Supporting Material

Reprogramming urokinase into an antibody-recruiting anticancer agent

Charles E. Jakobsche, Patrick J. McEnaney, Andrew X. Zhang, David A. Spiegel

Department of Chemistry, Yale University, New Haven, CT

Email: david.spiegel@yale.edu

Table of Contents

1. General Information S2
- Abbreviations S2
- General Procedures S2
- Standard Synthetic Procedures S3

2. Synthetic Procedures S4
- Synthesis of Compound **1** S4
- Synthesis of Compound **2** S12
- Synthesis of Compound **3** S16

3. Spectra S20
- From the Synthesis of Compound **1** S20
- From the Synthesis of Compound **2** S31
- From the Synthesis of Compound **3** S37

4. Biological Evaluation S44
- Buffers and Solutions S44
- Proteins, Antibodies, and Reagents S45
- Equipment, Materials, and Software S46
- Catalytic Inhibition S47
- In-Gel Fluorescence S48
- ELISA S50
- Cell Culture S53
- Flow Cytometry S54
- Phagocytosis Assay (ADCP) S58
- Cytotoxicity Assay (ADCC) S61

5. ADCP Flow Cytometry Data S64
- ARM-U_{DNP} to Target A172 Cells S65
- ARM-U_{Fluor} to Target A172 Cells S72
- ARM-U_{DNP} to Target HT-29 Cells S79
- ARM-U_{Fluor} to Target HT-29 Cells S86
- ARM-U_{DNP} to Target A172 Cells using Serum S94

1. General Information

Abbreviations

AcOH = acetic acid
AMC = 7-amino-4-methylcoumarin
Boc = *tert*-butoxycarbonyl
BSA = bovine serum albumin
Cbz = benzyloxycarbonyl
DCM = dichloromethane
DMF = *N*,*N*-dimethylformamide
DMSO = dimethylsulfoxide
DNP = 2,4-dinitrophenyl
DPBS = Dulbecco's phosphate-buffered saline
EDC = 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA = ethylenediaminetetraacetic acid, disodium salt
EGTA = ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid
EtOAc = ethyl acetate
Fmoc = 9-fluorenylmethyloxycarbonyl
GFP = green fluorescent protein
HI-FBS = heat inactivated fetal bovine serum
HOBt = hydroxybenzotriazole
iPrOH = isopropyl alcohol
HMW = high molecular weight
LMW = low molecular weight
MeCN = acetonitrile
MeOH = methanol
NHS = N-hydroxysuccinimide
NMP = *N*-methylpyrollidinone
PBS = phosphate-buffered saline
Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfony
PBMC = peripheral blood mononuclear cells
Pd/C = 10% palladium on carbon
Quant. = quantitative conversion
TEA = triethylamine
TFA = trifluoroacetic acid
TFAA = trifuloroacetic anhydride
THF = tetrahydrofurane
TiPS = triisopropylsilane
uPA = ukokinase plasminogen activator

General Procedures

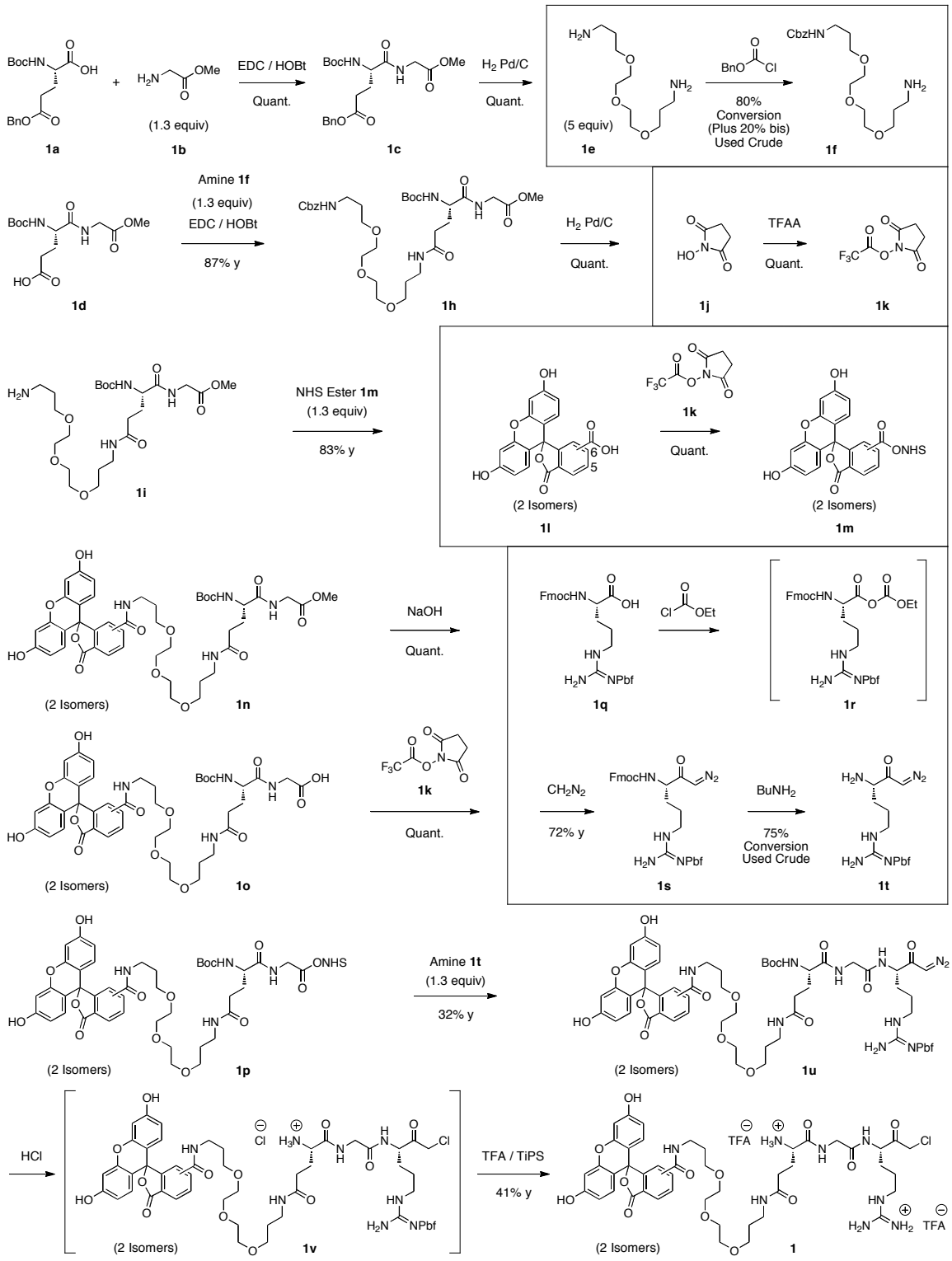
Column chromatography was performed with 60 Å 40–63 μm silia–P flash silica gel. **Solvents** for reactions (acetonitrile, DMF, DCM, ether, THF, and toluene) were dried using a Glass Contour purification system. Other solvents were used as received. **Bases** (triethylamine, Hünig's base) were dried by distillation from calcium hydride. **Chemicals** were purchased from Aldrich or Advanced ChemTech and used as received unless noted otherwise. **NMR Spectra** were measured in $CDCl_3$ at ambient temperature unless otherwise noted. **^{1}H NMR** spectra were recorded on either a 500 or 400 MHz Bruker spectrometer. Chemical shifts are reported in ppm (δ) relative to tetramethylsilane using the solvent as a reference ($CDCl_3$ = 7.26 ppm, DMSO-d_6 = 2.49 ppm, D_2O = 4.80 ppm, CD_3OD = 3.30). The following is an example data point: chemical shift (multiplicity [s = singlet, d = doublet, t = triplet, q = quartet, pent = pentet, sext = sextet, sept = septet, oct = octet, m = multiplet, br = broad, and combinations thereof], coupling constants [Hz], integration, assignment [if any]). **^{13}C NMR** spectra were recorded on a 500 MHz (125 MHz) Bruker spectrometer with complete proton decoupling. Chemical shifts are reported in ppm (δ) relative to tetramethylsilane using the solvent or MeOH as a reference ($CDCl_3$ = 77.0 ppm, DMSO-d_6 = 39.5 ppm, CD_3OD = 49.0 ppm, MeOH = 49.5). **^{19}F NMR** spectra were recorded on a 400 MHz (375 MHz) Bruker spectrometer without proton decoupling. Chemical shifts are reported in ppm (δ) relative to trichlorofluoromethane. **IR** spectra were recorded on a Nicolet 6700 FT–IR spectrometer with OMNIC software, using a thin film from solution evaporation. Spectra are partially reported (υ_{max}, cm^{-1}). **HRMS** was performed at The Keck Center at the Yale Medical School. Unless otherwise noted, data were obtained by positive mode electrospray ionization. **TLC** was performed on 60 Å F_{254} precoated silica gel plates. Samples were visualized by either ultraviolet irradiation, potassium permanganate staining, or cerium ammonium molybdenate staining. **LC/MS** were run on a Waters Acquity LC system using an Acquity UPLC BEH C_{18} column, a gradient of 20–100% (over 3 min) MeCN in water + 0.1% formic acid, and a flow of 0.8 mL/min. **Yield** refers to isolated material.

Standard Synthetic Procedures

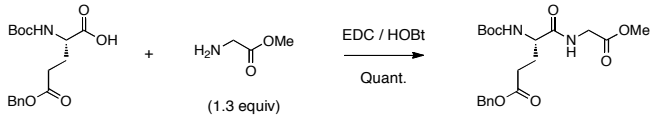
Hydrogenolysis Into a reaction vessel were added the starting benzyl ester or benzyloxycarbamate and methanol or 2-propanol (0.2 M, methanol generally gives better solubility and reactivity, but sometimes produces a methyl ester byproduct). Pd/C (10%, approximately 50–100 mg/mmol substrate) was added into another flask. The palladium-containing flask was capped with a septum and flushed with nitrogen. Methanol or isopropanol (equal to previous volume) was added by syringe, the flask was uncapped, and the slurry was transferred to the substrate solution. The mixture was capped and flushed with nitrogen (5 min). A hydrogen-filled balloon was attached such that its needle remained submerged, the flask was flushed briefly with hydrogen (30–60 sec), and the reaction was stirred at ambient temperature under the balloon's pressure (3 h). The balloon was removed, the flask was flushed with nitrogen, the mixture was filtered through Celite, and volatiles were removed under reduced pressure. Due to cleanliness of the reaction, the crude product was usually not purified further.

2. Synthetic Procedures

Scheme S1



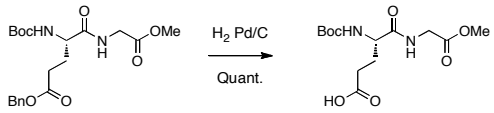
Scheme S1 shows the synthetic strategy used to access compound (**1**).



1a **1b** **1c**

Synthesis of amide 1c Into a flask were added Boc–Glu(Bn)–OH (**1a**, 4.50 g, 13.4 mmol, 1 equiv), H–Gly–OMe hydrochloride (**1b**, 2.18 g, 17.4 mmol, 1.3 equiv), EDC hydrochloride (3.34 g, 17.4 mmol, 1.3 equiv), HOBt hydrate (2.66 g, 17.4 mmol, 1.3 equiv), DCM (67 mL, 0.2 M), DMF (30 mL, 0.4 M), and TEA (2.60 mL, 18.8 mmol, 1.4 equiv). The crude mixture was stirred (8 h) diluted with EtOAc (80 mL), washed with a 1:1:1 mixture of sodium bicarbonate (saturated), sodium carbonate (10%), and brine (120 mL), washed with sodium chloride (50% saturated, 2 x 80 mL), washed with a 1:1 mixture of citric acid (10%) and brine (60 mL), and dried with sodium sulfate. Volatiles were removed under reduced pressure to yield nearly pure amide **1c** (quantitative), half of which was used directly in the next step. For analysis, an aliquot was purified by column chromatography (40 mL silica gel, [4:3] EtOAc/hexane). ^{1}H NMR signals were assigned by ^{1}H–^{1}H COSY.

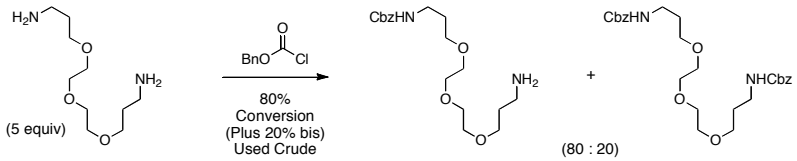
^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.37–7.32 (m 5H, Bn), 6.82–6.73 (brs, 1H, Gly-NH), 5.32 (d, J = 7.7 Hz, 1H, Boc-NH), 5.13 (s, 2H, Bn), 4.25 (q, J = 7.0 Hz, 1H, Glu-α), 4.05 (dd, J_1 = 18.2 Hz, J_2 = 5.5 Hz, 1H, Gly-α), 3.99 (dd, J_1 = 18.3 Hz, J_2 = 5.5 Hz, 1H, Gly-α), 3.74 (s, 3H, OMe), 2.62–2.45 (m, 2H, Glu-γ), 2.22–2.12 (m, 1H, Glu-β), 2.01–1.91 (m, 1H, Glu-β), 1.43 (s, 9H, Boc); **^{13}C NMR** ($CDCl_3$, 125 MHz) δ 173.06, 171.91, 155.64, 135.66, 128.48, 128.19, 128.14, 80.02, 66.44, 53.40, 52.23, 41.03, 30.28, 28.21, 27.84; **IR** (film, cm^{-1}) 1733, 1711, 1662, 1515, 1368, 1210, 1155, 730, 698; **HRMS** calculated for $[C_{20}H_{28}N_2O_7H]^+$, requires m/z = 409.1969, found m/z = 409.1967 (ESI); **TLC** (4:3) EtOAc/hexane, UV/permanganate, R_f = 0.29.



1c **1d**

Synthesis of acid 1c The standard hydrogenolysis was followed using crude peptide **1c** (half, 6.7 mmol, 1.0 equiv), Pd/C (10%, 335 mg, 50 mg/mmol substrate), and MeOH (67 mL, 0.1 M) for 3 h to yield nearly pure acid **1d** (quantitative), which was used directly in the next step. For analysis, an aliquot was purified by column chromatography (40 mL silica gel, [11:9:1] DCM/EtOAc/AcOH). ^{1}H NMR signals were assigned by ^{1}H–^{1}H COSY.

^{1}H NMR (DMSO-d_6, 500 MHz) δ 8.23 (t, J = 5.7 Hz, 1H, Gly-NH), 6.94 (d, J = 9.3 Hz, 1H, Boc-NH), 3.98–3.92 (m, 1H, Glu-α), 3.88 (dd, J_1 = 17.4 Hz, J_2 = 6.0 Hz, 1H, Gly-α), 3.77 (dd, J_1 = 17.5 Hz, J_2 = 7.8 Hz, 1H, Gly-α), 3.61 (s, 3H, OMe), 2.30–2.22 (m, 2H, Glu-γ), 1.89–1.81 (m, 1H, Glu-β), 1.75–1.65 (m, 1H, Glu-β), 1.37 (s, 9H, Boc); **^{13}C NMR** (DMSO-d_6, 125 MHz) δ 174.06, 172.29, 170.25, 155.29, 78.13, 53.41, 51.67, 40.57, 30.10, 28.18, 27.28; **IR** (film, cm^{-1}) 3318, 2974, 1708, 1667, 1516, 1246, 1213, 1160; **HRMS** calculated for $[C_{13}H_{22}N_2O_7H]^+$, requires m/z = 319.1500, found m/z = 319.1499 (ESI); **TLC** (11:9:1) DCM/EtOAc/AcOH, permanganate (faint), R_f = 0.35.



