Supporting Information

Application of Strain-Promoted Azide-Alkyne Cycloaddition and Tetrazine Ligation to Targeted Fc-Drug Conjugates

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General Materials and Methods

All experiments involving moisture-sensitive compounds were conducted under dry conditions (positive argon pressure) using standard syringe and septa protocols. All anhydrous solvents were obtained commercially (Aldrich) and used directly. HPLC grade hexanes, EtOAc, CH2Cl2, and MeOH were used in flash chromatography. Analytical TLC was performed using Analtech precoated plates (Uniplate, silica gel GHLF, 250 microns) containing a fluorescence indicator. NMR spectra were recorded using a Varian Inova 400 MHz spectrometer. Coupling constants are reported in hertz, and peak shifts are reported in ppm. Low-resolution mass spectra (ESI) were measured with using an Agilent 1200 LC/MSD-SL system, and high-resolution mass spectra (ESI or APCI) were measured by the University of California Riverside Mass Spectrometry Facility, Department of Chemistry, University of California, Riverside. Reaction

products requiring HPLC purification were purified using a Waters PrepLC 4000 preparative HPLC system having photodiode array detection and using a Phenomenex C_{18} column (250 mm x 21 mm; 5 μm particle size, 110 Å pore size) at a flow rate of 10 mL/min. HPLC solvent A was water (0.1% TFA) and solvent B was acetonitrile (0.1% TFA).

Synthetic Procedures:

Compound 4. To a solution of **1**[1] (0.497 g, 2.92 mmol) and HOAt (0.397 g, 2.92 mmol) in dry DMF (6 mL) was added DIC (452 µL, 2.92 mmol) and the resulting mixture was then stirred at room temperature. After 10 min *tert*-butyl piperazine-1-carboxylate was added and stirring was continued overnight. The reaction mixture was concentrated under vacuum and then diluted with CH_2CH_2 and filtered. The filtrate was diluted with CH_2CH_2 (~ 60 mL) and washed sequentially with 0.1 M HCl (20 mL), water (20 mL), and brine (20 mL). The organic phase was dried over Na_2SO_4 and then concentrated under vacuum. The crude product was purified by flash chromatography (EtOAc/Hex, 3:7) to yield **4** as a pale yellow oil (0.504 g, 51%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.79-3.67 (m, 2 H), 3.64-3.49 (m, 3 H), 3.45 (t, 3 H, J = 4.6 Hz), 2.75-2.59 (m, 1 H); 2.40-2.18 (m, 3 H); 1.98-1.89 (m, 2 H); 1.87-1.69 (m, 3.10); 1.65-1.59 (m, 1 H); 1.47 (s, 9 H). LRMS (ESI^+): calc for $C_{18}H_{27}FN_2O_3$ [M + Na^+], 361.2; found 361.1.

Compound 5. Compound **4** (0.504 g, 1.49 mmol) was deprotected over 30 min at room temperature in a mixture of TFA/CH_2Cl_2 (1:1, 5 mL) containing 5% TIS. The mixture was concentrated to an oily residue under vacuum and then diluted with hexane and concentrated again under vacuum. The residue was diluted with $CHCl_3$ and concentrated again under vacuum twice. The deprotected amine was then dissolved in THF/ACN (5 mL, 87:13) containing Et_3N (623 µL, 4.47 mmol). To this solution was added *tert*-butyl 2-bromoacetate (264 µL, 1.79 mmol) and let the mixture was stirred overnight at room temperature. The crude reaction

mixture was filtered and then concentrated under vacuum. The crude product was purified by flash chromatography (EtOAc/Hex, 2:3) to afford **5** as a colorless oil (0.372 g, 71%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.86-3.77 (m, 2 H), 3.73-3.63 (m, 2 H), 3.13 (s, 2 H), 2.73-2.63 (m, 2 H), 2.62-2.53 (m, 3 H), 2.40-2.17 (m, 3 H), 1.99-1.88 (m, 2 H), 1.87-1.68 (m, 3 H), 1.65-1.54 (m, 1 H), 1.47 (s, 9 H). MS (ESI^+): calc for $C_{19}H_{29}FN_2O_3$ [M + H^+], 353.2; found 353.2.

