.95

mole-eq relative to the amino acid) and DIEA (2.0 mole-eq relative to the amino acid) with shaking (1 min). The resin was washed with NMP, and the solution of HATU-activated amino acid was added to the washed resin. Coupling reactions were shaken at RT and allowed to proceed from 2 h to overnight, depending on the eq used and steric bulk of the amino acid. Coupling reactions were routinely checked for completion using a Kaiser test. Once completed, the resin was filtered and washed with NMP, followed by Fmoc-deprotection using 20% (v/v) piperidine in DMF with shaking (10 min). Cleavage of the Lys ε-amine Dde group was performed by treatment with 2% (v/v) hydrazine monohydrate in NMP (2 h, twice) with allyl alcohol (200 eq based on resin) for peptides **17** and **6**. Deprotection of the Lys ε-amine Alloc group was performed by treatment with $Pd(PPh_3)_4$ (0.30 eq based on resin) and $PhSiH_3$ (10 eq based on resin) in $CHCl_3$, which was well-degassed by bubbling with argon gas (20 min, 3 times). Following Alloc-deprotection, the resin was treated with 0.50% (w/v) sodium diethyldithiocarbamate trihydrate in DMF (20 min, 3 times) to scavenge residual Pd metal. Coupling with diglycolic anhydride **9** was performed in the presence of *N*,*N*-diisopropylethylamine (DIEA; 4.0 eq based on resin) in NMP (3 h) and the resin was subsequently coupled with 4-(5-(methylsulfonyl)-1,3,4-oxadiazol-2-yl)aniline (**18**, Ambeed, 2.0 eq based on resin) using HATU (0.95 mole-eq relative to the amino acid) and DIEA (2.0 mole-eq relative to the amino acid) with shaking (RT, 3 to 4 h). Cleavage of the finished resin with global deprotection was performed using a cocktail of trifluoroacetic acid (TFA)/triisopropylsilane (TIPS)/H_2O = 95:2.5:2.5 (4.0 mL, 2 h). The mixture was filtered, and the filtrate was added to cold diethyl ether (Et_2O). The resulting precipitate was washed with cold Et_2O (3 times). Crude peptides were dissolved in 0.1% TFA containing MeCN and H_2O and purified using preparative RP-HPLC. Further purification was conducted using semi-preparative RP-HPLC when needed. HPLC eluents were A: 0.1% TFA in H_2O; B: 0.1% TFA in MeCN.

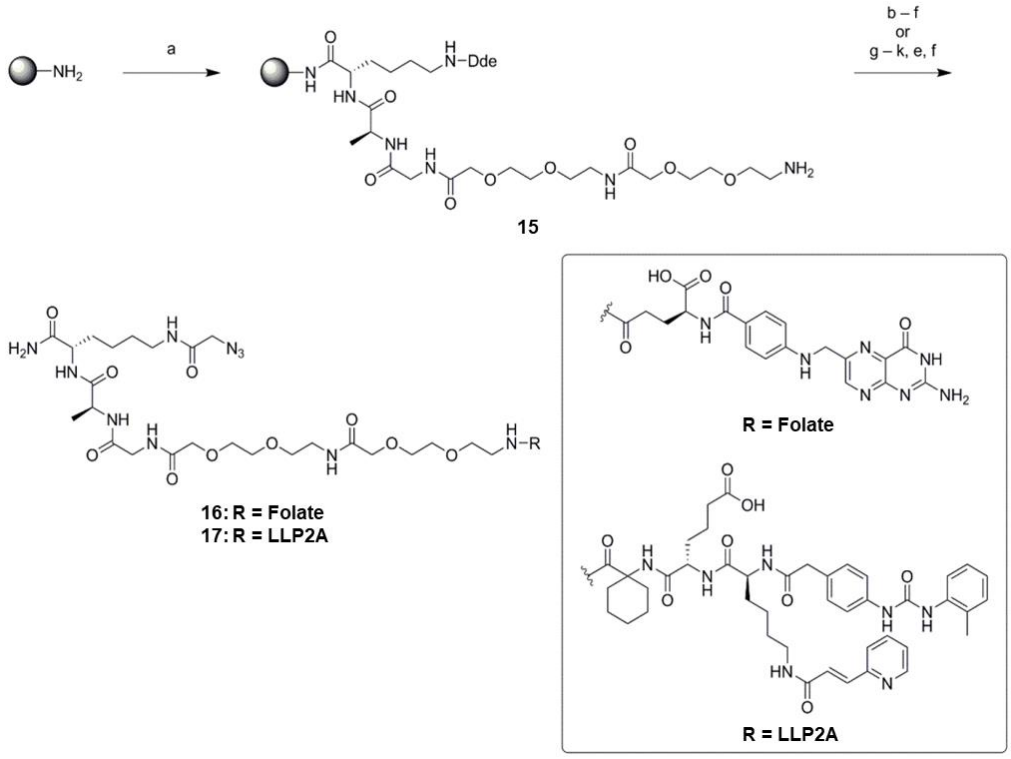
General procedure for Cu-catalyzed azide-alkyne cycloaddition reactions