1e **1f** **1g**

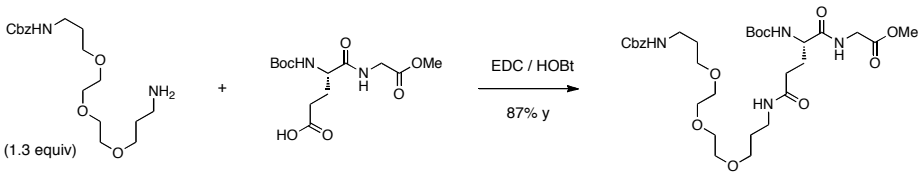
Synthesis of carbamate 1e[1] Into a flask were added THF (200 mL, 0.1 M), 4,7,10-trioxa-1,13-tridecanediamine (**1e**, 22.0 mL, 100 mmol, 5 equiv), triethylamine (2.77 mL, 20.0 mmol, 1.0 equiv), and MeOH (70 mL, 0.3 M). The

[1] Carbamate-formation procedure and compound data from: Harris, T. D., Kalogeropoulos, S., Nguyen, T., Dwyer, G., Edwards, D. S., Liu, S., Bartis, J., Ellars, C., Onthank, D., Yalamanchili, P., Heminway, S., Robinson, S., Lazewatsky, J., Barrett, J. (2006) *Bioconj. Chem. 17*, 1294.

flask was fitted with an addition funnel, maintained under a nitrogen atmosphere, and cooled in an ice bath (0 ºC). Benzyl chloroformate (2.84 mL, 20.0 mmol, 1 equiv) was dissolved in THF (100 mL, 0.2 M) and added dropwise (over 45 min) to the reaction mixture. The reaction was allowed to warm to ambient temperature and stirred (30 min). Volatiles were removed under reduced pressure. The crude mixture was diluted with brine (200 mL) and sodium carbonate (10% aqueous, 40 mL), extracted with ether (150 + 2 x 100 mL), washed with brine (100 mL), and dried with sodium sulfate. Volatiles were removed under reduced pressure, to yield a mixture (approximately 4:1) of monocarbamate **1f** and dicarbonate **1g**, which was used directly for the next step. Product analysis is consistent with reported data.[1]

Crude monocarbamate **1f**: **^{1}H NMR** ($CDCl_3$, 500 MHz) δ 7.36–7.28 (m 5H, Bn), 5.58–5.52 (brs, 1H, NHZ), 5.09 (s, 2H, Bn), 3.65–3.45 (m, 12H, 6xCH_2–O), 3.33–3.27 (m, 2H, CH_2–NHZ), 2.77 (t, J = 6.8 Hz, 2H, CH_2–NH_2), 1.80–1.66 (m, 4H, 2xC–CH_2–C).

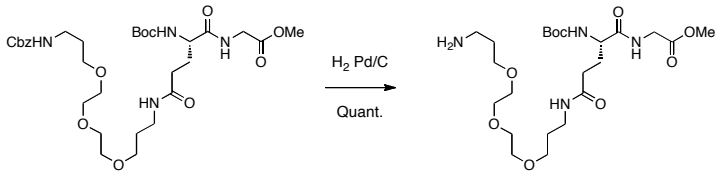
Crude dicarbamate **1g**: **^{1}H NMR** ($CDCl_3$, 500 MHz) δ 7.36–7.28 (m 10H, 2 x Bn), 5.36–5.30 (brs, 2H, 2xNHZ), 5.09 (s, 4H, 2 x Bn), 3.65–3.45 (m, 12H, 6xCH_2–O), 3.33–3.27 (m, 4H, 2 x CH_2–NHZ), 1.80–1.66 (m, 4H, 2 x C–CH_2–C).



1f **1d** **1h**

Synthesis of amide 1h Into a flask were added crude acid **1d** (6.70 mmol, 1 equiv), crude amine **1f** (8.71 mmol, 1.3 equiv), EDC hydrochloride (1.67 g, 8.71 mmol, 1.3 equiv), HOBt hydrate (1.33 g, 8.71 mmol, 1.3 equiv), and DCM (35 mL, 0.2 M). The crude mixture was stirred (15 h) diluted with DCM (40 mL), washed with a mixture (1:1, 50 mL) of sodium bicarbonate (saturated) and sodium carbonate (10%), washed with a mixture (1:1, 50 mL) of citric acid (10%) and brine (60 mL), and dried with sodium sulfate. Volatiles were removed under reduced pressure, and the crude mixture was purified by column chromatography (250 mL silica gel, [4:1 to 1:1] DCM/acetone) to yield amide **1h** (3.82 g, 5.84 mmol, 87% yield from glutamic acid **1a**). ^{1}H NMR signals were assigned by ^{1}H–^{1}H COSY.

^{1}H NMR (DMSO-d_6, 500 MHz) δ 8.20 (t, J = 6.0 Hz, 1H, Gly-NH), 7.74 (t, J = 5.8 Hz, 1H, Glu-γ-NH), 7.38–7.28 (m 5H, Bn), 7.20 (t, J = 5.6 Hz, 1H, Cbz-NH), 6.88 (d, J = 8.0 Hz, 1H, Boc-NH), 4.99 (s, 2H, Bn), 3.95–3.90 (m, 1H, Glu-α), 3.87 (dd, J_1 = 17.2 Hz, J_2 = 3.8 Hz, 1H, Gly-α), 3.79 (dd, J_1 = 17.7 Hz, J_2 = 6.0 Hz, 1H, Gly-α), 3.61 (s, 3H, OMe), 3.51–3.42 (m, 8H, 4 x CH_2–O), 3.37 (q, J = 6.0 Hz, 4H, 2 x CH_2–C–C–N), 3.08–3.00 (m, 4H, 2 x CH_2–NHCO), 2.14–2.07 (m, 2H, Glu-γ), 1.90–1.80 (m, 1H, Glu-β), 1.73–1.65 (m, 1H, Glu-β), 1.64–1.55 (m, 4H, 2 x C–CH_2–C), 1.37 (s, 9H, Boc); **^{13}C NMR** (DMSO-d_6, 125 MHz) δ 172.41, 171.53, 170.24, 156.10, 155.24, 137.27, 128.33, 127.75, 127.72, 78.09, 69.76, 69.55, 68.09, 67.95, 65.14, 53.81, 51.65, 40.56, 37.64, 35.82, 31.77, 29.64, 29.35, 28.18, 28.01; **IR** (film, cm^{-1}) 3315, 2935, 2867, 1700, 1656, 1529, 1248; **HRMS** calculated for $[C_{31}H_{50}N_4O_{11}H]^+$, requires m/z = 655.3549, found m/z = 655.3513 (ESI); **TLC** (1:1) DCM/acetone, permanganate, R_f = 0.32.

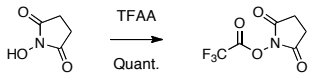


1h **1i**

Synthesis of amine 1i The standard hydrogenolysis was followed using carbamate **1h** (1.31 g, 2.00 mmol, 1 equiv, methanol azeotrope to remove any remaining acetone), Pd/C (10%, 160 mg, 80 mg/mmol substrate), and MeOH (20 mL, 0.1 M) for 2.5 h to yield nearly pure amine **1i** (quantitative), which was used directly in the next step. ^{1}H NMR signals were assigned by ^{1}H–^{1}H COSY.

Crude Amine **1i**: **^{1}H NMR** (DMSO-d_6, 500 MHz. major signals) δ 8.22 (t, J = 7.6 Hz, 1H, Gly-NH), 7.80–7.67 (m, 1H, Glu-γ-NH), 6.90 (d, J = 8.2 Hz, 1H, Boc-NH), 3.95–3.88 (m, 1H, Glu-α), 3.87 (dd, J_1 = 17.6 Hz, J_2 = 5.3 Hz, 1H, Gly-α), 3.79 (dd, J_1 = 17.4 Hz, J_2 = 5.6 Hz, 1H, Gly-α), 3.61 (s, 3H, OMe), 3.52–3.35 (m, 12H, 6 x

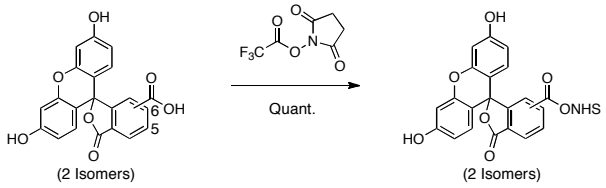
CH_2–O), 3.05 (q, J = 6.3 Hz, 2H, CH_2–NHCO), 2.56 (t, J = 6.8 Hz, 2H, CH_2–NH_2), 2.15–2.05 (m, 2H, Glu-γ), 1.90–1.80 (m, 1H, Glu-β), 1.73–1.64 (m, 1H, Glu-β), 1.64–1.52 (m, 4H, 2 x C–CH_2–C), 1.37 (s, 9H, Boc); **^{13}C NMR** (DMSO-d_6, 125 MHz, major signals) δ 172.35, 171.46, 170.19, 155.18, 78.02, 69.78, 69.76, 69.52, 69.49, 68.42, 68.06, 53.77, 51.61, 40.52, 38.71, 35.78, 33.09, 31.73, 29.33, 28.15; **IR** (film, cm^{-1}) 3305, 2933, 2872, 1658, 1524, 1160, 1095; **HRMS** calculated for $[C_{23}H_{44}N_4O_9H]^+$, requires m/z = 521.3181, found m/z = 521.3172 (ESI). **LC/MS** elutes at 0.65 min, calculated for $[C_{23}H_{44}N_4O_9H]^+$, requires m/z = 521.32, found m/z = 521.22 (ESI).



1j **1k**

Synthesis of trifluoroacetate 1k[2] Into a flame-dried flask backfilled with argon were added *N*-hydroxysuccinimide (2.30 g, 20.0 mmol, 1 equiv) and THF (40 mL, 0.5 M, producing a cloudy solution). The flask was capped with a rubber septum and maintained in an ambient temperature water bath. Trifluoroacetic anhydride (5.68 mL, 40.0 mmol, 2 equiv) was added by syringe dropwise (over 5 min). The mixture was stirred (2 h) and diluted with toluene (20 mL). Volatiles were removed under reduced pressure (3 x 20 mL toluene azeotrope) to yield mostly pure trifluoroacetate **1k** (quantitative, as fluffy white solid), which was used directly for the next step. Acetate **1k** is somewhat moisture sensitive, so it was used within a few hours of its synthesis.

Crude **1k**: **^{1}H NMR** ($CDCl_3$, 400 MHz, major signal) δ 2.91 (s, 4H); **^{13}C NMR** ($CDCl_3$, 125 MHz, major signals) δ 167.14, 153.66 (q, J = 46.0 Hz), 113.87 (q, J = 286.1 Hz), 25.51; **^{19}F NMR** ($CDCl_3$, 375 MHz, major signal) δ -72.69.



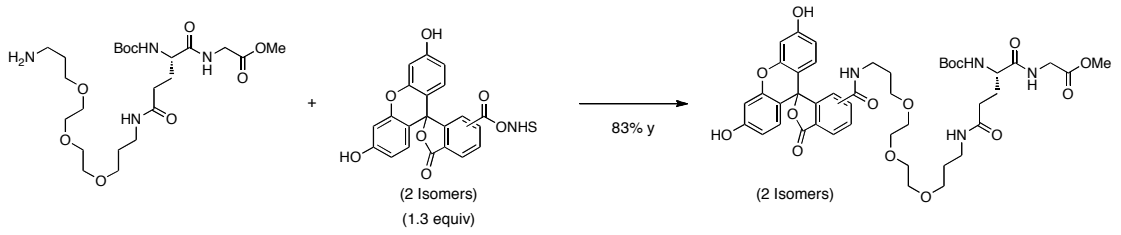
1l **1k** **1m**

Synthesis of succinimide 1m Crude trifluoroacetate **1k** (20.0 mmol, 5 equiv) was placed in a flask. The flask was backfilled with argon, capped with a rubber septum, and maintained under an argon atmosphere. To the flask were added THF (15 mL, 0.2 M) and 5(6)-carboxyfluorescein (**1l**, 1.13 g, 3.00 mmol, 1 equiv, 1:1 mixture of two isomers, producing a suspension). Pyridine (1.69 mL, 21.0 mmol, 7 equiv) was added by syringe dropwise (over 3 min, slightly exothermic). The mixture was stirred in the dark (3 h, producing a homogeneous solution), quenched with water (5 mL, 5 min), and diluted with DCM (45 mL) and water (50 mL). The organic phase was isolated, the aqueous phase was further extracted with a mixture of DCM and THF (3:1, 25 mL), and the combined organic phases were washed with sodium bisulfate (0.1 M aqueous, 40 mL) and dried with sodium sulfate. Volatiles were removed under reduced pressure to yield nearly pure succinimide **1m** (1:1 mixture of isomers, quantitative), which was used directly for the next step. Compound analysis is consistent with commercially available material.[3]

Crude ester **1m**: **^{1}H NMR** (DMSO-d_6, 500 MHz, major signals) δ 10.18 (s, 1H, 2 x OH), 10.17 (s, 1H, 2 x OH), 8.52 (dd, J_1 = 1.5 Hz, J_2 = 0.5 Hz, 0.5H), 8.41 (dd, J_1 = 8.1 Hz, J_2 = 1.6 Hz, 0.5H), 8.37 (dd, J_1 = 8.1 Hz, J_2 = 1.5 Hz, 0.5H), 8.24 (dd, J_1 = 8.0 Hz, J_2 = 0.7 Hz, 0.5H), 7.90 (dd, J_1 = 1.4 Hz, J_2 = 0.7 Hz, 0.5H), 7.54 (dd, J_1 = 8.1 Hz, J_2 = 0.7 Hz, 0.5H), 6.70–6.63 (m, 4H), 6.56–6.52 (m, 2H), 2.95–2.90 (brs, 2H), 2.88–2.84 (brs, 2H).

[2] a) Procedure from: Dey, S., Pappin, J. C., Purkayastha, S., Pillai, S., Coull, J. M. U.S. Patent 2005/0148771, July 7, 2005. b) also see: Sadakibara, S., Inukai, N. (1964) *Bull. Chem. Soc. Jap. 37*, 1231.

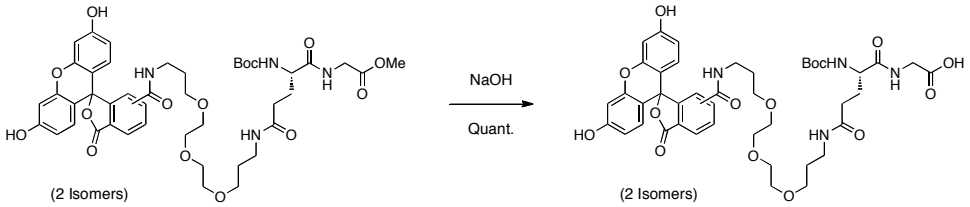
[3] 5(6)-Carboxyfluorescein N-hydroxysuccinimide ester can be purchased from Sigma–Aldrich



1i **1m** **1n**

Synthesis of amide 1n Into a flask were added crude amine **1i** (0.76 mmol, 1 equiv), DCM (8 mL, 0.1M) and a solution of crude ester **1m** (1.0 mmol, 1.3 equiv) in THF (8 mL, 0.1 M). The mixture was stirred in the dark (slightly exothermic, changing from yellow to red, 17 h). As analysis showed only partial consumption of starting materials, Hünig's base (170 μL, 1.00 mmol, 1.3 equiv) was added, and the mixture was stirred (additional 3 h). Analysis showed nearly full consumption of amine **1i**. The reaction was quenched by adding butylamine (0.2 mL, 2 mmol, 3 equiv, stirred 1 h) and diluted with THF (20 mL), DCM (40 mL), and citric acid (5% aqueous, 40 mL). The organic phase was isolated, the aqueous phase was further extracted with a mixture of DCM and THF (3:1, 40 mL), and the combined organic phases were dried with sodium sulfate. Volatiles were removed under reduced pressure, and the crude material was purified by column chromatography (60 mL silica gel, [1:1 to 5:1] acetone/EtOAc) to yield amide **1n** (1:1 mixture of isomers, 552 mg, 629 mmol, 83% yield from benzyloxy carbamate **1h**). ^{1}H NMR signals were assigned by ^{1}H–^{1}H COSY.

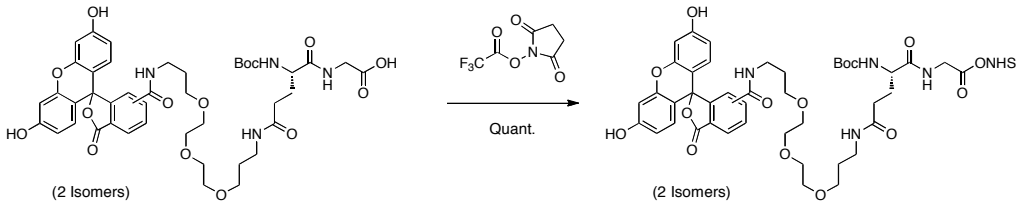
^{1}H NMR (CD_3OD, 500 MHz) δ 8.42–8.40 (m, 0.5H, Ar), 8.19 (dd, J_1 = 8.0 Hz, J_2 = 1.6 Hz, 0.5H, Ar), 8.12 (dd, J_1 = 8.1 Hz, J_2 = 1.4 Hz, 0.5H, Ar), 8.05 (dd, J_1 = 8.1 Hz, J_2 = 0.5 Hz, 0.5H, Ar), 7.64–7.63 (m, 0.5H, Ar), 7.29 (dd, J_1 = 8.1 Hz, J_2 = 1.3 Hz, 0.5H, Ar), 6.68–6.50 (m, 6H, Ar), 4.10–4.05 (m, 1H, Glu-α), 3.98 (d, J = 17.6 Hz, 1H, Gly-α), 3.89 (d, J = 17.7 Hz, 1H, Gly-α), 3.70–3.35 (m, 17H, OMe, 6 x CH_2–O, CH_2–NHCO), 3.25–3.15 (m, 2H, CH2–NHCOAr), 2.29 (q, J = 7.7 Hz, 2H, Glu-γ), 2.10–2.00 (m, 1H, Glu-β), 1.93–1.83 (m, 2H, Glu-β, C–CH_2–C), 1.77 (pent, J = 6.3 Hz, 1H, C–CH_2–C), 1.70 (pent, J = 6.5 Hz, 1H, C–CH_2–C), 1.65 (pent, J = 6.5 Hz, 1H, C–CH_2–C), 1.43 (s, 9H, Boc); **^{13}C NMR** (CD_3OD, 125 MHz) δ 175.04, 174.89, 171.60, 170.53, 168.12, 167.84, 161.29, 157.68, 153.95, 153.93, 142.38, 137.91, 135.50, 130.40, 130.31, 130.25, 130.13, 128.54, 126.14, 125.66, 124.77, 123.88, 113.70, 110.88, 110.82, 103.63, 103.61, 80.72, 71.47, 71.45, 71.24, 71.15, 71.13, 71.04, 70.89, 70.22, 70.15, 69.86, 69.79, 55.42, 52.63, 41.82, 39.07, 38.96, 37.90, 37.85, 30.30, 30.28, 30.03, 29.36, 28.71, 26.26; **IR** (film, cm^{-1}) 1751, 1632, 1617, 1447, 1245, 1215, 1179, 1107; **HRMS** calculated for $[C_{44}H_{54}N_4O_{15}H]^+$, requires m/z = 879.3658, found m/z = 879.3667 (ESI); **TLC** (6:1) acetone/EtOAc, yellow, R_f = 0.50.