Compound 6. To a solution of **5** (0.372 g, 1.05 mmol) in a mixture of CH_2Cl_2 (7 mL) and TIS (0.5 mL) was added TFA (6 mL). The mixture was stirred 1 h at room temperature and then was concentrated under vacuum. The residue was triturated in ether to afford TFA salt **6** as a colorless solid (0.278 g, 64%). ^{1}H NMR (400 MHz, CD_3OD): δ 4.15 -4.03 (m, 2 H), 4.01 (s, 2 H), 3.97-3.80 (m, 2 H), 3.58-3.33 (m, 4 H), 2.76-2.50 (m, 2 H), 2.45-2.06 (m, 3 H), 2.04-1.56 (m, 6 H). ^{13}C NMR (100 MHz, CD_3OD) 168.4, 166.7, 111.2 (d, J = 10.5 Hz), 92.9 (d, J = 182.7 Hz), 87.8 (d, J = 31.0), 57.4, 53.5, 46.2 (d, J = 24.3 Hz), 45.6, 35.0, 30.42, 27.4, 21.1. HRMS (ESI +): calc for $C_{15}H_{22}N_2O_3F$ [M + H^+], 297.1609; found 297.1609.

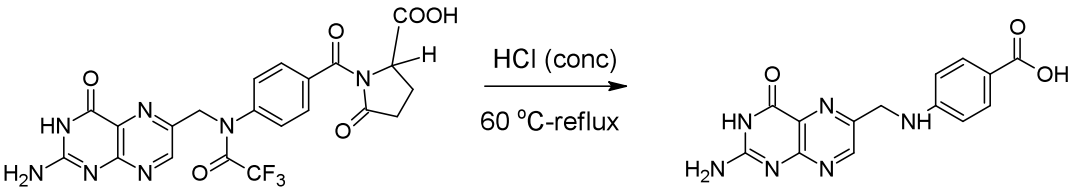
Compound 8. To a round bottom flask containing Boc-β-alanine (2.57 g, 13.6 mmol) was added a mixture of TFA/CH_2Cl_2 (40 mL, 1:1) and the contents were stirred for 1h. The reaction mixture was concentrated under vacuum remove TFA. The residue was triturated in toluene and then concentrated again under vacuum. The β-alanine TFA salt was dissolved in THF (60 mL) and then mixed with Et_3N (2.75 g, 27.2 mmol). To this mixture was added a solution of anhydride **7** (2.75g, 13.6 mmol, prepared according literature procedure[2]) in THF (10 mL). The reaction mixture was then refluxed overnight under argon. The mixture was cooled to room temperature and then concentrated under vacuum. The residue was diluted with 0.1 M HCl (50 mL) and then extracted with EtOAc (150 mL). The organic phase was washed with brine, dried over Na_2SO_4, and then concentrated under vacuum to afford the title compound as a

partially pure (approx 78% pure by ^{1}H NMR) colorless solid (2.11 g, 57%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.90-5.84 (m, 4 H), 3.73 (t, 2 H, *J* = 7.3 Hz), 3.16 (brs, 2 H), 2.83-2.78 (m, 4 H), 2.60 (t, 2 H, *J* = 7.3 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): 178.8, 172.8, 137.8, 128.1, 43.90, 43.1, 36.5, 34.1, 31.5. HRMS (ESI +): calc for $C_{15}H_{15}NO_4Na$ [M + Na^+] 296.0893; found 296.0899.

Compound 11. To a solution of **2** (0.100 g, 0.387 mmol) in dry THF (1.5 mL) was added p-nitrophenol (0.054 g, 0.387 mmol) followed by DCC (0.080 g, 0.387 mmol). The resulting mixture was stirred overnight at room temperature and then filtered. The filtrate was concentrated under vacuum and the crude product was purified by flash chromatography (EtOAc: Hex, 1:9) to afford **11** as a pale yellow solid (0.077 g, 52%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.33 (d, 2 H, *J* = 8.8 Hz), 8.16 (d, 2 H, *J* = 8.1 Hz), 7.52 (d, 2 H, *J* = 8.1 Hz), 7.42 (d, 2 H, *J* = 8.8 Hz), 4.79 (d, 1 H, *J* = 12.8 Hz), 4.52 (d, 1 H, *J* = 12.8 Hz), 4.32-4.23 (m, 1 H), 2.36-2.25 (m, 1 H), 2.24-2.12 (m, 2 H), 2.11-1.80 (m, 4 H), 1.77-1.60 (m, 2 H), 1.56-1.41 (m, 1 H).