Azido-containing peptides targeting either FOLR1 or integrin $\alpha_4\beta_1$ (1.0 eq) were dissolved in H_2O (5.0 mM) and mixed with **14** (1.1 eq) in DMSO (5.0 mM based on the peptide). Separately, 4.0% (w/v) $CuSO_4 \cdot 5H_2O$ in H_2O (0.13 eq), 0.10 M tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) in DMSO (0.25 eq), and 0.50 M sodium ascorbate in H_2O (5.0 eq) were combined and the mixture was added to the peptide solution and incubated in the dark (1 to 6 d). The resulting crude peptide was purified using preparative RP-HPLC using gradients consisting of A: 0.1% TFA in H_2O; B: 0.1% TFA in MeCN.

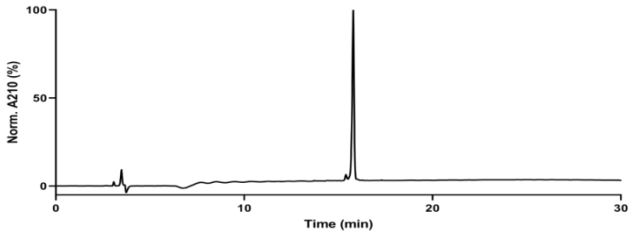
Synthesis of azido-folate and azido-LLP2A peptides

The synthesis of azido peptides targeting either FOLR1 or integrin $\alpha_4\beta_1$ were conducted using standard Fmoc-based solid phase peptide synthesis (SPPS) protocols using Fmoc-L-Lys(Dde)-OH, which can be selectively deprotected by treatment with hydrazine and functionalized at the final step of peptide resin construction (**Scheme S2**). Folate-containing peptide (**16**) was synthesized from Sieber Amide resin (Novabiochem, 0.71 mmol/g)-bound peptide (**15**) using a TFA-protected pteroic acid analog having an α-protected glutamic acid residue.[2] Following removal of the Lys ε-amine Dde protecting group with 2% (v/v) hydrazine monohydrate in NMP, the azide group was incorporated by coupling with azidoacetic acid. The finished resin was cleaved with a TFA cocktail