1n **1o**

Synthesis of acid 1o Into a flask were added ester **1n** (832 mg, 0.948 mmol, 1 equiv), dioxane (10 mL, 0.1 M), water (4 mL), and sodium hydroxide (1 M aqueous, 6 mL, 6 mmol, 6 equiv). The mixture was stirred in the dark (4 h) and neutralized with hydrochloric acid (2 M aqueous, until the color changed from brown to pale orange, approximately pH 4). Volatiles were removed by lyophilization to yield mostly pure acid **1o**, which was used directly for the next step.

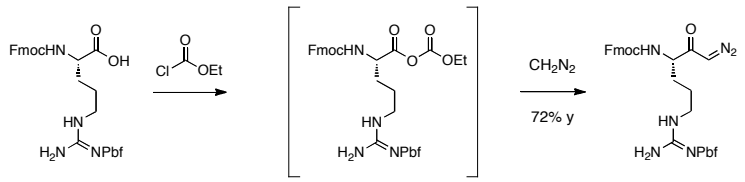
^{1}H NMR (CD_3OD, 500 MHz, major signals) δ 8.42 (d, J = 1.9 Hz, 0.5H, Ar), 8.05 (d, J = 8.9 Hz, 0.5H, Ar), 8.02 (dd, J_1 = 8.2 Hz, J_2 = 1.6 Hz, 0.5H, Ar), 7.97 (dd, J_1 = 7.8 Hz, J_2 = 1.9 Hz, 0.5H, Ar), 7.66 (d, J = 1.3 Hz, 0.5H, Ar), 7.31 (d, J = 7.9 Hz, 0.5H, Ar), 7.05–7.00 (m, 2H, Ar), 6.55–6.50 (m, 4H, Ar), 4.08–4.02 (m, 1H, Glu-α), 3.75–3.72 (m, 2H, Gly-α), 3.70–3.40 (m, 14H, 6 x CH_2–O, CH_2–NHCO), 3.27–3.18 (m, 2H, CH2–NHCOAr), 2.27 (q, J = 7.4 Hz, 2H, Glu-γ), 2.12–2.00 (m, 1H, Glu-β), 1.96–1.83 (m, 3H, Glu-β, C–CH_2–C), 1.77–1.68 (m, 2H, C–CH_2–C), 1.43 (s, 9H, Boc); **LC/MS** elutes at 0.89 min, calculated for $[C_{43}H_{52}N_4O_{15}H]^+$, requires m/z = 865.35, found m/z = 865.35 (ESI).



1o **1k** **1p**

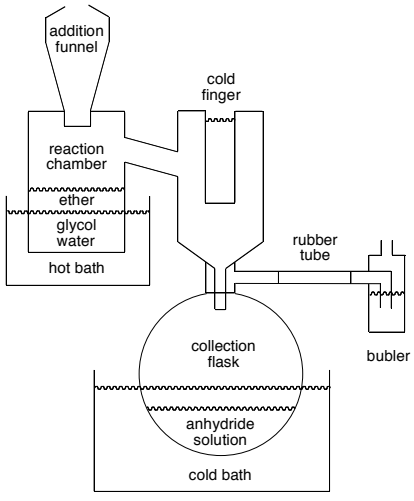
Synthesis of ester 1p Into a flask were added crude acid **1o** (0.948 mmol, 1 equiv), THF (20 mL, 0.05 M, producing a suspension), pyridine (398 μL, 4.93 mmol, 5.2 equiv), and trifluoroacetate **1k** (1.00 g, 4.74 mmol, 5 equiv). The suspension was maintained under an argon atmosphere, stirred in the dark (2 h, mostly dissolving and showing full conversion by LC/MS), quenched with water (10 mL, 3 min), and diluted with additional water (30 mL) and DCM (40 mL). The organic phase was isolated and the aqueous phase was further extracted with a mixture of DCM and THF (2:1, 30 mL). The combined organic phases were washed with sodium bisulfate (0.1 M, 60 mL) and dried with sodium sulfate. Volatiles were removed under reduced pressure to yield mostly pure ester **1p** (quantitative), which was used directly for the next step. Ester **1p** decomposes on silica gel.

^{1}H NMR (CD_3OD, 500 MHz, major signals) δ 8.41 (s, 0.5H, Ar), 8.19 (d, J = 8.4 Hz, 0.5H, Ar), 8.14 (d, J = 8.5 Hz, 0.5H, Ar), 8.07 (d, J = 9.0 Hz, 0.5H, Ar), 7.64 (s, 0.5H, Ar), 7.30 (d, J = 8.6 Hz, 0.5H, Ar), 6.70–6.67 (m, 2H, Ar), 6.64–6.51 (m, 4H, Ar), 4.36 (d, J = 18.5 Hz, 1H, Gly-α), 4.26 (d, J = 17.9 Hz, 1H, Gly-α), 4.10–4.04 (m, 1H, Glu-α), 3.67–3.35 (m, 14H, 6 x CH_2–O, CH_2–NHCO), 3.25–3.15 (m, 2H, CH2–NHCOAr), 2.81 (s, 4H, NHS), 2.27 (q, J = 8.8 Hz, 2H, Glu-γ), 2.09–1.97 (m, 1H, Glu-β), 1.95–1.62 (m, 5H, Glu-β, 2 x C–CH_2–C), 1.41 (s, 9H, Boc); **LC/MS** elutes at 1.03 min, calculated for $[C_{47}H_{55}N_5O_{17}H]^+$, requires m/z = 962.37, found m/z = 962.32 (ESI).



1q **1r** **1s**

Synthesis of ketone 1s[4] Into a flame-dried flask backfilled with argon was added Fmoc–Arg(Pbf)–OH (**1q**, 1.95 g, 3.0 mmol, 1 equiv). The flask was capped with a septum and maintained under an argon atmosphere. THF (15 mL, 0.2 M) was added, and the flask was cooled in a water/methanol/dry ice bath (-30 ºC). N-Methylmorpholine (395 μL, 3.60 mmol, 1.2 equiv) was added, and the flask was allowed to equilibrate (10 min). Ethyl chloroformate (317 μL, 3.30 mmol, 1.1 equiv) was added dropwise by syringe (producing precipitate). The mixture was stirred (1 h), quickly filtered into a fire-polished, argon-filled, -30 ºC flask, and chased with cold THF (15 mL). The filtrate was maintained at -30 ºC, and used directly for the next step. Mixed anhydride **1r** decomposes if it is allowed to warm to ambient temperature.

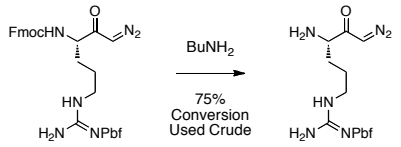


Diazomethane was generated in specially prepared fire-polished glassware behind a strong blast shield. Extra caution was used while working with this explosive and toxic gas. Potassium hydroxide (1.01 g, 18.0 mmol, 6 equiv) was dissolved in water (5 mL, exothermic) and poured into the reaction chamber. To the hydroxide solution were added diethylene glycol ethyl ether (10 mL) and ether (10 mL, producing a biphasic mixture). No stirbar was used. The reaction chamber was flushed with argon. The attached cold finger was cooled with a mixture of iPrOH and dry ice. The anhydride-containing flask (-30 ºC) was used as the collection flask, and the vent was passed through a bubbler filled with acetic acid. Diazald (1.93 g, 9.00 mmol, 3 equiv) was dissolved in

[4] Procedure modified from and compound data from: Rueping, M., Mahajan, Y. R., Jaun, B., Seebach, D. (2004) *Chem. Eur. J. 10*, 1607.

ether (20 mL) and added to the addition funnel. The reaction chamber was warmed in an oil bath (65 ºC, oil level at ether–water interface). As the ether began to distill, the Diazald solution was added dropwise (over 20 min) to maintain a constant amount of ether in the reaction chamber. Within a few minutes the distillate became yellow. The Diazald solution was chased with ether (2 x 5 mL, after which the distillate had returned to colorless). The oil bath was removed, and the system was maintained under argon (1 h). The cold bath (-30 ºC) was exchanged for an ice bath (0 ºC, additional 2 h). The glassware was removed from the collection flask. The reaction was quenched with acetic acid (dropwise from a blunt plastic syringe until bubbling ceased), diluted with a mixture (1:1, 40 mL) of sodium bicarbonate (saturated aqueous) and brine, extracted with ether (50 mL), washed with ammonium chloride (50% saturated aqueous), and dried with sodium sulfate. Volatiles were removed under reduced pressure, and the crude material was purified by column chromatography (100 mL silica gel, [1:1 to 2:1] EtOAc/DCM) to yield ketone **1s** (1.45 g, 2.15 mmol, 72% yield). Ketone **1s** is stable for at least several months at -20 ºC.

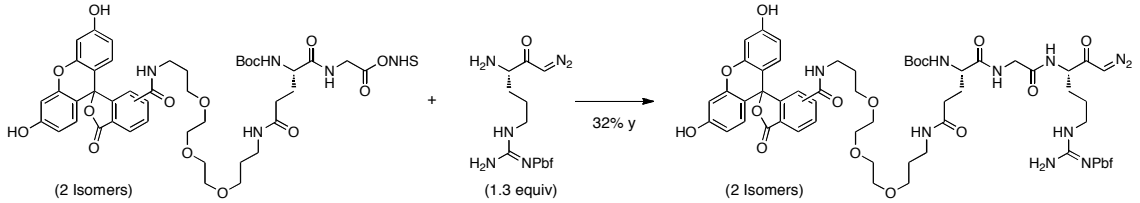
Compound analysis is consistent with published data.[4] **^{1}H NMR** ($CDCl_3$, 500 MHz) δ 7.74 (d, J = 7.6 Hz, 2H, Fmoc), 7.56 (d, J = 7.7 Hz, 2H, Fmoc), 7.37 (t, J = 7.4 Hz, 2H, Fmoc), 7.28–7.25 (m, 2H, Fmoc), 6.15–5.95 (brm, 3H, 3 x guan-NH), 5.86 (d, J = 8.2 Hz, 1H, Fmoc-NH), 5.55–5.50 (brs, 1H, CH=N_2), 4.43–4.35 (m, 2H, Fmoc), 4.22–4.12 (m, 2H, Fmoc, Arg-α), 3.32–3.15 (m, 2H, Arg-δ), 2.91 (s, 2H, Pbf), 2.58 (s, 3H, Pbf), 2.50 (s, 3H, Pbf), 2.07 (s, 3H, Pbf), 1.85–1.77 (m, 1H, Arg-β/γ), 1.65–1.50 (m, 3H, Arg-β/γ), 1.43 (s, 6H, Pbf); **TLC** (3:2) EtOAc/DCM, UV/permanganate, R_f = 0.22.



1s **1t**

Synthesis of amine 1t Into a flask were added carbamate **1s** (323 mg, 0.480 mmol, 1 equiv) and DCM (13 mL, 0.04 M). The mixture was capped with a rubber septum, flushed with argon, and cooled in an ice bath (0 ºC). Butylamine (1 mL, 10 mmol, 20 equiv) was added. The mixture was stirred (5 min), transferred into a cold room (-4 ºC), stirred (14 h), and diluted with cold toluene (10 mL). Volatiles were removed under reduced pressure (2 x toluene azeotrope), and the crude material (approximately 75% conversion) was used directly in the next step. Retention of stereochemistry was shown by coupling crude amine **1t** to each enantiomer of Boc–Phe–ONHS; the two diastereomeric products are readily distinguishable by ^{1}H NMR, and each reaction produced product with a diastereomeric ratio of >20:1. Several other Fmoc deprotection conditions produced significant amounts of epimerization of the Arg stereocenter or decomposition.

Crude amine **1t**: **^{1}H NMR** (CD_3OD, 500 MHz) δ 4.06–4.02 (m, 1H, Arg-α), 3.20–3.14 (m, 2H, Arg-δ), 2.99 (s, 2H, Pbf), 2.56 (s, 3H, Pbf), 2.50 (s, 3H, Pbf), 2.07 (s, 3H, Pbf), 1.76–1.68 (m, 1H, Arg-β/γ), 1.66–1.50 (m, 3H, Arg-β/γ), 1.44 (s, 6H, Pbf); **LC/MS** elutes at 0.73 min, calculated for $[C_{20}H_{30}N_6O_4SH]^+$, requires m/z = 451.56, found m/z = 451.06 (ESI).

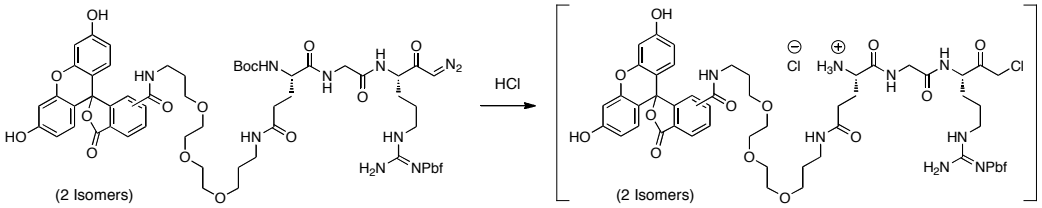


1p **1t** **1u**

Synthesis of peptide 1u Into a flask were added crude amine **1t** (0.48 mmol, 1.3 equiv, approximately 75% pure), THF (5 mL, 0.1 M), crude NHS ester **1p** (0.38 mmol, 1 equiv), Hünig's base (125 μL, 0.72 mmol, 1.9 equiv, causing precipitation), and DMF (0.3 mL, redissolving the precipitate). The mixture was stirred in the dark (3 h), quenched with methanol (0.5 mL, 30 min), diluted with DCM (40 mL) and THF (5 mL), washed with sodium bisulfate (0.1 M aqueous, 40 mL), and dried with sodium sulfate. Volatiles were removed under reduced pressure, and the crude material was passed through a silica gel column (50 mL silica gel, 1:1 DCM/acetone + 7–16% MeOH). The product streaked through the column and did not elute cleanly. Product-containing fractions (>90% pure, 156 mg, 0.120 mmol, 32% yield, 1:1 mixture of isomers at fluorescein) were collected and used directly for

the next step. Diazomethyl ketone **1u** is not stable to acidic HPLC conditions (1:1 water/MeCN + 0.1% TFA causes full decomposition within 15 h). ^{1}H NMR signals were assigned by ^{1}H–^{1}H COSY and by comparison to signals from the starting materials. ^{13}C NMR signals were assigned by ^{1}H–^{13}C HMQC.