Synthesis of 18a-d:

N^{10}-(TFA)pteroic acid used in the synthesis of **18a-d** (Scheme S2) was prepared from pteroic acid **30** (Scheme S1) according to published procedures.[3] Compound **30** was prepared from N^{10}-(TFA)pyrofolic acid **29** by a modification of a literature method[4] as described below:



29 **30**

Scheme S1. Synthesis of pteroic acid **30.**

Compound 30. Compound **29** (13.66 g, 26.3 mmol) was dissolved in concentrated HCl (150 mL) and stirred overnight at 60 ^{o}C. The reaction mixture was heated to reflux and stirred 2.5h. The reaction mixture was cooled to room temperature and then poured into ~ 1 L ice. The ice-cold mixture was stirred 1 h and then filtered. The filter cake was washed several times with ACN and then concentrated under vacuum to afford **30** as a yellow-brown solid (8.45 g, 100%). The MS and ^{1}H NMR were consistent with expected structure and published spectra.[3]

Folate resin 33. Fmoc-Lys(Mtt)-Wang resin (0.500 g, 0.36 mmol) was swollen 1.5 h in DMF. The resin was deprotected with 20% piperidine in DMF (2 x 15 min) and then shaken with Fmoc-Lys(ivDde)-OH (0.9 mmol, 0.517 g), HATU (0.9 mmol, 0.342 g), and DIEA (0.9 mmol, 0.116 g) in DMF overnight. The resin was deprotected with 20% piperidine and then shaken overnight with previously described Fmoc-PEGSU linker[5] (0.508 g, 1.08 mmol), HATU (0.411 g, 1.08 mmol), and DIEA (376 uL, 2.16 mol). Unreacted resin-bound amine was capped with 1-acetylimidazole (10% w/v in DMF) for 30 min. The resin was deprotected with 20% piperidine and then shaken again with Fmoc-PEGSU linker (0.847 g, 1.8 mmol), HOAt (0.245 g, 1.8 mmol), and DIC (279 uL, 1.8 mmol) in DMF for 2-3 h. Unreacted resin-bound amine was capped with 1-acetylimidazole (10% w/v in DMF) for 30 min. The resin was deprotected with 20% piperidine and then shaken with Fmoc-Glu-OtBu (0.766 g, 1.8 mmol), HOAt (0.245 g, 1.8 mmol), and DIC (279 uL, 1.8 mmol) in DMF for 4.5 h. Unreacted resin-bound amine was capped with 1-acetylimidazole (10% w/v in DMF) for 30 min. The resin was deprotected with 20% piperidine and then shaken with N^{10}-TFA pteroic acid (0.441 g, 1.08 mmol), HATU (0.411 g, 1.08 mmol) and DIEA (376 uL, 2.16 mmol) 2 h. The resin was washed with DMF, CH_2Cl_2, and ether and then was dried 10 h under high vacuum to afford **20** (0.712 g).

General Procedure for the synthesis of 18a-c. Resin **33** (0.075 g, 0.038 mmol) was swollen 1.5 h in DMF and then TFA and ivDde protecting groups were removed with 2% hydrazine monohydrate in DMF (3 x 15 min). The resin was then shaken several hours with a mixture of **6**, **8**, or biotin-OPfp[6] (5-10 equiv), HOAt (5-10 equiv), and DIC (5-10 equiv for **6**, and **8** only) in DMF. The resin was washed with DMF and CH_2Cl_2 and then swollen 1 h in CH_2Cl_2. The resin was deprotected by shaking with a mixture of TFA/TIS/DCM (1/5/94, 5 x 2 min). The resin was then sequentially washed with DCM, DMF, 10% DIEA in DMF, and DMF. After swelling 1h in DMF, the resin was shaken with a mixture of 3-maleimidopropionic acid (5 equiv), HOAt (5 equiv), and DIC (5 equiv) several hours. After a final wash with DMF, DCM, and ether, the resin was dried under vacuum. The crude product was cleaved from the resin over 2 h in a mixture of TFA/TIS/H2O (95/2.5/2.5) and then precipitated with ether. The crude product was purified by preparatory HPLC and then lyophilized to afford **18a-c** as yellow solids:

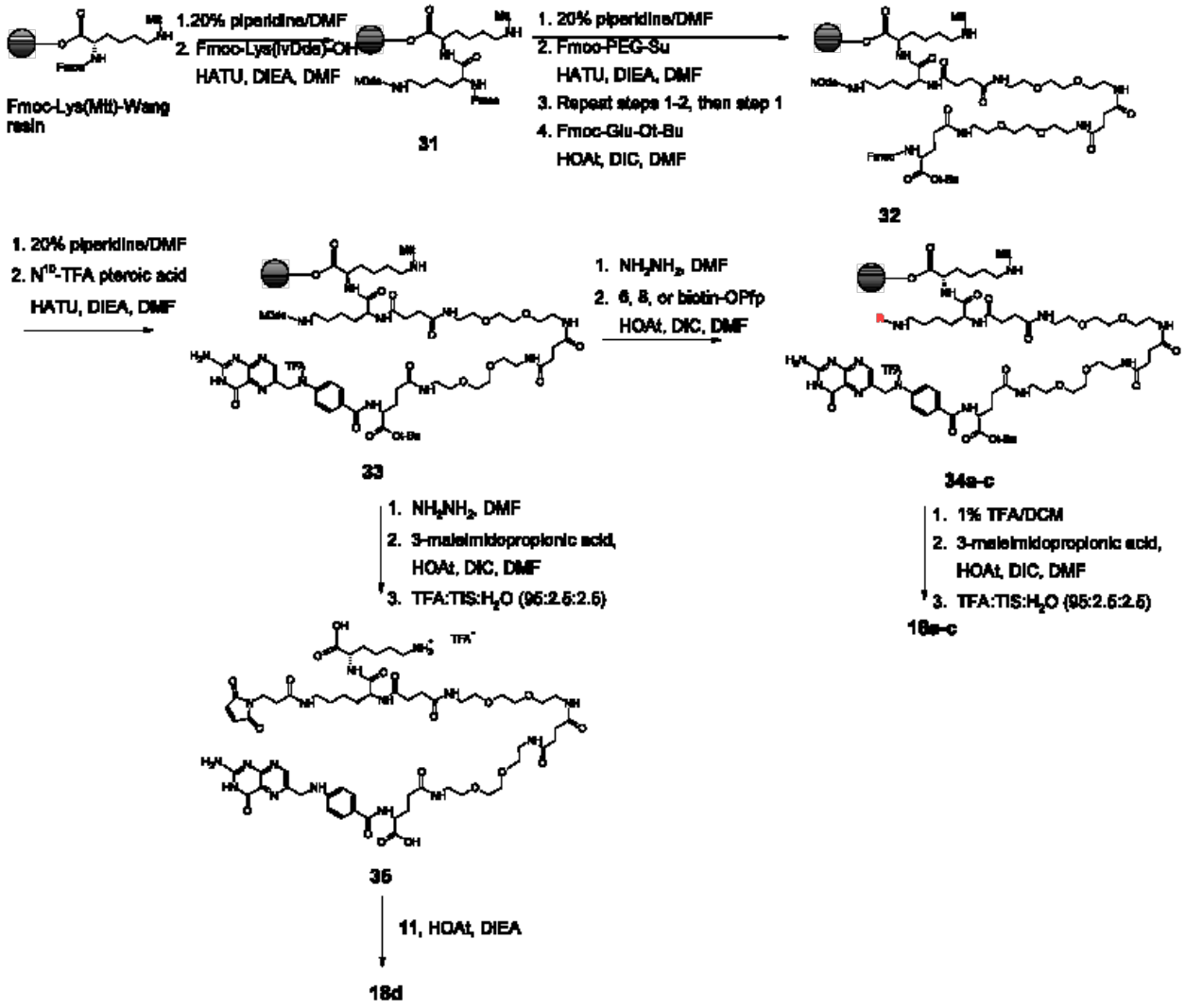
22a: HRMS (ESI +): calc for $C_{73}H_{104}FN_{18}O_{21}$ $[M+H^+]$ 1587.7601; found 1587.7582.

22b: HRMS (ESI +): calc for $C_{73}H_{97}N_{17}O_{22}$ $[M+H^+]$ 1564.7067; found 1564.7051.

22c: HRMS (ESI +): calc for $C_{68}H_{98}N_{18}O_{21}S$ $[M+H^+]$ 1535.6947; found 1535.6931.