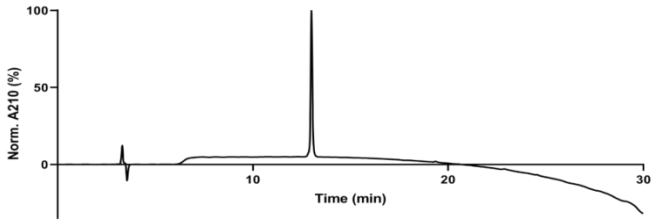
and purified by RP-HPLC using gradients consisting of A: 0.1% TFA in H_2O; B: 0.1% TFA in MeCN to afford **16** (**Scheme S2**). The LLP2A-containing peptide (**17**) was synthesized from Sieber Amide resin (Novabiochem, 0.71 mmol/g)-bound peptide (**15**) by sequentially coupling with Fmoc-1-aminocyclohexane carboxylic acid (Fmoc-Ach-OH), Fmoc-L-α-aminoadipic acid δ-tert-butyl ester (Fmoc-L-Aad(O*t*-Bu)-OH), and Fmoc-L-Lys(Alloc)-OH. The resulting resin was coupled with 4-(*N'*-(2-methylphenyl)urea)phenylacetic acid (MPUPA) NHS ester, and then the Alloc group was selectively removed by treatment with $Pd(PPh_3)_4/PhSiH_3$, followed by coupling with *trans*-3-(3-pyridyl)-acrylic acid.[3] The Lys ε-amine Dde protecting group was removed by treating with 2% (v/v) hydrazine in NMP in the presence of allyl alcohol (200 eq) to avoid undesired hydrogenation of acrylic acid moiety.[4] The azide group was subsequently incorporated by treatment with azidoacetic acid and the finished resin was treated with a TFA cocktail and purified by RP-HPLC using gradients consisting of A: 0.1% TFA in H_2O; B: 0.1% TFA in MeCN to obtain peptide (**17**) (**Scheme S2**).



Scheme S2. Synthesis of azido peptides targeting either FOLR1 (**16**) or integrin $\alpha_4\beta_1$ (**17**). (a) Fmoc-SPPS; (b) Fmoc-L-Glu(OH)-O*t*-Bu, HATU, DIEA, NMP then 20% (v/v) piperidine in DMF; (c) *N*-10-TFA-pteroic acid, HATU, DIEA, NMP; (d) 2% (v/v) hydrazine monohydrate in NMP; (e) azidoacetic acid NHS ester, DIEA, NMP; (f) TFA/TIPS/H_2O = 95:2.5:2.5; (g) Fmoc-SPPS using Fmoc-Ach-OH, Fmoc-L-Aad(O*t*-Bu)-OH, and Fmoc-L-Lys(Alloc)-OH; (h) MPUPA-NHS ester, DIEA, NMP; (i) $Pd(PPh_3)_4$, $PhSiH_3$, $CHCl_3$; (j) *trans*-3-(3-pyridyl)-acrylic acid, HATU, DIEA, NMP; (k) 2% (v/v) hydrazine monohydrate in NMP, allyl alcohol.



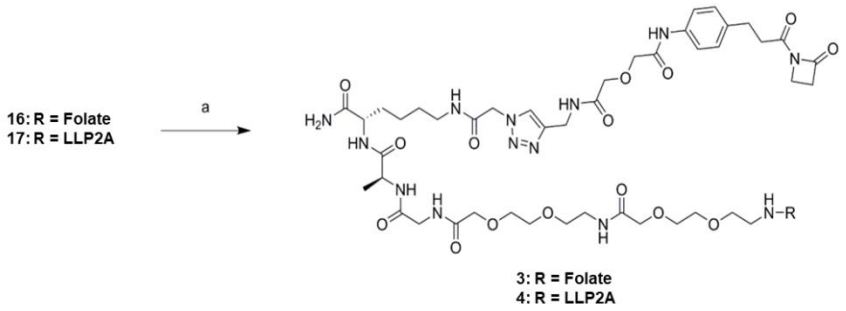
Chromatogram 1. Analytical HPLC of azido-folate peptide **16**. Analytical HPLC conditions: linear gradient elution (1 to 50% of B in A over 30 min).



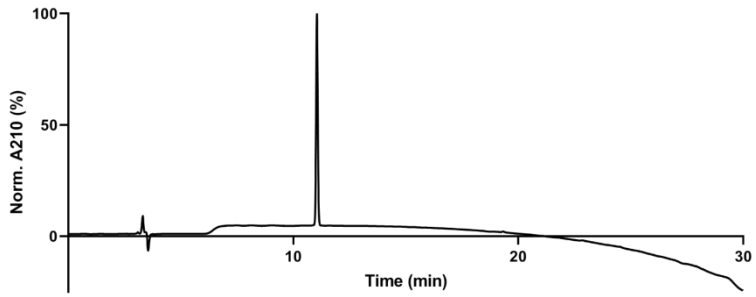
Chromatogram 2. Analytical HPLC of azido-LLP2A peptide **17**. Analytical HPLC conditions: linear gradient elution (10 to 100% of B in A over 30 min).