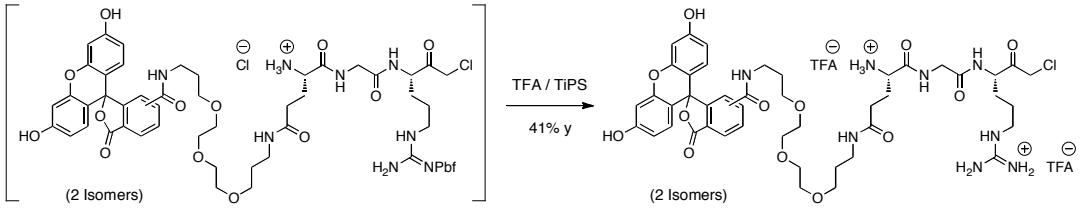
^{1}H NMR (CD_3OD, 500 MHz, major signals) δ 8.46 (s, 0.5H, fluor), 8.20 (d, J = 7.9 Hz, 0.5H, fluor), 8.14 (d, J = 8.0 Hz, 0.5H, fluor), 8.07 (d, J = 7.9 Hz, 0.5H, fluor), 7.68 (s, 0.5H, fluor), 7.31 (d, J = 7.9 Hz, 0.5H, fluor), 6.72–6.64 (m, 4H, phenol), 6.58–6.54 (m, 2H, phenol), 6.00 (s, 1H, CH=N_2), 4.38–4.30 (brs, 1H, Arg-α), 4.06–4.00 (m, 1H, Glu-α), 3.91 (s, 2H, Gly-α), 3.66–3.12 (m, 18H, 6 x CH_2–O, Arg-δ, 2 x CH_2–NH), 2.97 (s, 2H, Pbf), 2.58 (s, 3H, Pbf), 2.51 (s, 3H, Pbf), 2.31 (d, J = 7.4 Hz, 2H, Glu-γ), 2.06 (s, 3H, Pbf), 1.99–1.47 (m, 10H, Glu-β, Arg-β, Arg-γ, 2 x C–CH_2–C), 1.43 (s, 6H, Pbf), 1.40 (s, 9H, Boc); **^{13}C NMR** (CD_3OD, 125 MHz, major signals) δ 196.25, 175.22, 174.73, 174.72, 171.63, 170.70, 168.07, 167.79, 162.77, 159.74, 157.95, 157.90, 154.40, 141.65, 139.26, 137.73, 134.85, 134.29, 133.40, 130.52, 130.33, 126.66, 126.17, 125.96, 125.31, 124.49, 118.35, 114.64, 114.55, 111.41, 111.29, 103.68, 87.61, 80.84, 71.36, 71.17, 71.15, 71.09, 71.04, 70.96, 70.83, 70.14, 70.08, 69.72, 57.76, 55.91, 54.79, 43.88, 43.69, 41.31, 38.98, 38.88, 37.84, 37.80, 33.11, 30.28, 30.24, 30.02, 29.41, 28.74, 28.72, 26.78, 19.64, 18.44, 12.57; **IR** (film, cm^{-1}) 3342, 2972, 2928, 2874, 2442, 1642, 1610, 1450, 1428, 1109, 1084, 974; **HRMS** calculated for $[C_{63}H_{80}N_{10}O_{18}SH]^+$, requires m/z = 1297.5446, found m/z = 1297.5412 (ESI); **TLC** (1:1) DCM/acetone + 10% MeOH, UV/permanganate, R_f = 0.38 (streaks); **LC/MS** elutes at 1.46 min, calculated for $[C_{63}H_{80}N_{10}O_{18}SH]^+$, requires m/z = 1297.54, found m/z = 1297.16 (ESI).



1u **1v**

Synthesis of chloromethyl ketone 1v Into a flask were added mostly pure diazomethyl ketone **1u** (0.120 mmol, 1 equiv) and THF (3 mL, 0.04 M, producing a nearly homogeneous solution). The flask was capped with a rubber septum and vented with a needle, and hydrochloric acid (4.0 M in dioxane, 3 mL, 0.04 M in substrate) was added by syringe (producing a clumpy goo). The mixture was stirred (1 h) in the dark and diluted with toluene (4 mL). Volatiles were removed under reduced pressure (3 x toluene azeotrope). Analysis by LC/MS showed chloromethyl ketone **1v** as the major product, and the crude mixture was used directly for the next step.

LC/MS elutes at 1.14 min, calculated for $[C_{58}H_{73}ClN_8O_{16}SH]^+$, requires m/z = 1205.46, found m/z = 1205.34 (ESI).



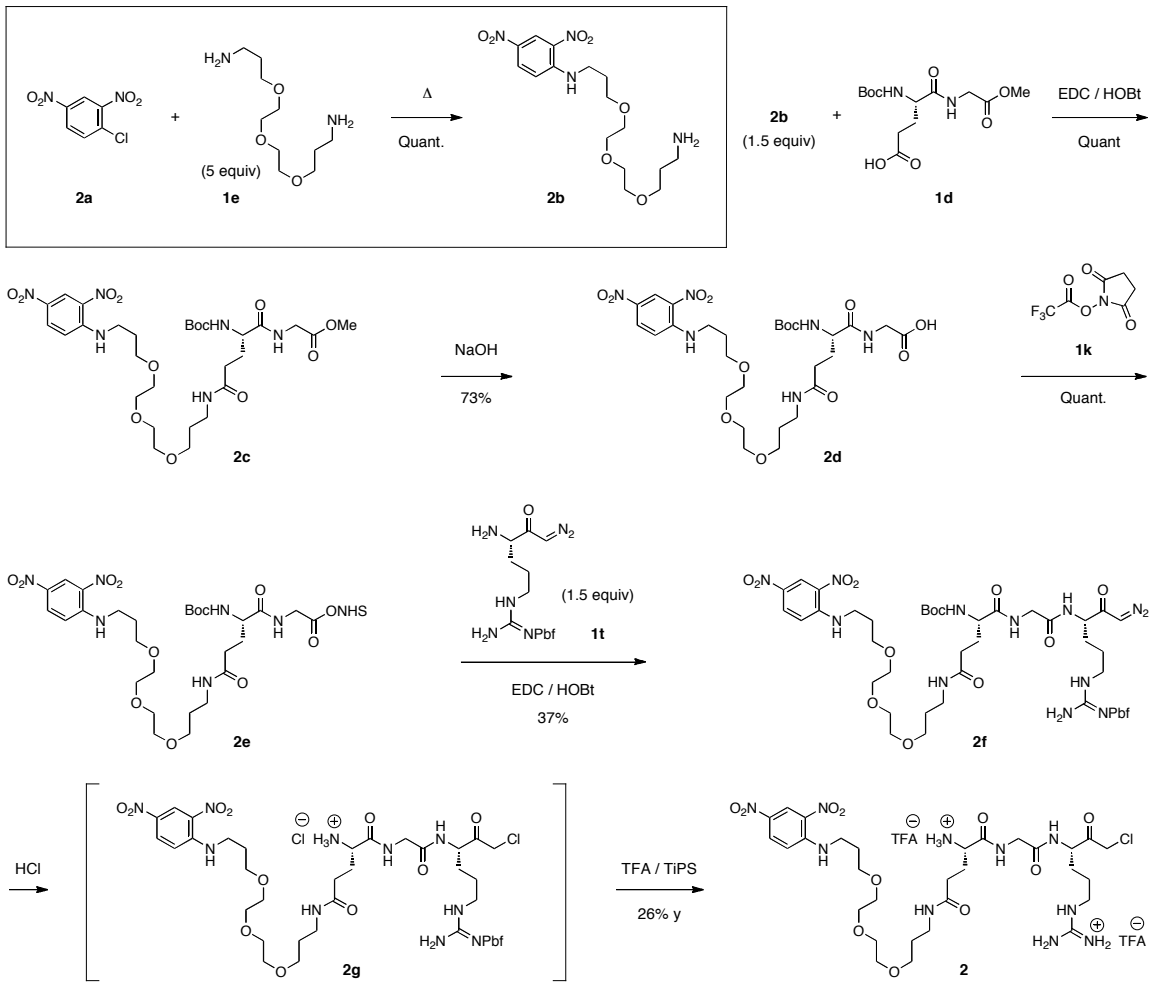
1v **1**

Synthesis of guanidinium 1 Into a flask were added crude peptide **1v** (0.120 mmol, 1 equiv), and a mixture of TFA, TiPS, and water (38:1:1, 2 mL, 0.06 M, producing a homogeneous solution). The mixture was stirred in the dark (1.5 h) and diluted with toluene (3 mL). Volatiles were removed under reduced pressure (2 x toluene azeotrope). The crude mixture was dissolved in water (2 mL) and washed with EtOAc (2 x 2 mL). The aqueous phase was purified by semi-preparative RP-HPLC (C_{18} column, water + 0.1% TFA with 15–35% MeCN over 30 min, product isomers elute at 14.5 and 15.4 min) to yield pure guanidinium **1** (58 mg, 0.049 mmol, 41% yield) as a mixture (approximately 1:1) of regioisomers at fluorescein and as a yellow solid. The regioisomeric ratio was measured by ^{1}H NMR. ^{1}H NMR signals were assigned by ^{1}H–^{1}H COSY. Material was stored at -20 ºC as a stock solution in water, and is stable for at least several months.

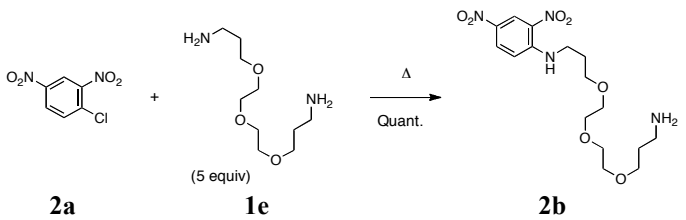
^{1}H NMR (D_2O, 500 MHz) δ 8.47 (d, J = 4.9 Hz, 0.5H, fluor), 8.19–8.15 (m, 0.5H, fluor), 8.06–7.98 (m, 1H, fluor), 7.47 (s, 0.5H, fluor), 7.22 (t, J = 7.5 Hz, 0.5H, fluor), 7.03–6.67 (m, 6H, 2 x phenol), 4.55–4.43 (m, 3H,

Arg-α, CH_2–Cl), 4.10–3.93 (m, 3H, Glu-α, Gly-α), 3.62–3.08 (m, 18H, 6 x CH_2–O, Arg-δ, 2 x CH_2–NHCO), 2.43–2.32 (m, 2H, Glu-γ), 2.17–2.10 (m, 2H, Glu-β), 1.92–1.44 (m, 8H, Arg-β, Arg-γ, 2 x C–CH_2–C); **^{13}C NMR** (D_2O + 1.5% MeOH, 125 MHz) δ 203.95, 174.27, 171.40, 170.35, 168.79, 168.48, 167.50, 166.82, 163.3 (q, J = 35.5 Hz), 157.32, 156.94, 141.92, 140.51, 139.19, 136.76, 132.89, 131.91, 131.69, 130.32, 130.04, 129.87, 129.31, 128.92, 127.16, 118.50, 116.9 (q, J = 291.7 Hz), 114.81, 114.72, 114.37, 103.17, 70.21, 70.05, 69.91, 69.87, 69.39, 69.21, 68.94, 57.44, 53.15, 47.30, 42.73, 41.06, 38.19, 37.10, 37.36, 28.82, 27.30, 27.23, 24.93; **IR** (film, cm^{-1}) 3285, 1671, 1638, 1201, 1180, 1127; **HRMS** calculated for $[C_{45}H_{57}ClN_8O_{13}H]^+$, requires m/z = 953.3806, found m/z = 953.3774 (ESI); **RP-HPLC** C_{18} column, 15–35% MeCN in water + 0.1% TFA, flow = 1.0 mL/min, monitored at 214 nm, isomers elute overlapping at 14.6 and 15.4 min.

Scheme S2

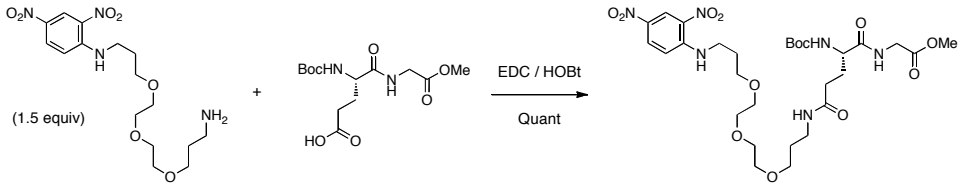


Scheme S2 shows the synthetic strategy used to access compound **2**.



Synthesis of aniline 2b Into a flask were added 1-chloro-2,4-dinitrobenzene (**2**, 4.05 g, 20.0 mmol, 1 equiv), 4,7,10-trioxa-1,13-tridecanediame (**1e**, 22.0 mL, 100 mmol, 5 equiv), and ethanol (100 mL, 0.2 M). The mixture was heated to reflux (2 h, changing from yellow to red), concentrated under reduced pressure, diluted with water (100 mL), brine (100 mL), and sodium bicarbonate (saturated aqueous, 50 mL), extracted with DCM (150 + 50 mL), washed with sodium chloride (50% saturated, 2 x 100 mL), and dried with sodium sulfate. Volatiles were removed under reduced pressure to yield mostly pure aniline **2b**, which was used directly in the next step.

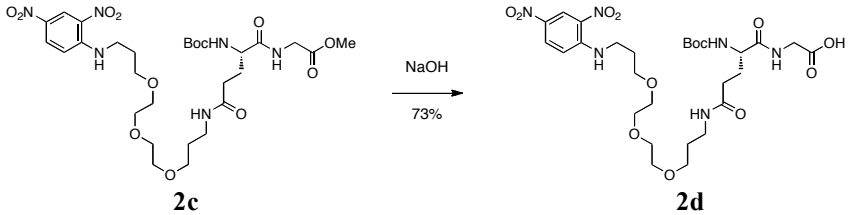
^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.14 (s, 1H, Ar), 8.92 (s, 1H, Ar-NH), 8.27 (d, J = 11.6 Hz, 1H, Ar), 6.97 (d, J = 10.5 Hz, 1H, Ar), 3.78–3.51 (m, 14H, 6 x CH_2–O, CH_2–NHAr), 2.78 (t, J = 8.3 Hz, 2H, CH_2–NH_2), 2.08–2.00 (m, 2H, C–CH_2C), 1.68–1.76 (m, 2H, C–CH_2C), 1.42–1.00 (brs, 2H, NH_2); **LC/MS** elutes at 0.61 min, calculated for $[C_{16}H_{26}N_4O_7H]^+$, requires m/z = 387.19, found m/z = 387.13 (ESI).



2b **1d** **2c**

Synthesis of amide 2c Into a flask were added crude acid **1d** (1.57 g, 4.93 mmol, 1 equiv), crude amine **2b** (2.85 g, 7.39 mmol, 1.5 equiv), EDC hydrochloride (1.14 g, 5.92 mmol, 1.2 equiv), HOBt hydrate (904 mg, 5.91 mmol, 1.2 equiv), and DCM (49 mL, 0.1 M). The mixture was stirred (15 h), diluted with DCM (50 mL), washed with a mixture (1:1:1, 60 mL) of water, brine, and sodium bicarbonate (saturated aqueous) and with a mixture (1:1:1, 60 mL) of citric acid (10% aqueous), water, and brine, and dried with sodium sulfate. Volatiles were removed under reduced pressure, and the crude material was purified by column chromatography (120 mL silica gel, [4:1 to 1:1] DCM/acetone) to yield amide **2c** (3.39 g, quantitative). ^{1}H NMR signals were assigned by ^{1}H–^{1}H COSY and by comparison to signals from the starting materials.

^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.13 (d, J = 2.7 Hz, 1H, Ar), 8.92 (t, J = 4.7 Hz, 1H, NH-Ar), 8.26 (dd, J_1 = 5.6 Hz, J_2 = 2.7 Hz, 1H, Ar), 7.50–7.40 (brs, 1H, Gly-NH), 6.97 (d, J = 9.6 Hz, 1H, Ar), 6.56 (t, J = 6.2 Hz, 1H, NH-Glu-γ), 5.82 (d, J = 6.9 Hz, 1H, NH-Boc), 4.20 (q, J = 6.9 Hz, 1H, Glu-α), 4.10 (dd, J_1 = 18.0 Hz, J_2 = 6.0 Hz, 1H, Gly-α), 3.98 (dd, J_1 = 18.3 Hz, J_2 = 5.2 Hz, 1H, Gly-α), 3.72 (s, 3H, OMe), 3.71–3.52 (m, 14H, 6 x CH_2–O, CH_2–NHAr), 3.35 (q, J = 6.0 Hz, 2H, CH_2–NH), 2.42–2.32 (m, 2H, Glu-γ), 2.07–1.98 (m, 4H, Glu-β, C–CH_2–C), 1.81–1.73 (m, 2H, C–CH_2–C), 1.42 (s, 9H, Boc); **^{13}C NMR** ($CDCl_3$, 125 MHz) δ 172.61, 172.15, 170.12, 155.64, 148.30, 135.38, 130.14, 130.10, 124.12, 113.85, 79.64, 70.48, 70.34, 70.26, 69.92, 69.73, 69.09, 53.64, 52.10, 41.82, 40.92, 37.83, 32.33, 28.88, 28.75, 28.48, 28.17; **IR** (film, cm^{-1}) 3326, 2929, 2872, 1659, 1618, 1523, 1336; **HRMS** calculated for $[C_{29}H_{45}N_6O_{13}H]^+$, requires m/z = 687.3196, found m/z = 687.3195 (ESI); **TLC** (3:2) DCM/acetone, yellow, R_f = 0.38.

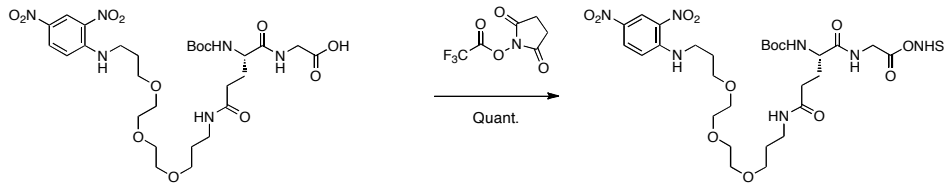


2c **2d**

Synthesis of acid 2d Into a flask were added ester **2c** (641 mg, 0.934 mmol, 1 equiv), dioxane (9 mL, 0.1 M), water (3.4 mL), and sodium hydroxide (1 M, 5.6 mL, 5.6 mmol, 6 equiv). The mixture was stirred (2 h), cooled in an ice bath (0 ºC), neutralized with sodium bisulfate (2 M, approximately 3 mL, until the mixture became dark brown, approximately pH 6) and sodium bicarbonate (saturated aqueous, 0.5 mL, to pH 7, becoming red). Volatiles were removed by lyophilization. The crude mixture was mixed with DCM (25 mL), insolubles were removed by vacuum filtration, and the red filtrate was collected. Volatiles were removed under reduced pressure to yield nearly pure amide **2d** (459 mg, 0.683 mmol, 73% yield from Boc–Glu(Bn)–OH), which was used directly for the next step.