Compound 18d. Folate resin **33** (0.100 g, 0.047 mmol) was swollen 3 h in DMF and then TFA and ivDde protecting groups were removed with 10% N2H4 monohydrate in DMF (3 X 10 min). The resin was then shaken with 3-maleimidopropionic acid (0.040 g, 0.235 mmol), HOAt (0.032 g, 0.235 mmol), and DIC (36.4 uL, 0.235 mmol) for 2.5 h. The resin was washed with DMF, CH_2Cl_2, and ether and then was dried overnight under high vacuum. Crude product was cleaved from the resin over 2 h in a mixture of TFA/TIS/H2O (95/2.5/2.5) and then precipitated with ether. Crude product was purified by preparatory HPLC and then lyophilized to afford **35** as a yellow solid (7.1 mg, 11%). To a solution of compound **35** (6 mg) in dry DMF

(0.6 mL) was added **11** (0.0032 g, 0.00843 mmol), HOAt (0.0011 g. 0.0084 mmol), and DIEA (0.0016 g, 0.013 mmol). The mixture was stirred overnight protected from light and then the crude product was purified by preparatory HPLC to afford **18d** as a yellow solid. HRMS (ESI +): calc for $C_{74}H_{101}N_{16}O_{21}$ [M + H^+] 1549.7322; found 1549.7278.



Scheme S2. Synthesis of bifunctional FA conjugates **18a-d** for Fc-Sec programming.

Compounds 13 and 14. Tetrazine **12**[7] (0.011 g, 0.024 mmol) and **6** (0.008 g, 0.020 mmol) were dissolved in a mixture of ACN/H_2O (1:1.8, 300 uL) and stirred overnight. Subsequent purification by HPLC yielded an inseparable mixture of **13** and **14** as an off-white foam (0.0056 g, 28%). ^{1}H NMR (400 MHz, CD_3OD): δ 9.04 (d, 2 H, *J* = 4.8 Hz), 8.96 (d, 1 H, *J* = 5.0 Hz), 7.77-7.67 (m, 2 H), 7.62 (d, 2 H, *J* = 8.4 Hz), 7.53 (brs, 1 H), 4.16 (s, 2 H), 4.07 (s, 1 H), 4.02-3.47 (m, 8 H), 3.44-3.24 (m, 5 H), 3.19-2.95 (m, 3 H), 2.94-2.62 (m, 2 H), 2.60-2.34 (m, 1 H), 2.07-1.93 (m, 1 H), 1.91-1.68 (m, 3 H), 1.66-1.53 (m, 2 H), 1.51-1.33 (m, 1 H). ^{13}C NMR (100 MHz, CD_3OD): δ 171.8, 168.1, 162.1, 161.7, 161.3, 159.0, 136.7, 132.3, 130.9, 128.7, 127.3, 122.8, 57.0, 53.1, 44.4, 40.9, 28.9, 23.9. HRMS (ESI +): calc for $C_{32}H_{38}N_8O_4F$ [M + H^+] 617.2995; found 617.3000.

Compounds 16 and 17. To a solution of tetrazine **15**[7] (0.017 g, 0.032 mmol) in DMF:MeOH:H_2O (1.1 mL, 3.3:3:1) was added **2** (0.0075 g, 0.029 mmol) and the mixture was stirred overnight at room temperature. HPLC analysis (10% B to 50% B linear gradient over 25 min) of the crude reaction showed that the regioisomeric cycloaddition products were formed in 1:1 ratio (retention times = 22.8 and 23.9 min). Though they could be separated by preparatory HPLC, fractions containing **16** and **17** were combined and lyophilized to afford a mixture of regioisomers as a pale pink solid (17 mg, 79%). (400 MHz, CD_3OD): δ 9.03 (brs, 1 H), 8.74 (brs, 1 H), 7.91 (t, 2 H, *J* = 8.6 Hz), 7.70 (s, 2 H), 7.59-7.48 (m, 2 H), 7.47-7.40 (m, 1 H), 7.25 (d, 1 H, *J* = 8.2 Hz), 7.13 (d, 1 H, *J* = 8.1 Hz), 5.11-4.98 (m, 1 H), 4.54-4.31 (m, 2 H), 4.18 (s, 2 H), 4.09-3.68 (m, 4 H), 3.51 (brs, 4 H), 3.16-3.00 (m, 1 H), 2.94-2.75 (m, 1 H), 2.64-2.44 (m, 1 H), 2.31-2.05 (m, 1 H), 1.94-1.58 (m, 4 H), 1.55-1.28 (m, 2 H), 1.22-0.94 (m, 1 H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.8, 171.7, 169.6, 167.9, 161.5, 161.0, 161.1, 159.0, 158.2, 144.4, 140.5, 136.2, 135.9, 131.4, 131.1, 130.9, 130.6, 128.7, 128.5, 128.3, 78.1, 77.6, 71.9, 71.4, 57.0, 53.5,