Synthesis of β-lactam-hapten-folate 3 and β-lactam-hapten-LLP2A 4

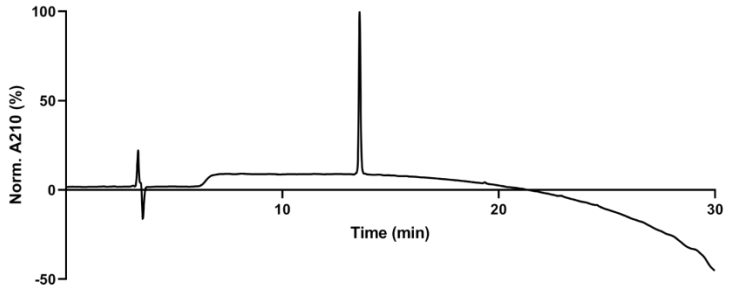
Compounds **3** and **4** (**Figure 4**) were synthesized utilizing Cu-catalyzed azide-alkyne cycloaddition reactions of azido peptides **16** and **17**, respectively, and **14** (**Scheme S3**).



Scheme S3. Synthesis of β-lactam-hapten-folate **3** and β-lactam-hapten-LLP2A **4**. (a) **14**, $CuSO_4 \cdot 5H_2O$, TBTA, sodium ascorbate, DMSO/H_2O = 1:1, 54% for **3** and 66% for **4**.



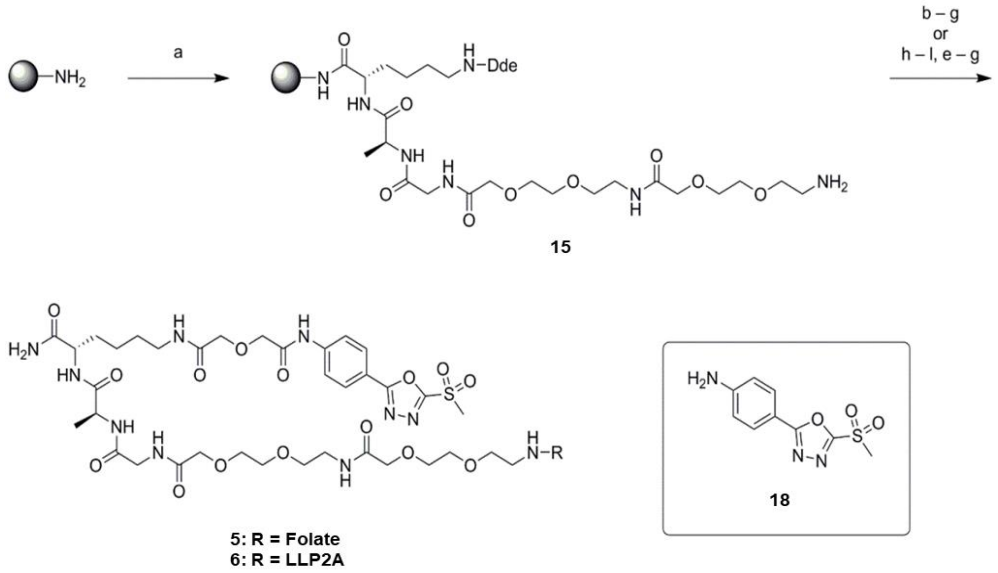
Chromatogram 3. Analytical HPLC of β-lactam-hapten-folate **3**. Analytical HPLC conditions: linear gradient elution (10 to 100% of B in A over 30 min).