^{1}H NMR (CD_3OD, 400 MHz) δ 9.03 (d, J = 1.8 Hz, 1H, Ar), 8.29 (dd, J_1 = 10.2 Hz, J_2 = 1.7 Hz, 1H, Ar), 7.20 (d, J = 10.4 Hz, 1H, Ar), 4.05 (t, J = 7.8 Hz, 1H, Glu-α), 3.77 (d, J = 18.3 Hz, 1H, Gly-α), 3.71 (d, J = 16.6 Hz,

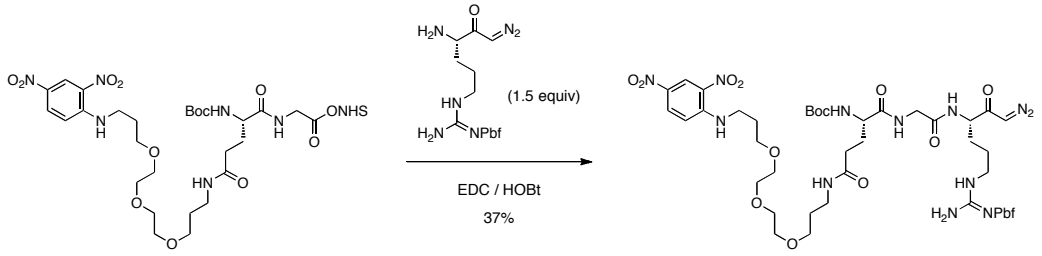
1H, Gly-α), 3.68–3.54 (m, 12H, 6 x CH_2–O), 3.41 (t, J = 6.2 Hz, 2H, CH_2–NHAr), 3.22 (t, J = 7.7 Hz, 2H, CH_2–NH), 2.26 (t, J = 7.7 Hz, 2H, Glu-γ), 2.10–1.84 (m, 4H, Glu-β, C–CH_2–C), 1.73 (quint, J = 6.5 Hz, 2H, C–CH_2C), 1.43 (s, 9H, Boc); **LC/MS** elutes at 1.22 min, calculated for $[C_{28}H_{44}N_6O_{13}H]^+$, requires m/z = 673.30, found m/z = 673.28 (ESI).



2d **1k** **2e**

Synthesis of ester 2e Into a dried flask flushed with argon were added crude acid **2d** (1.82 mmol, 1 equiv), THF (18 mL, 0.1 M), pyridine (622 μL, 8.19 mmol, 4.5 equiv), and trifluoroacetate **1k** (1.54 g, 7.28 mmol, 4 equiv). The mixture was maintained under an argon atmosphere, stirred in the dark (3 h), quenched with water (5 mL, 5 min), and diluted with additional water (35 mL) and DCM (40 mL). The organic phase was isolated and the aqueous phase was further extracted with a mixture (2:1, 20 mL) of DCM and THF. The combined organic phases were washed with a mixture (1:1, 40 mL) of citric acid (10% aqueous) and brine and dried with sodium sulfate. Volatiles were removed under reduced pressure to yield mostly pure ester **2e** (quantitative), which was used directly for the next step. Ester **2e** decomposes on silica gel.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.12 (d, J = 2.6 Hz, 1H, Ar), 8.90 (t, J = 5.2 Hz, 1H, NH-Ar), 8.25 (dd, J_1 = 9.6 Hz, J_2 = 2.6 Hz, 1H, Ar), 7.73–7.67 (brs, 1H, Gly-NH), 6.96 (d, J = 9.7 Hz, 1H, Ar), 6.88–6.81 (brs, 1H, NH), 5.85 (d, J = 6.4 Hz, 1H, Boc-NH), 4.47 (dd, J_1 = 18.2 Hz, J_2 = 6.4 Hz, 1H, Gly-α), 4.32–4.20 (m, 2H, Gly-α, Glu-α), 3.74–3.51 (m, 14H, 6 x CH_2–O, CH_2–NHAr), 3.34 (q, J = 6.3 Hz, 2H, CH_2–NH), 2.82 (s, 4H, NHS), 2.34 (t, J = 6.6 Hz, 2H, Glu-γ), 2.09–1.88 (m, 4H, Glu-β, C–CH_2–C), 1.76 (quint, J = 6.1 Hz, 2H, C–CH_2C), 1.41 (s, 9H, Boc); **LC/MS** elutes at 1.37 min, calculated for $[C_{32}H_{47}N_7O_{15}H]^+$, requires m/z = 770.32, found m/z = 770.34 (ESI).

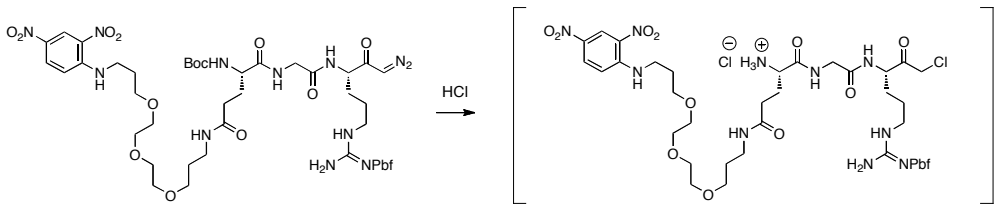


2e **1t** **2f**

Synthesis of peptide 2f Into a flask were added crude amine **1t** (0.48 mmol, 1.2 equiv, approximately 75% pure), THF (5 mL, 0.1 M), crude NHS ester **2e** (0.42 mmol, 1 equiv), and Hünig's base (125 μL, 0.72 mmol, 1.7 equiv). The mixture was stirred (3 h), diluted with DCM (40 mL), washed with citric acid (5% aqueous, 40 mL), and dried with sodium sulfate. Volatiles were removed under reduced pressure, and the crude material was purified by column chromatography (50 mL silica gel, 1:1 DCM/acetone + 1–5% MeOH) to yield amide **2f** (166 mg, 0.150 mmol, 36% yield from methylester **1d**) plus additional slightly impure material (107 mg). ^{1}H NMR signals were assigned by ^{1}H–^{1}H COSY and by comparison to signals from the starting materials.

^{1}H NMR ($CDCl_3$, 400 MHz) δ 9.11 (d, J = 2.7 Hz, 1H, Ar), 8.93 (t, J = 4.9 Hz, 1H, NH-Ar), 8.25 (dd, J_1 = 9.5 Hz, J_2 = 2.8 Hz, 1H, Ar), 7.76 (t, J = 6.1 Hz, 1H, Gly-NH), 7.54 (d, J = 8.5 Hz, 1H, Arg-NH), 6.98 (d, J = 9.6 Hz, 1H, Ar), 6.86 (t, J = 5.2 Hz, 1H, Glu-γ-NH), 6.40–6.32 (brs, 2H, 2 x Guan-NH), 6.23–6.10 (brs, 2H, Guan-NH, Boc-NH), 5.79–5.71 (brs, 1H, CH=N_2), 4.46–4.38 (brs, 1H, Arg-α), 4.13 (q, J = 6.4 Hz, 1H, Glu-α), 4.05 (dd, J_1 = 17.0 Hz, J_2 = 5.9 Hz, 1H, Gly-α), 3.90 (dd, J_1 = 16.7 Hz, J_2 = 5.6 Hz, 1H, Gly-α), 3.72–3.50 (m, 14H, 6 x CH_2–O, CH_2–NHAr), 3.30 (q, J = 6.2 Hz, 2H, CH_2–NH), 3.19 (q, J = 5.9 Hz, 2H, Arg-δ), 2.94 (s, 2H, Pbf), 2.56 (s, 3H, Pbf), 2.50 (s, 3H, Pbf), 2.34 (t, J = 6.9 Hz, 2H, Glu-γ), 2.08 (s, 3H, Pbf), 2.08–1.99 (m, 4H, Glu-β, C–CH_2–C), 1.94–1.82 (m, 1H, Arg-β), 1.75 (quint, J = 6.2 Hz, 2H, C–CH_2C), 1.70–1.60 (m, 1H, Arg-β), 1.59–1.50 (m, 2H, Arg-γ), 1.45 (s, 6H, Pbf), 1.39 (s, 9H, Boc); **^{13}C NMR** ($CDCl_3$, 125 MHz) δ 194.01, 173.25, 172.67, 169.60, 158.50, 156.26,

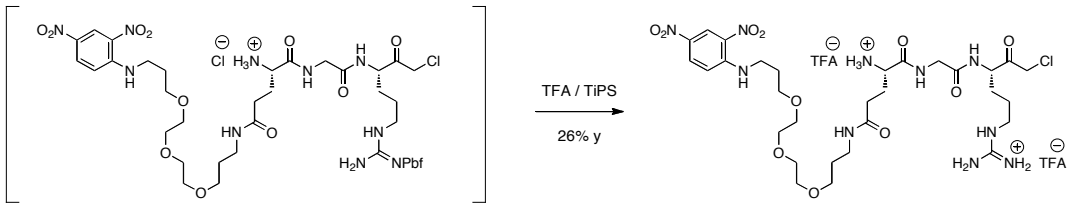
156.08, 148.29, 137.99, 135.45, 132.80, 131.95, 130.13, 129.97, 124.47, 124.04, 117.28, 113.95, 86.25, 79.92, 70.40, 70.26, 70.18, 69.84, 69.37, 69.14, 56.41, 54.71, 53.92, 43.03, 41.87, 40.33, 37.60, 32.26, 28.79, 28.53, 28.42, 28.40, 28.15, 27.98, 25.29, 19.13, 17.80, 12.30; **IR** (film, cm^{-1}) 3334, 2974, 2925, 2868, 1618, 1544, 1520, 1336, 1099, 903; **HRMS** calculated for $[C_{48}H_{72}N_{12}O_{16}SH]^+$, requires *m/z* = 1105.4983, found *m/z* = 1105.4956 (ESI); **TLC** (1:1) DCM/acetone + 4% MeOH, permanganate, R_f = 0.23.



2f **2g**

Synthesis of chloromethyl ketone 2g Into a flask were added diazomethyl ketone **2f** (166 mg, 0.150 mmol, 1 equiv) and THF (3 mL, 0.05 M). The flask was capped with a rubber septum and vented with a needle, and hydrochloric acid (4.0 M in dioxane, 3 mL, 0.05 M in substrate) was added by syringe. The mixture was stirred (1.5 h) and diluted with toluene (4 mL). Volatiles were removed under reduced pressure (3 x toluene azeotrope). Analysis by LC/MS showed chloromethyl ketone **2g** as the major product, and the crude mixture was used directly for the next step.

LC/MS elutes at 1.36 min, calculated for $[C_{43}H_{65}ClN_{10}O_{14}SH]^+$, requires *m/z* = 1013.42, found *m/z* = 1013.23 (ESI).

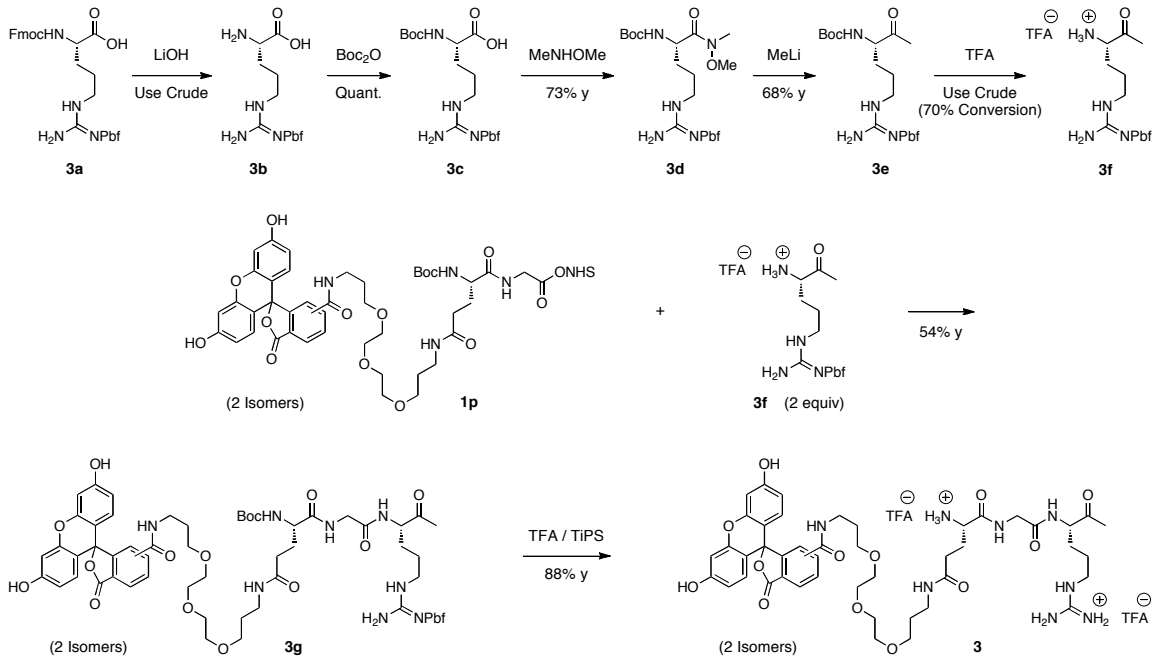


2g **2**

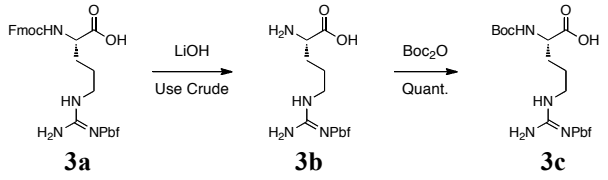
Synthesis of guanidinium 2 Into a flask were added crude peptide **2g** (0.150 mmol, 1 equiv), and a mixture of TFA, TiPS, and water (38:1:1, 2 mL, 0.07 M). The mixture was stirred (1.5 h) and diluted with toluene (3 mL). Volatiles were removed under reduced pressure (2 x toluene azeotrope). The crude mixture was dissolved in water (2 mL) and washed with EtOAc (2 x 2 mL). The aqueous phase was purified by semi-preparative RP-HPLC (C_{18} column, water + 0.1% TFA with 15–35% acetonitrile over 30 min, product elutes at 19.3 min) to yield pure guanidine **2** (38 mg, 0.038 mmol, 26% yield) as a yellow solid. ^{1}H NMR signals were assigned by ^{1}H–^{1}H COSY. Material was stored at -20 ºC as a stock solution in water, and is stable for at least several months.

^{1}H NMR (D_2O, 500 MHz) δ 8.73 (dd, J_1 = 15.7 Hz, J_2 = 3.6 Hz, 1H, Ar), 8.09 (dd, J_1 = 9.0 Hz, J_2 = 2.7 Hz, 1H, Ar), 7.00 (dd, J_1 = 9.8 Hz, J_2 = 6.3 Hz, 1H, Ar), 4.48 (dd, J_1 = 9.0 Hz, J_2 = 4.3 Hz, 1H, Arg-α), 4.55 (d, J = 17.2 Hz, 1H, CH_2–Cl), 4.51 (d, J = 17.1 Hz, 1H, CH_2–Cl), 4.11–3.95 (m, 3H, Glu-α, Gly-α), 3.68–3.14 (m, 18H, 6 x CH_2–O, Arg-δ, 2 x CH_2–NH), 2.42–2.36 (m, 2H, Glu-γ), 2.18–2.12 (m, 2H, Glu-β), 1.98–1.91 (m, 3H, Arg-β, Arg-γ, C–CH_2–C), 1.74–1.54 (m, 5H, Arg-β, Arg-γ, C–CH_2–C); **^{13}C NMR** (D_2O + 1.5% MeOH, 125 MHz) δ 204.06, 174.29, 171.41, 170.36, 163.2 (q, J = 35.4 Hz), 157.36, 149.23, 135.36, 130.80, 129.88, 124.71, 116.7 (q, J = 291.7 Hz), 115.39, 57.46, 53.16, 47.83, 42.76, 41.69, 41.09, 37.15, 31.36, 28.88, 28.43, 27.32, 27.28, 24.96; **IR** (film, cm^{-1}) 3359, 1662, 1618, 1331, 1172, 1127; **HRMS** calculated for $[C_{30}H_{49}ClN_{10}O_{11}H]^+$, requires *m/z* = 761.3344, found *m/z* = 761.3324 (ESI); **RP-HPLC** C_{18} column, 15–35% MeCN in water + 0.1% TFA, flow = 1.0 mL/min, monitored at 214 nm, elutes at 19.3 min.