38.4, 38.2, 31.7, 30.9, 28.0, 27.2, 26.0, 24.3, 22.6. HRMS (ESI +): calc for $C_{35}H_{37}N_6O_6$ [M + H^+] 637.2769; found 637.2783.

Programming Fc-Sec.

The cloning, expression, and purification of Fc*-Sec-His (50 kDa), which contains mutation Asn297Ala to eliminate the unique N-glycosylation site in the C_H2 domain of Fc, was described previously[8]. For programming, i.e. selective conjugation at the C-terminal selenocysteine, 200 μg Fc*-Sec-His was diluted in 15 mL 100 mM sodium acetate (pH 5.2) and concentrated to 1mL (4 μM) using a 10-kDa cutoff centrifugal filter device. Freshly prepared dithiothreitol (DTT; 100 mM in H_2O) followed by maleimide-folate derivatives **18a-d** (4 mM in DMSO) were added to the protein at final concentrations of 0.1 mM and 40 μM, respectively, and incubated for 1 h at room temperature in the dark on a Glas-Col mini rotator (Terre Haute, IN). The mixture was subsequently diluted in 15 mL 100 mM sodium acetate (pH 5.2) and concentrated to 0.5 mL using a 30-kDa cutoff centrifugal filter device. This step was repeated once with 15 mL 100 mM sodium acetate (pH 5.2) and subsequently twice with 15 mL phosphate-buffered saline (PBS; pH 7.4) to remove free compounds. The final programmed Fc*-Sec-His protein concentration was determined by A_{280} absorbance using a NanoDrop 2000 spectrophotometer (Thermo Scientific, West Palm Beach, FL).