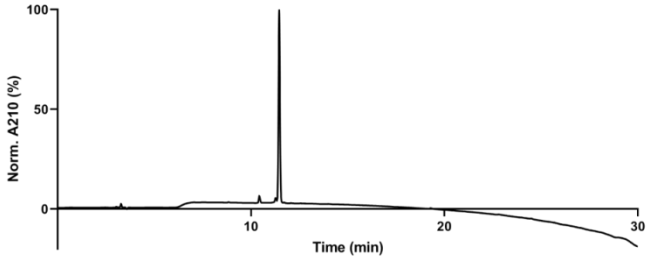
Chromatogram 4. Analytical HPLC of β-lactam-hapten-LLP2A **4**. Analytical HPLC conditions: linear gradient elution (10 to 100% of B in A over 30 min).

Synthesis of MS-PODA-folate 5 and MS-PODA-LLP2A 6

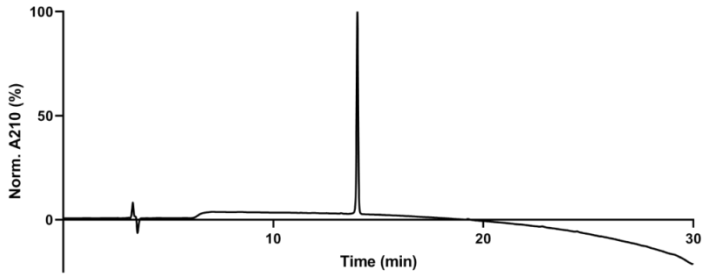
Compounds **5** and **6** (**Figure 4**) were synthesized by procedures described above for the synthesis of **16** and **17**, except that following the removal of the Lys ε-amine Dde protecting group, coupling was performed with the diglycolic anhydride **9** and the commercially available MS-PODA aniline derivative **18** rather than with azidoacetic acid NHS ester (**Scheme S4**).



Scheme S4. Synthesis of MS-PODA-folate **5** and MS-PODA-LLP2A **6**. (a) Fmoc-SPPS; (b) Fmoc-L-Glu(OH)-O*t*-Bu, HATU, DIEA, NMP then 20% (v/v) piperidine in DMF; (c) *N*-10-TFA-pteroic acid, HATU, DIEA, NMP; (d) 2% (v/v) hydrazine monohydrate in NMP; (e) diglycolic anhydride, DIEA, NMP; (f) **18**, HATU, DIEA, NMP; (g) TFA/TIPS/H_2O = 95:2.5:2.5; (h) Fmoc-SPPS using Fmoc-Ach-OH, Fmoc-L-Adp(O*t*-Bu)-OH, and Fmoc-L-Lys(Alloc)-OH; (i) MPUPA-NHS ester, DIEA, NMP; (j) $Pd(PPh_3)_4$, $PhSiH_3$, $CHCl_3$; (k) *trans*-3-(3-pyridyl)-acrylic acid, HATU, DIEA, NMP; (l) 2% (v/v) hydrazine monohydrate in NMP, allyl alcohol.



Chromatogram 5. Analytical HPLC of MS-PODA-folate **5**. Analytical HPLC conditions: linear gradient elution (10 to 100% of B in A over 30 min).