Scheme S3



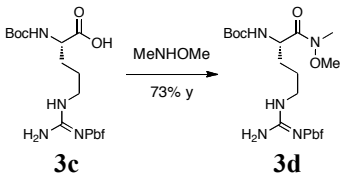
Scheme S3 shows the synthetic strategy used to access compound **3**.



Synthesis of carbamate 3c Into a flask were added Fmoc–Arg(Pbf)–OH (1.98 g, 3.05 mmol, 1 equiv), dioxane (10 mL, 0.3 M), water (5 mL, 0.6 M), and lithium hydroxide (2 M aqueous, 4.6 mL, 9.2 mmol, 3 equiv). The mixture was stirred (2.5 h, producing precipitate), diluted with water (10 mL), and washed with EtOAc (20 mL). The aqueous phase was used directly in the next step.

To the aqueous phase containing crude amine **3b** was added sodium hydroxide (1 M aqueous, 6 mL, 6 mmol, 2 equiv). Di-*tert*-butyl dicarbonate (864 mg, 3.97 mmol, 1.3 equiv) was dissolved in dioxane (20 mL, 0.15 M) and added. The mixture was stirred (19 h), concentrated under reduced pressure, diluted with water (30 mL) and sodium carbonate (5% aqueous, 3 mL, producing pH = 9), washed with ether (40 mL), acidified with hydrochloric acid (1 M aqueous, until pH = 1), extracted with ethyl acetate (3 x 30 mL), and dried with sodium sulfate. Volatiles were removed under reduced pressure to yield pure carbamate **3c** (quantitative), a fraction of which was used directly in the next step. Product analysis is consistent with commercially available material.[5]

^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.58–6.30 (m, 3H), 5.65–5.55 (brs, 1H), 4.25 (q, J = 6.6 Hz, 1H), 3.25–3.10 (brs, 2H), 2.93 (s, 2H), 2.53 (s, 3H), 2.47 (s, 3H), 2.07 (s, 3H), 1.95–1.55 (m, 4H), 1.44 (s, 6H), 1.41 (s, 9H).

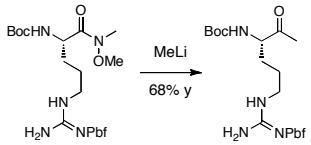


Synthesis of amide 3d Into a flask were added one third of crude acid **3c** (1.0 mmol, 1 equiv), *N*,*O*-dimethylhydroxylamine hydrochloride (195 mg, 2.00 mmol, 2 equiv), EDC hydrochloride (250 mg, 1.30 mmol, 1.3

[5] Boc–Arg(Pbf)–OH can be purchased from Sigma–Aldrich.

equiv), HOBt hydrate (230 mg, 1.50 mmol, 1.5 equiv), acetonitrile (4 mL, 0.3 M), and *N*-methylmorpholine (165 μL, 1.50 mmol, 1.5 equiv). The mixture was stirred (18 h), diluted with ethyl acetate (40 mL), washed with sodium chloride (50% saturated aqueous, 30 mL), a mixture (1:1, 30 mL) of citric acid (5% aqueous) and brine, a mixture (1:1, 30 mL) of sodium bicarbonate (saturated) and sodium carbonate (10% aqueous), and again with sodium chloride (50% aqueous), and dried with sodium sulfate. Volatiles were removed under reduced pressure to yield nearly pure amide **3d** (530 mg, 0.93 mmol, 93% yield from carbamate **3a**). Alternately, amide **3d** can be prepared by first performing the amide coupling and then exchanging the amine protecting group. The crude material obtained from 1.5 mmol of starting carbamate **3a** was purified by column chromatography (50 mL silica gel, 3:1 EtOAc/DCM) to yield amide **3d** (623 mg, 1.09 mmol, 73% yield). ^{1}H NMR signals were assigned by ^{1}H–^{1}H COSY.

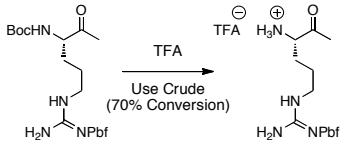
^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.40–2.22 (brs, 1H, guanidineNH), 6.18–6.06 (brs, 2H, guanidine-NH_2), 5.47 (d, J = 8.3 Hz, 1H, Boc-NH), 4.68–4.58 (m, 1H, Arg-α), 3.72 (s, 3H, OMe), 3.40–3.30 (m, 1H, Arg-δ), 3.21–3.10 (m, 1H, Arg-δ), 3.18 (s, 3H, NMe), 2.94 (s, 2H, Pbf), 2.57 (s, 3H, Pbf), 2.51 (s, 3H, Pbf), 2.08 (s, 3H, Pbf), 1.75–1.52 (m, 4H, Arg-β, Arg-γ), 1.45 (s, 6H, Pbf), 1.41 (s, 9H, Boc); **^{13}C NMR** ($CDCl_3$, 125 MHz) δ 172.58, 158.56, 156.15, 138.27, 133.05, 132.20, 124.45, 117.33, 86.24, 80.01, 61.56, 49.67, 43.19, 40.78, 32.03, 30.71, 28.70, 28.53, 29.29, 28.12, 24.89, 19.19, 17.82, 12.39; **IR** (film, cm^{-1}) 3338, 2978, 2929, 1712, 1650, 1621, 1548, 1168, 1107; **HRMS** calculated for $[C_{26}H_{42}N_5O_7SH]^+$, requires m/z = 570.2956, found m/z = 570.2957 (ESI); **TLC** (2:1) EtOAc/DCM, permanganate, R_f = 0.28.



3d **3e**

Synthesis of ketone 3e Into a flame-dried flask backfilled with argon were added amide **3d** (566 mg, 1.00 mmol, 1 equiv) and THF (20 mL, 0.05 M). The flask was capped with a rubber septum, flushed with argon, and cooled in a dry ice/acetone bath (-78 ºC, 10 min). Methyllithium (1.6 M in ether, 3.13 mL, 5.00 mmol, 5 equiv), was added dropwise (over 5 min, becoming orange). The mixture was stirred (1.5 h), quenched with ammonium chloride (saturated aqueous, 5 mL, becoming colorless), allowed to warm to ambient temperature, diluted with water (30 mL) and brine (30 mL), extracted with EtOAc (50 + 20 mL), and dried with sodium sulfate. Volatiles were removed under reduced pressure, and the crude material was purified by column chromatography (50 mL silica gel, 3:2 EtOAc/DCM) to yield ketone **3e** (388 mg, 0.682 mmol, 68% yield). ^{1}H NMR signals were assigned by ^{1}H–^{1}H COSY and by comparison to signals from the starting materials.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.22–6.05 (brs, 1H, guanidineNH), 6.05–5.97 (brs, 2H, guanidine-NH_2), 5.43 (d, J = 7.1 Hz, 1H, Boc-NH), 4.33–4.25 (m, 1H, Arg-α), 3.43–3.34 (m, 1H, Arg-δ), 3.22–3.13 (m, 1H, Arg-δ), 2.95 (s, 2H, Pbf), 2.58 (s, 3H, Pbf), 2.52 (s, 3H, Pbf), 2.19 (s, 3H, Me), 2.09 (s, 3H, Pbf), 1.88–1.79 (m, 1H, Arg-β/Arg-γ), 1.70–1.52 (m, 3H, Arg-β, Arg-γ), 1.45 (s, 6H, Pbf), 1.41 (s, 9H, Boc); **^{13}C NMR** ($CDCl_3$, 125 MHz) δ 207.42, 158.65, 156.26, 155.82, 138.08, 132.68, 132.02, 124.55, 117.41, 86.31, 79.74, 59.56, 43.09, 40.59, 28.48, 28.20, 26.57, 25.14, 19.18, 17.84, 12.34; **IR** (film, cm^{-1}) 3334, 1699, 1552, 1246, 1156, 1091; **HRMS** calculated for $[C_{25}H_{39}N_4O_6SH]^+$, requires m/z = 525.2741, found m/z = 525.2738 (ESI); **TLC** (3:1) EtOAc/DCM, UV, R_f = 0.38.

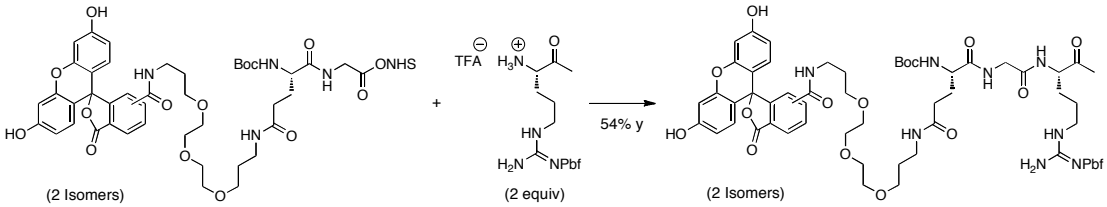


3e **3f**

Synthesis of amine 3f Into a flask were added ketone **3f** (388 mg, 0.740 mmol, 1 equiv) and DCM (3.6 mL, 0.2 M). The flask was capped with a rubber septum, flushed with argon, and cooled in an ice bath (0 ºC, 10 min). Trifluoroacetic acid (0.4 mL, giving a 10% solution in DCM) was added. The argon line was removed, and the mixture was stirred (3 h, approximately 30% conversion as measured by LC/MS). Additional trifluoroacetic acid (0.2 mL, giving a 15% solution in DCM) was added. The mixture was stirred (additional 1.5 h, approximately 75% conversion by LC/MS and trace amounts of Pbf-deprotected material), and diluted with toluene (5 mL). Volatiles

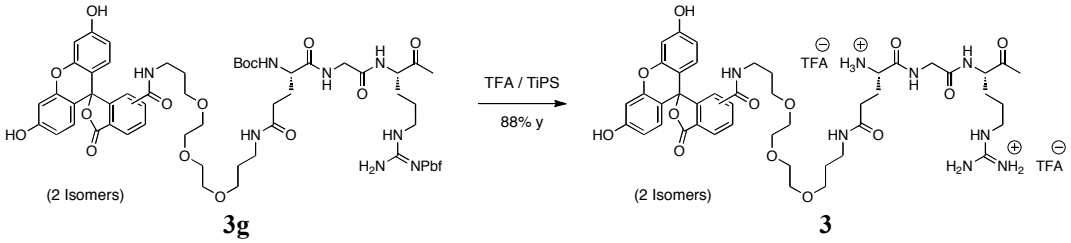
were removed under reduced pressure (2 x toluene azeotrope), and the crude material (approximately 70% conversion by ^{1}H NMR) was used directly for the next step. Retention of stereochemistry was shown by coupling amine **3f** to each enantiomer of Boc–Phe–ONHS; each produced a single diastereomer that could be readily distinguished by ^{1}H NMR.

^{1}H NMR (CD_3OD, 500 MHz, major signals) δ 4.19 (dd, J_1 = 8.1 Hz, J_2 = 4.1 Hz, 1H, Arg-α), 3.27–3.20 (m, 2H, Arg-δ), 3.00 (s, 2H, Pbf), 2.56 (s, 3H, Pbf), 2.50 (s, 3H, Pbf), 2.21 (s, 3H, Me), 2.08 (s, 3H, Pbf), 2.05–1.97 (m, 1H, Arg-β/Arg-γ), 1.84–1.47 (m, 3H, Arg-β, Arg-γ), 1.45 (s, 6H, Pbf); **LC/MS** elutes at 0.74 min, calculated for $[C_{20}H_{32}N_4O_4SH]^+$, requires m/z = 425.22, found m/z = 425.14 (ESI).



Synthesis of peptide 3g Into a flask were added 40% of crude NHS ester **1p** (0.37 mmol, 1 equiv), crude amine **3f** (70% pure, 0.740 mmol, 2 equiv), THF (3.7 mL, 0.1 M), and Hünig's base (258 μL, 1.48 mmol, 4 equiv, producing neutral pH). The mixture was stirred in the dark (2.5 h, 60% conversion by LC/MS, becoming slightly acidic). Additional Hünig's base (65 μL, 0.37 mmol, 1 equiv, producing slightly basic pH) was added. The mixture was stirred (additional 1 h, full conversion by LC/MS), diluted with THF (10 mL) and DCM (40 mL), washed with sodium hydrogen sulfate (0.1 M, 60 mL), and dried with sodium sulfate. Volatiles were removed under reduced pressure, and the crude material was purified by column chromatography (50 mL silica gel, DCM + 10–20% MeOH) to yield amide **3g** (253 mg, 0.199 mmol, 54% yield).

^{1}H NMR (CD_3OD, 500 MHz) δ 8.41 (s, 0.5H, Ar), 8.18 (dd, J_1 = 8.0 Hz, J_2 = 1.6 Hz, 0.5H, Ar), 8.12 (dd, J_1 = 8.0 Hz, J_2 = 1.4 Hz, 0.5H, Ar), 8.04 (d, J = 8.1 Hz, 0.5H, Ar), 7.63 (s, 0.5H, Ar), 7.28 (d, J = 8.0 Hz, 0.5H, Ar), 6.70–6.66 (m, 2H, Ar), 6.60 (t, J = 9.0 Hz, 2H, Ar), 6.55–6.50 (m, 2H, Ar), 4.36–4.30 (m, 1H, Arg-α), 4.00–3.95 (m, 1H, Glu-α), 3.87 (s, 2H, Gly-α), 3.64–3.34 (m, 14H, 6 x CH_2–O, CH_2–NH), 3.24–3.10 (m, 4H, CH_2–NHAr, Arg-δ), 2.95 (s, 2H, Pbf), 2.55 (s, 3H, Pbf), 2.48 (s, 3H, Pbf), 2.27 (q, J = 7.8 Hz, 2H, Glu-γ), 2.08 (s, 3H, Me), 2.05 (s, 3H, Pbf), 2.06–2.00 (m, 1H, Glu-β), 1.95–1.44 (m, 9H, Glu-β, 2 x C–CH_2–C, Arg-β, Arg-γ), 1.41 (s, 6H, Pbf), 1.38 (s, 9H, Boc); **^{13}C NMR** ($CDCl_3$, 125 MHz) δ 208.47, 175.18, 174.80, 171.61, 170.58, 168.11, 167.83, 161.60, 159.80, 158.02, 157.95, 154.04, 142.26, 139.31, 137.90, 135.40, 134.40, 133.46, 130.18, 128.77, 126.25, 126.00, 125.77, 124.88, 124.00, 118.39, 113.91, 111.00, 110.92, 103.63, 87.65, 80.86, 71.45, 71.24, 71.15, 71.04, 70.89, 70.23, 70.17, 69.86, 69.79, 59.94, 55.88, 43.93, 43.59, 41.37, 39.08, 38.97, 37.92, 37.86, 33.16, 30.28, 30.04, 28.76, 28.21, 26.81, 26.26, 19.62, 18.42, 12.54; **IR** (film, cm^{-1}) 3328, 2972, 2928, 2863, 1643, 1614, 1545, 1450, 1247, 1174, 1113; **HRMS** calculated for $[C_{68}H_{81}N_8O_{18}SH]^+$, requires m/z = 1271.5541, found m/z = 1271.5535 (ESI); **TLC** DCM + 15% MeOH, UV/permanganate, R_f = 0.32.



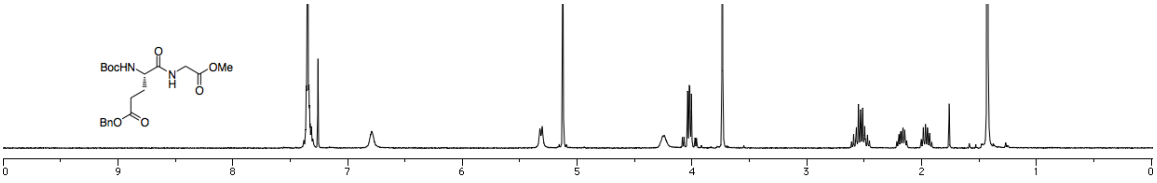
Synthesis of ketone 3 Into a flask were added protected ketone **3g** (127 mg, 1.00 mmol, 1 equiv) and a mixture of TFA, TiPS, and water (38:1:1, 3 mL, 0.3 M). The mixture was stirred in the dark (3.5 h) and diluted with toluene (5 mL). Volatiles were removed under reduced pressure (2 x toluene azeotrope). The crude mixture was diluted with deuterium oxide (3 mL) and deuterated methanol (1 mL) and washed with deuterated chloroform (2 x 2 mL). The aqueous phase, which contained mostly pure ketone **3**, which was purified by semi-preparative RP-HPLC (C_{18} column, water + 0.1% TFA with 15–40% MeCN over 30 min, product isomers elute at 12.1 and 12.5 min) to yield

pure ketone **3** (101 mg, 0.0881 mmol, 88% yield) as a mixture (approximately 1:1) of regioisomers at fluorescein and as a yellow solid. The regioisomeric ratio was measured by ^{1}H NMR. ^{1}H NMR signals were assigned by ^{1}H–^{1}H COSY. Material was stored at -20 ºC as an aqueous solution.