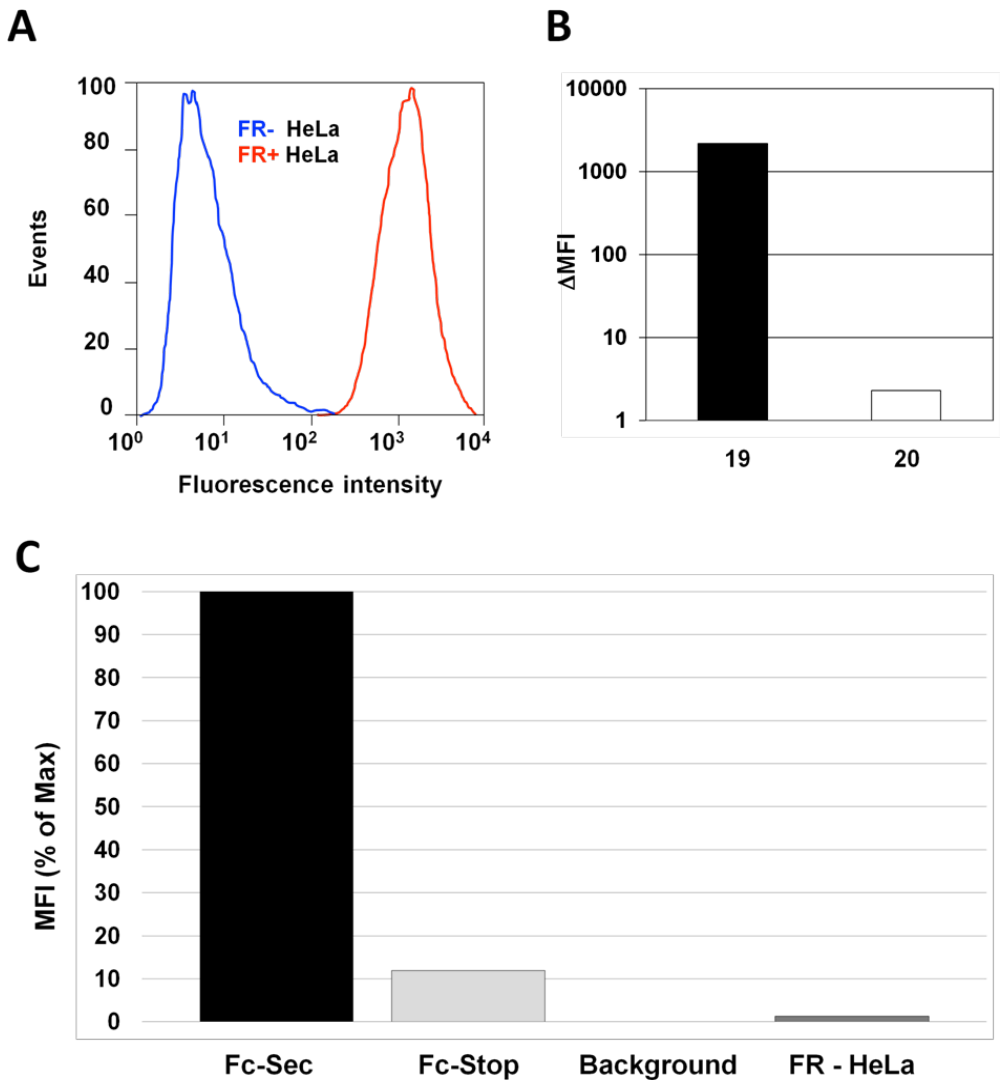


Figure S1. Programming Fc-Sec with **18d**. Fc-Sec (or Fc-Stop as shown in C) was first alkylated under previously established conditions (pH 5.5, 25 equiv DTT, 1:10 ratio Fc-Sec : alkylating agent) with either **18d** or biotin iodoacetamide to give **19** and **20** respectively, and then incubated with HeLa cells. The cells were stained with PE-coupled streptavidin and then analyzed by flow cytometry. (A) Histograms resulting from incubating **19** with (FR+) and (FR-) HeLa cells. (B) Mean fluorescence intensity (MFI) of histograms resulting from incubating **19** and **20** with (FR+) HeLa cells. (C) Fc-Sec and Fc-Stop[8] (a nearly identical Fc protein which does not contain C-terminal Sec residue) were alkylated with **18d** and then incubated with (FR

+) (Columns 1-3) and (FR-) HeLa cells. Maximum fluorescence signal defined as MFI of **19** in HeLa cells. All data shown in C were normalized to this value.

Arming Fc-Sec-folate

For arming, azide-biotin derivative **24a** or tetrazine-biotin derivative **24b** (4 mM in DMSO) at final concentrations of 40 μM (10:1 molar ratio) were added to 0.5 mL 200 μg/mL (4 μM) of the programmed Fc*-Sec-His protein and rotated in the dark for 3 h at room temperature and then overnight at 4°C. The mixture was subsequently diluted in 15 mL PBS and concentrated to 0.5 mL as described above. This step was repeated twice to remove free compounds. The final programmed and armed Fc*-Sec-His protein concentration was determined by A_{280} absorbance as described above. Following sterile filtration, the programmed and armed Fc*-Sec-His protein in PBS was stored refrigerated (4ºC) for short term use and frozen (-80ºC) in aliquots for long term use.

Flow cytometry.

Following trypsinization with 0.25 % (w/v) trypsin (Invitrogen; Carlsbad, CA), HeLa cells (American Type Culture Collection; Manassas, VA) maintained in 10% (v/v) fetal calf serum (FCS; Invitrogen) in regular RPMI 1640 or folic acid-deficient RPMI 1640 medium supplemented with penicillin-streptomycin (Invitrogen) were collected by centrifugation, resuspended in 1% (v/v) FCS/PBS, and aliquots of 50 μL containing 5 x 10^5 cells were distributed into a V-bottom 96-well plate (Corning, Corning, NY). The cells were then incubated with 10, 5.0, 3.3, 1.0, and 0.33 μg/mL programmed or programmed and armed Fc*-Sec-His protein for 45 min on ice. After washing twice with 1% (v/v) FCS/PBS, the cells were incubated with 2 μg/mL PE-coupled streptavidin (BD Biosciences Pharmingen; San Diego, CA) for 30 min on ice. After washing twice as before, the cells were resuspended in 400 μL 1%

(v/v) FCS/PBS and analyzed using a FACSCalibur instrument (BD Biosciences Immunocytometry Systems, San Jose, CA). A total of 20,000 events per sample were collected and data were analyzed using FlowJo analytical software (Tree Star, Ashland, OR).

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