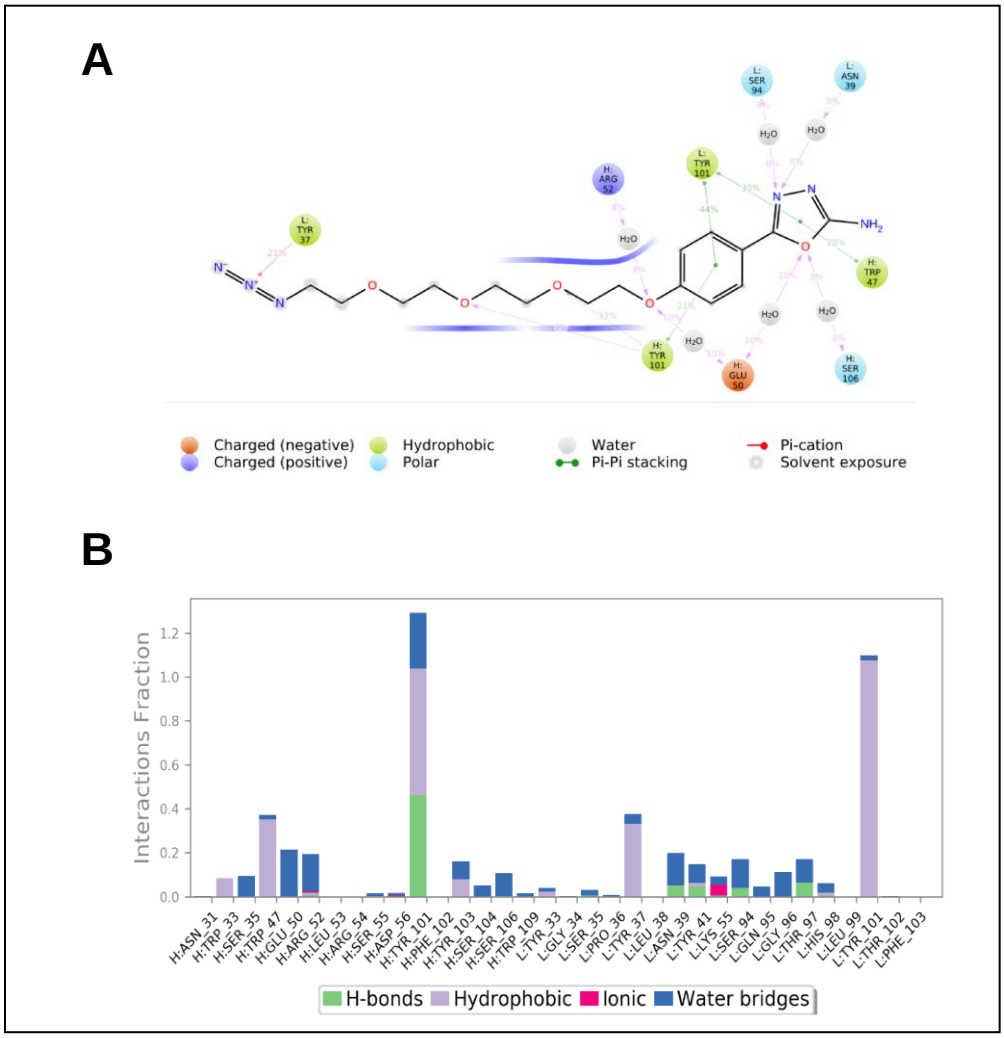


Chromatogram 6. Analytical HPLC of MS-PODA-LLP2A **6**. Analytical HPLC conditions: linear gradient elution (10 to 100% of B in A over 30 min).