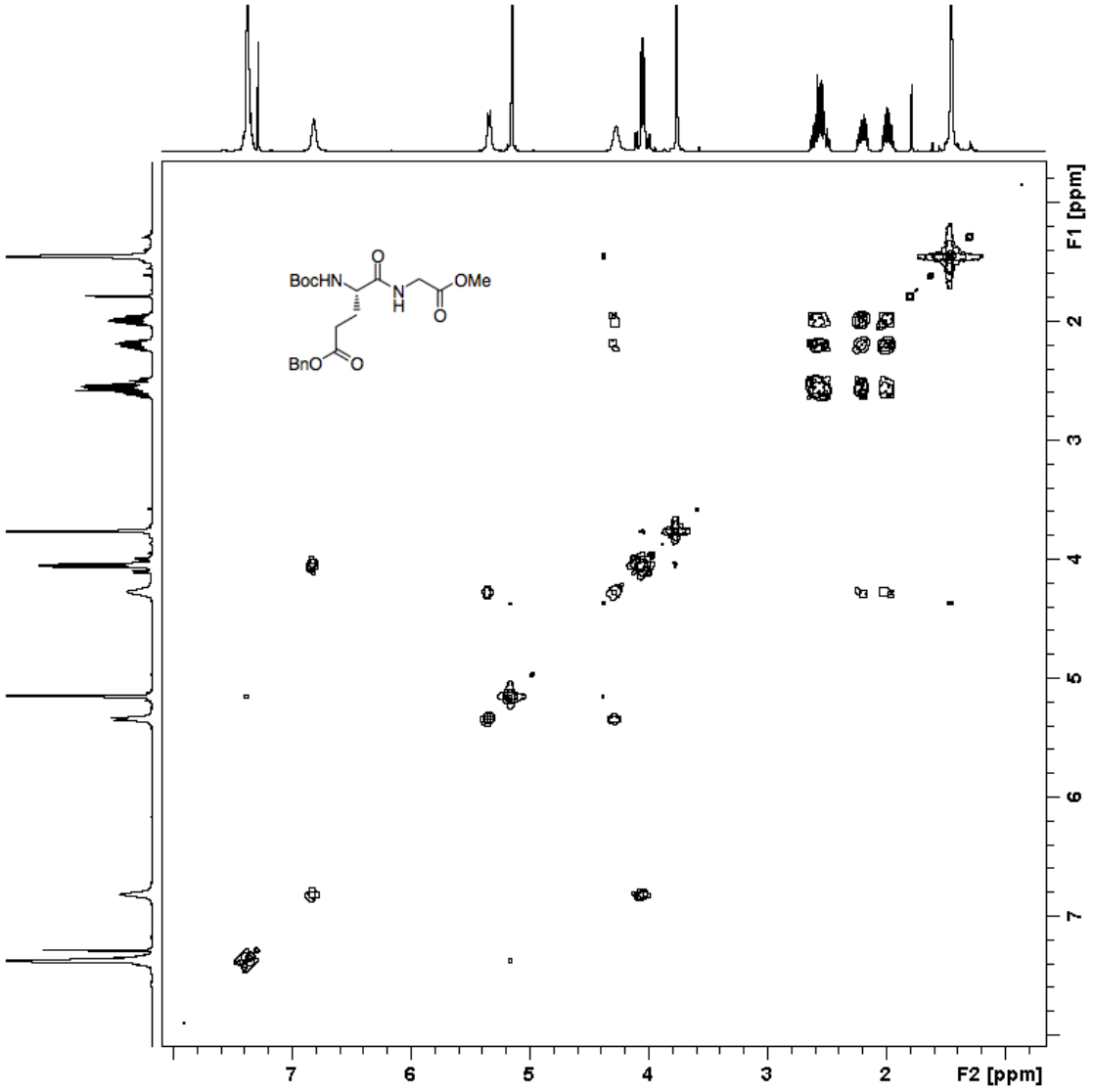
^{1}H NMR (D_2O, 400 MHz) δ 8.44 (d, J = 1.3 Hz, 0.5H, fluor), 8.15 (d, J = 1.2 Hz, 0.5H, fluor), 8.00 (d, J = 8.3 Hz, 0.5H, fluor), 7.96 (d, J = 8.9 Hz, 0.5H, fluor), 7.43 (s, 0.5H, fluor), 7.19 (d, J = 7.3 Hz, 0.5H, fluor), 7.00 (d, J = 9.1 Hz,1H, fluor), 6.92 (d, J = 9.0 Hz, 1H, fluor), 6.84–6.80 (m, 2H, fluor), 6.74 (dd, J_1 = 9.0 Hz, J_2 = 2.2 Hz, 1H, Ar), 6.67 (dd, J_1 = 9.4 Hz, J_2 = 2.1 Hz, 1H, fluor), 4.36–4.32 (m, 1H, Arg-α), 4.06–4.01 (m, 1H, Glu-α), 4.00 (d, J = 17.1 Hz, 1H, Gly-α), 3.94 (d, J = 16.7 Hz, 1H, Gly-α), 3.59–3.04 (m, 18H, 6 x CH_2–O, Arg-δ, 2 x CH_2–NHCO), 2.38–2.30 (m, 2H, Glu-γ), 2.13 (s, 3H, Me), 2.12–2.06 (m, 2H, Glu-β), 1.90–1.46 (m, 8H, Arg-β, Arg-γ, 2 x C–CH_2–C); **^{13}C NMR** (D_2O + 1.5% MeOH, 125 MHz) δ 211.61, 174.27, 174.25, 171.22, 170.31, 168.69, 168.39, 168.21, 167.87, 167.24, 163.05 (q, J = 35.6 Hz), 157.60, 157.33, 157.22, 141.24, 139.75, 139.13, 136.79, 132.79, 132.07, 131.97, 131.84, 130.59, 130.16, 129.87, 129.53, 129.09, 127.39, 118.73, 118.28, 116.26 (q, J = 291.7 Hz), 114.97, 114.65, 103.14, 103.14, 70.18, 70.03, 69.90, 69.86, 69.38, 69.20, 68.94, 68.90, 59.70, 53.19, 42.73, 41.11, 38.17, 37.07, 31.38, 28.96, 28.80, 27.28, 27.11, 26.72, 24.95; **IR** (film, cm^{-1}) 3297, 3077, 2941, 2876, 1659, 1634, 1593, 1540, 1197, 1176, 1131; **HRMS** calculated for $[C_{45}H_{57}N_8O_{13}H]^+$, requires m/z = 919.4196, found m/z = 919.4169 (ESI); **RP-HPLC** C_{18} column, 15–40% MeCN in water + 0.1% TFA, flow = 1.0 mL/min, monitored at 214 nm, isomers elute overlapping at 16.8 and 17.4 min.

3. Spectra

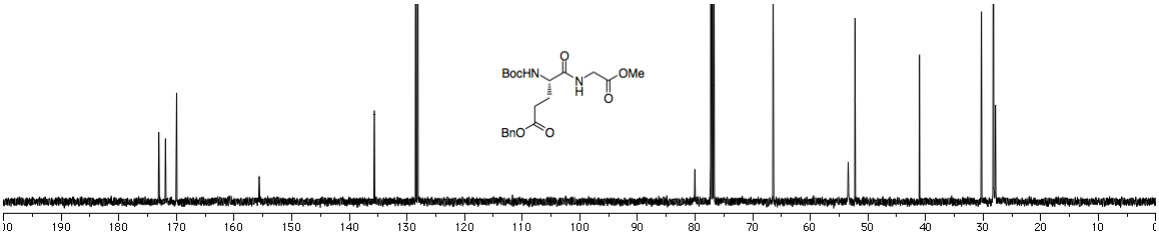
^{1}H NMR Spectrum of 1c



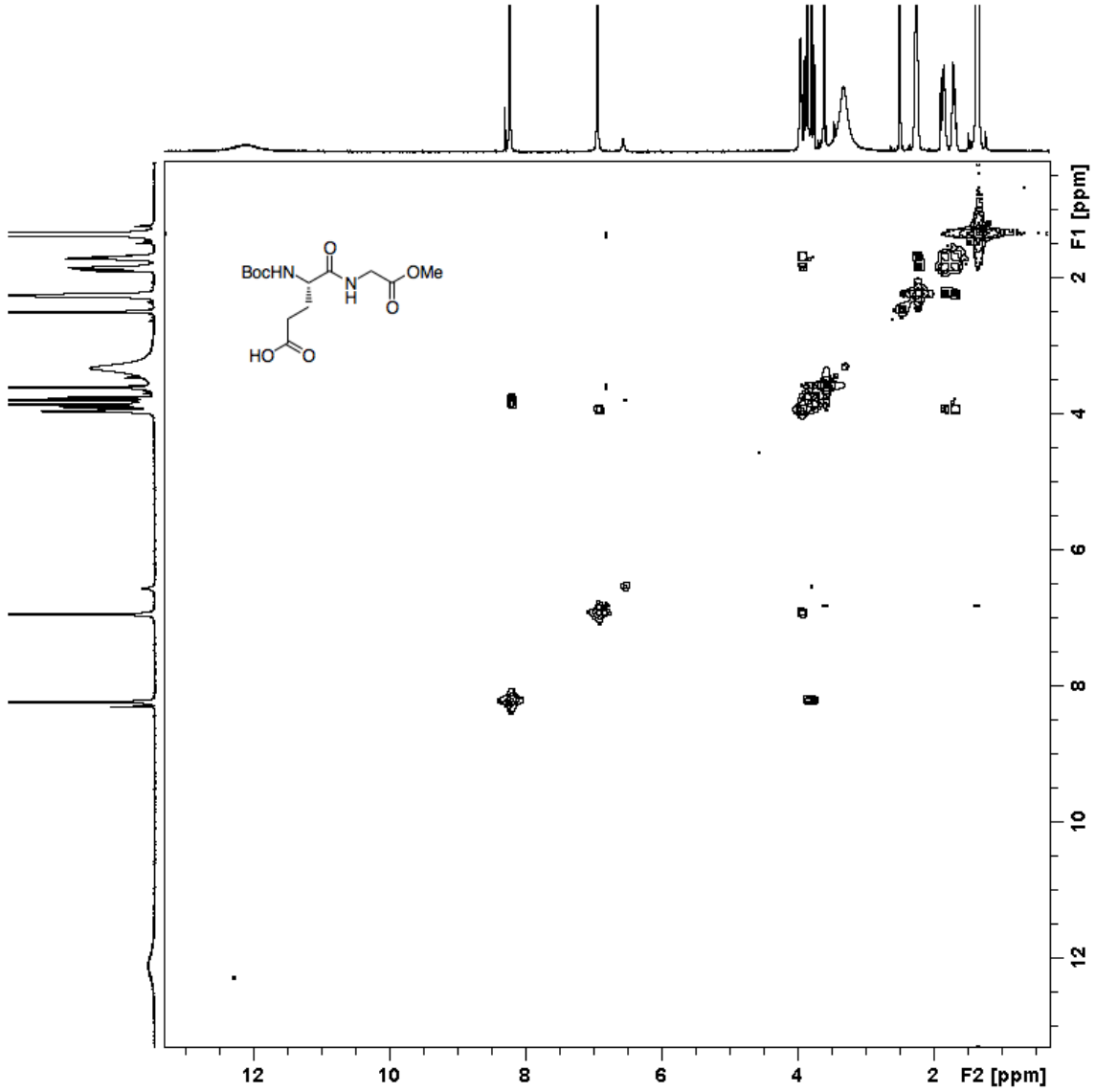
^{1}H–^{1}H COSY Spectrum of 1c



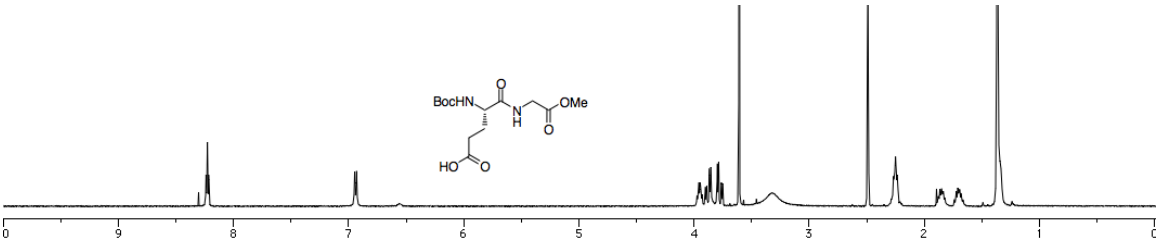
^{13}C NMR Spectrum of 1c



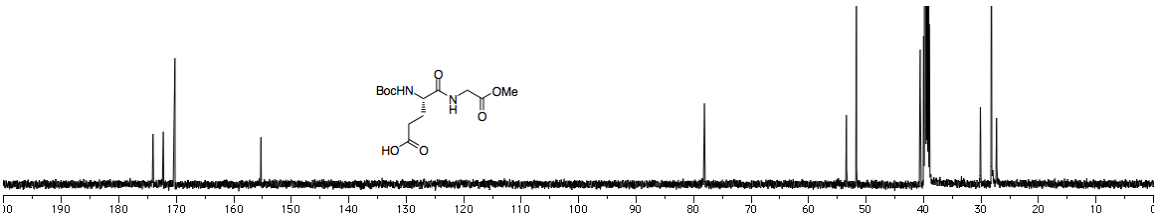
^{1}H–^{1}H COSY Spectrum of 1d (DMSO-d_6)



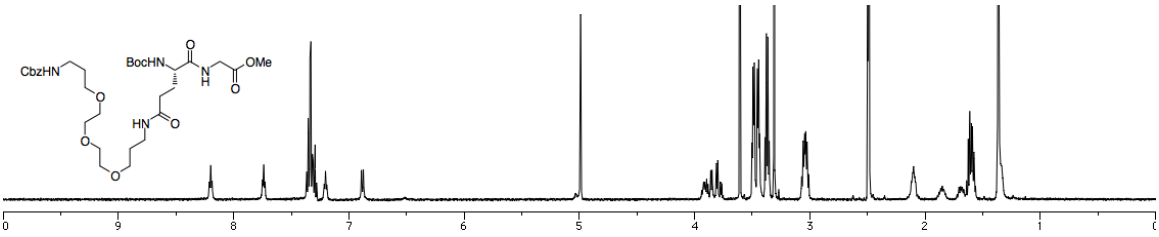
^{1}H NMR Spectrum of 1d (DMSO-d_6)



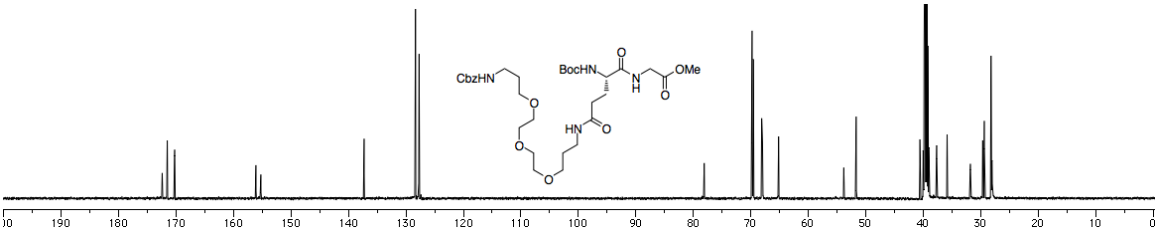
^{13}C NMR Spectrum of 1d (DMSO-d_6)