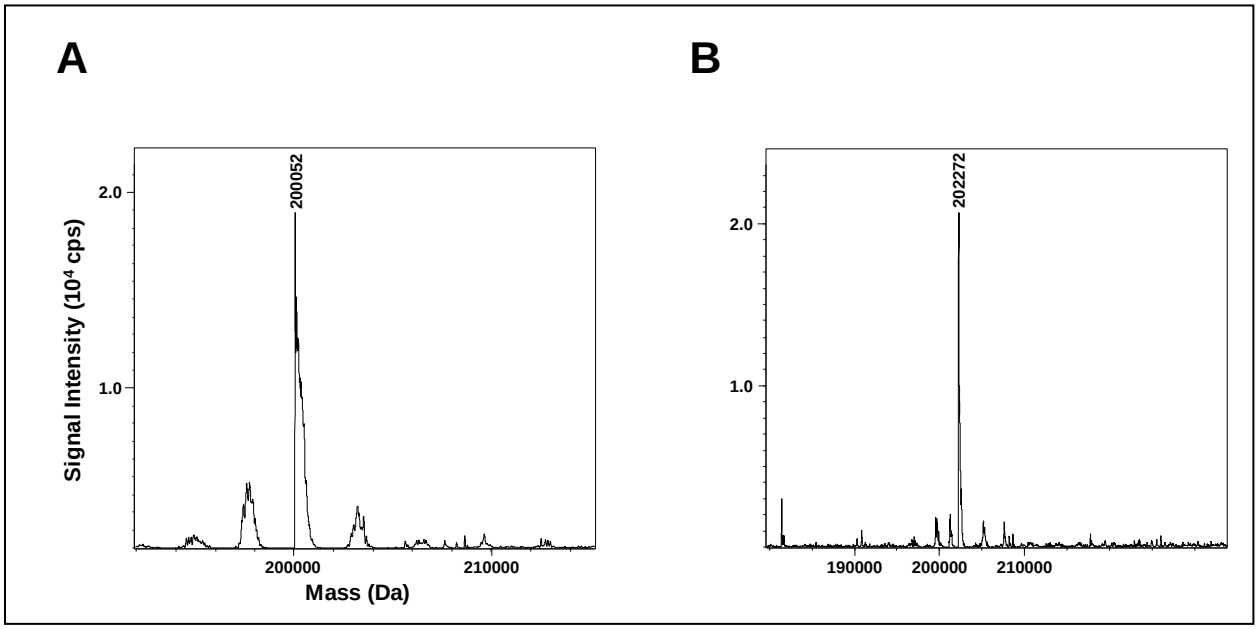
Table S1. Mass spectrometry analysis results and preparative and semi-preparative HPLC conditions of compounds **3**–**6**, **16**, and **17**.

	Calculated molecular weight (Da)	Found molecular weight (Da)	Preparative HPLC gradient	Semi-preparative HPLC gradient
16	1070.5 $[M + H]^+$	1070.3	10 to 30% of B in A over 30 min	
17	720.9 $[M +2H]^{2+}$	720.8	25 to 35% of B in A over 30 min	
3	721.3 $[M +2H]^{2+}$	721.3	10 to 40% of B in A over 30 min	
4	906.4 $[M +2H]^{2+}$	906.4	20 to 45% of B in A over 30 min	
5	1324.5 $[M + H]^+$	1324.3	5 to 50% of B in A over 30 min	10 to 40% of B in A over 30 min
6	1694.7 $[M + H]^+$	1694.5	5 to 55% of B in A over 30 min	15 to 45% of B in A over 30 min

SUPPLEMENTARY FIGURES



Supplementary Figure S1. Small molecule-antibody interactions in the *in silico* docking model of PODA-conjugated Lys99. (**A**) Starting with the crystal structure of h38C2_Arg Fab (PDB ID 6U85), Arg99 was replaced with an azido-$(PEG)_4$-PODA-derivatized Lys residue and subjected to energy minimization *in silico*. Shown are interactions that occur more than 8.0% of the simulation time in the selected trajectory (1.02 through 30.00 ns). (**B**) Small molecule-antibody interactions were categorized into the four types shown and displayed as stacked bar chart for each interacting V_H or V_L residue. The stacked bar charts are normalized over the course of the trajectory with a value of 1.0 suggesting that 100% of the simulation time the specific interaction is maintained. Tyr101 of V_H and Tyr 101 of V_L have values over 1.0 due to their multiple contacts shown in (A).



Supplementary Figure S2. Mass spectrometry analysis of nonreduced anti-HER2 DVD-ADC. MALDI-TOF analysis of the nonreduced and deglycosylated (PNGase F) unconjugated (**A**) and compound **8**-conjugated (**B**) anti-HER2 DVD-IgG1. The expected mass for the unconjugated DVD-IgG1 was 200,106 Da. The expected mass for the DVD-IgG1 with one conjugated compound **8** was 202,310 Da.

SUPPLEMENTARY REFERENCES

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[2] Walseng, E., Nelson, C. G., Qi, J., Nanna, A. R., Roush, W. R., Goswami, R. K., Sinha, S. C., Burke, T. R., Jr., and Rader, C. (2016) Chemically programmed bispecific antibodies in diabody format. *J. Biol. Chem. 291*, 19661-19673.
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