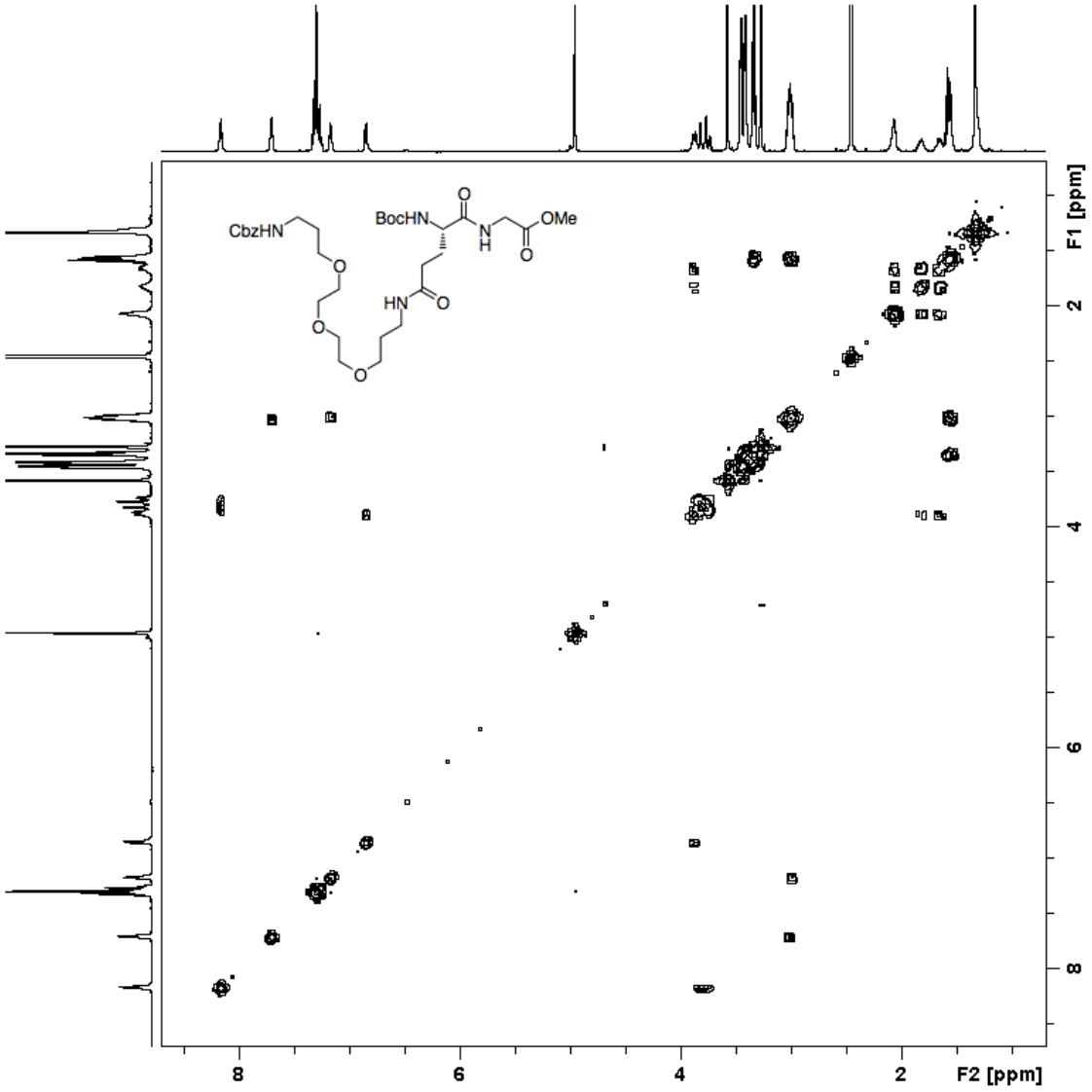
^{1}H NMR Spectrum of 1h (DMSO-d_6)



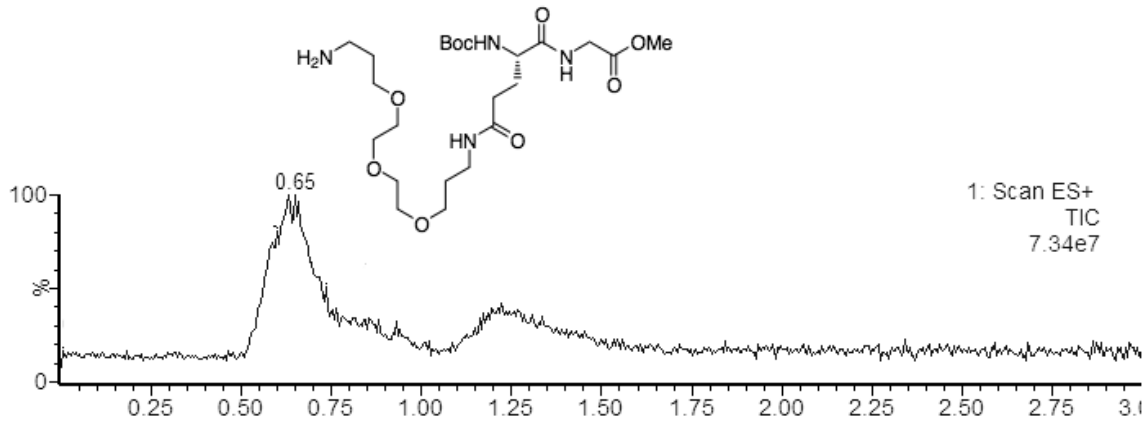
^{13}C NMR Spectrum of 1h (DMSO-d_6)



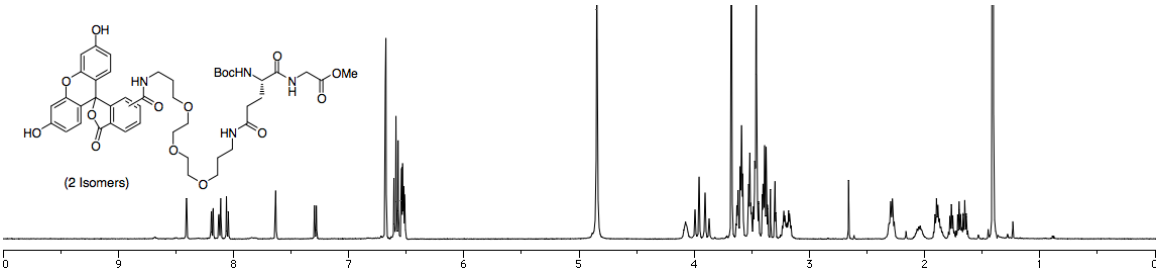
^{1}H–^{1}H COSY Spectrum of 1h (DMSO-d_6)



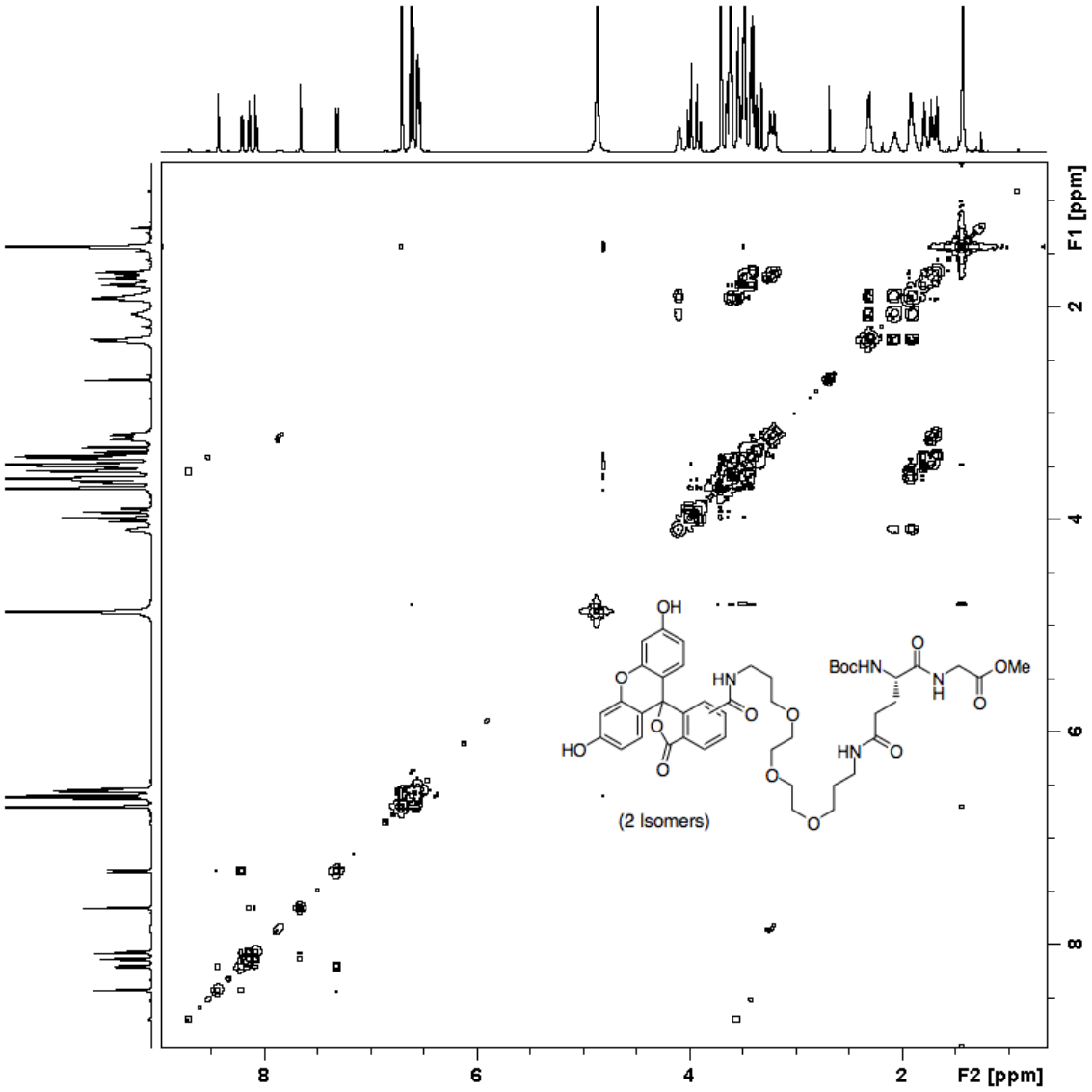
LC/MS Trace of Crude 1i



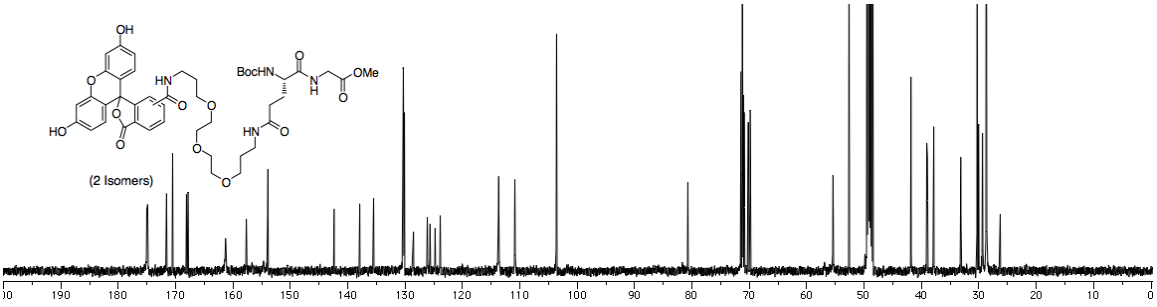
^{1}H NMR Spectrum of 1n (CD_3OD)



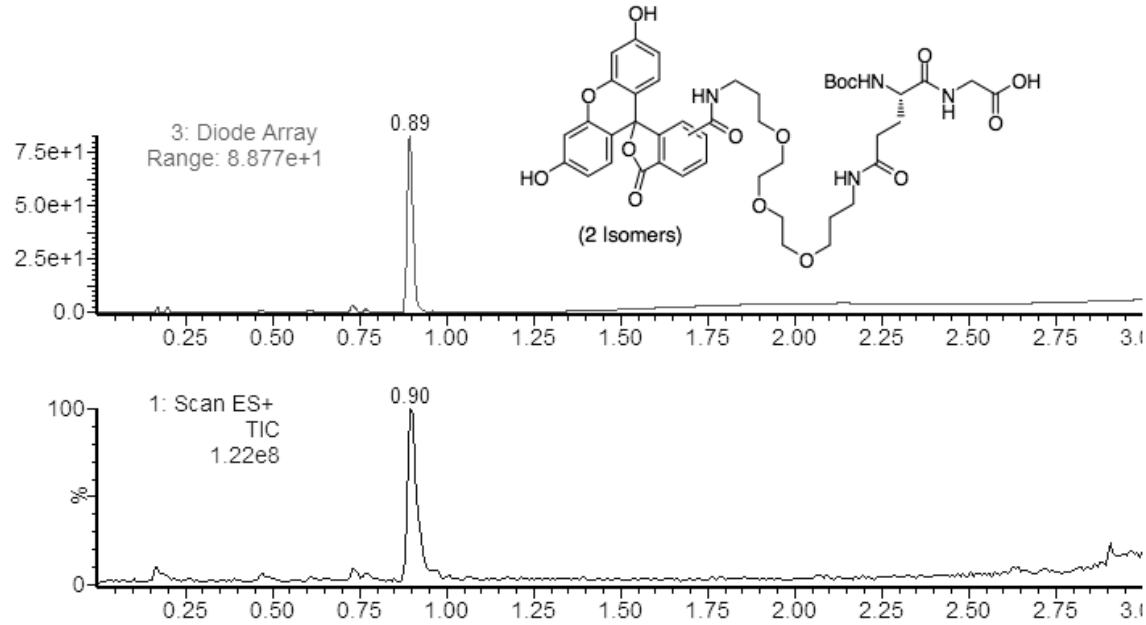
^{1}H–^{1}H COSY Spectrum of 1n (CD_3OD)



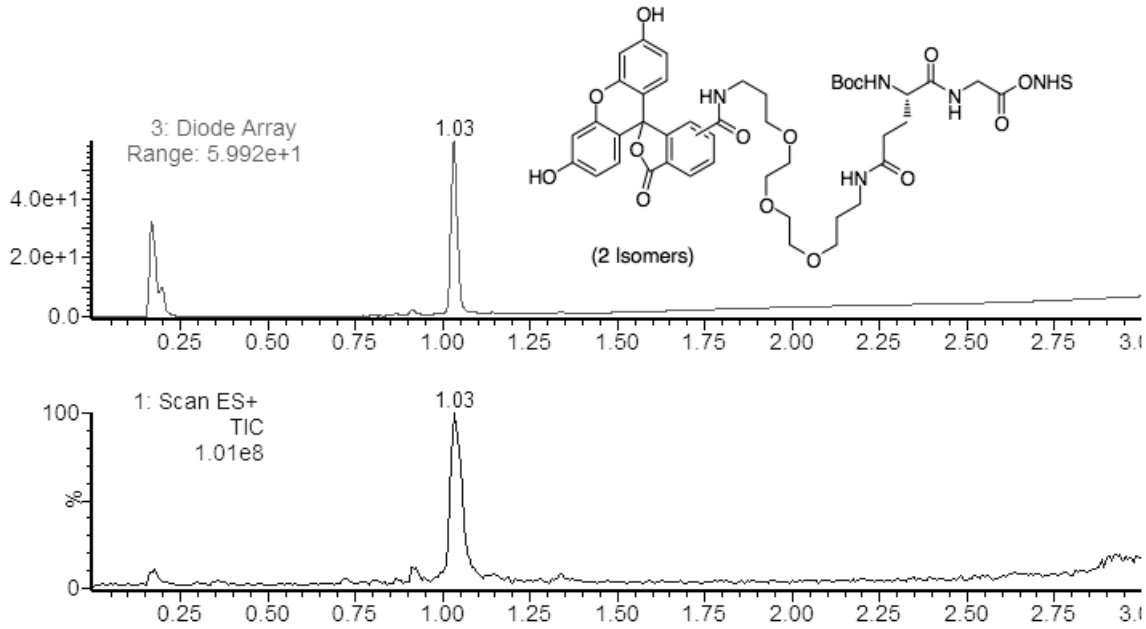
^{13}C NMR Spectrum of 1n (CD_3OD)



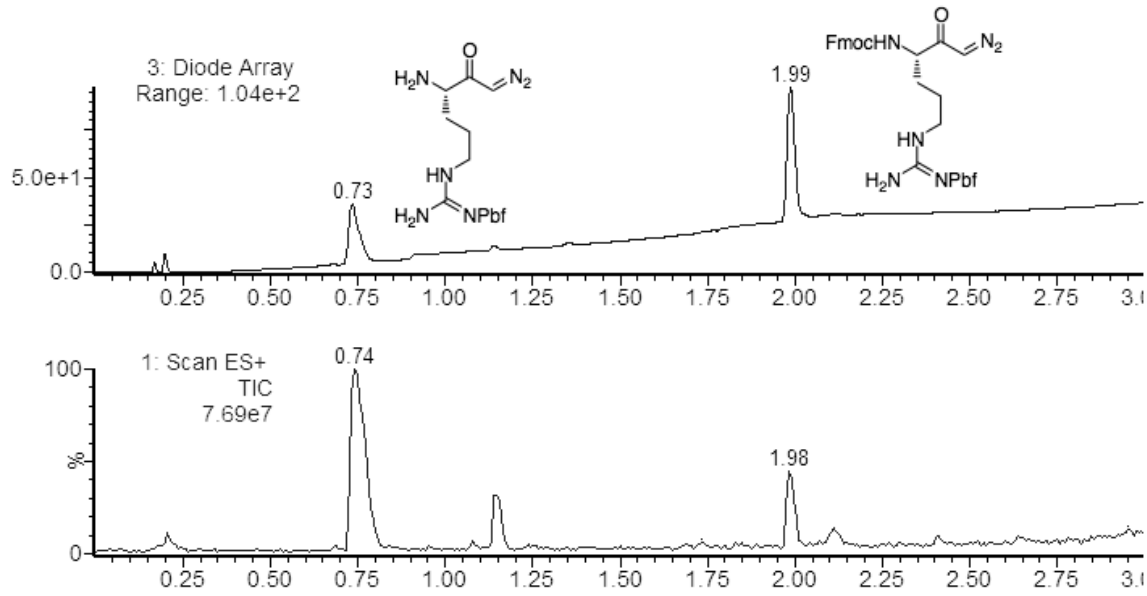
LC/MS Trace of Crude 1o



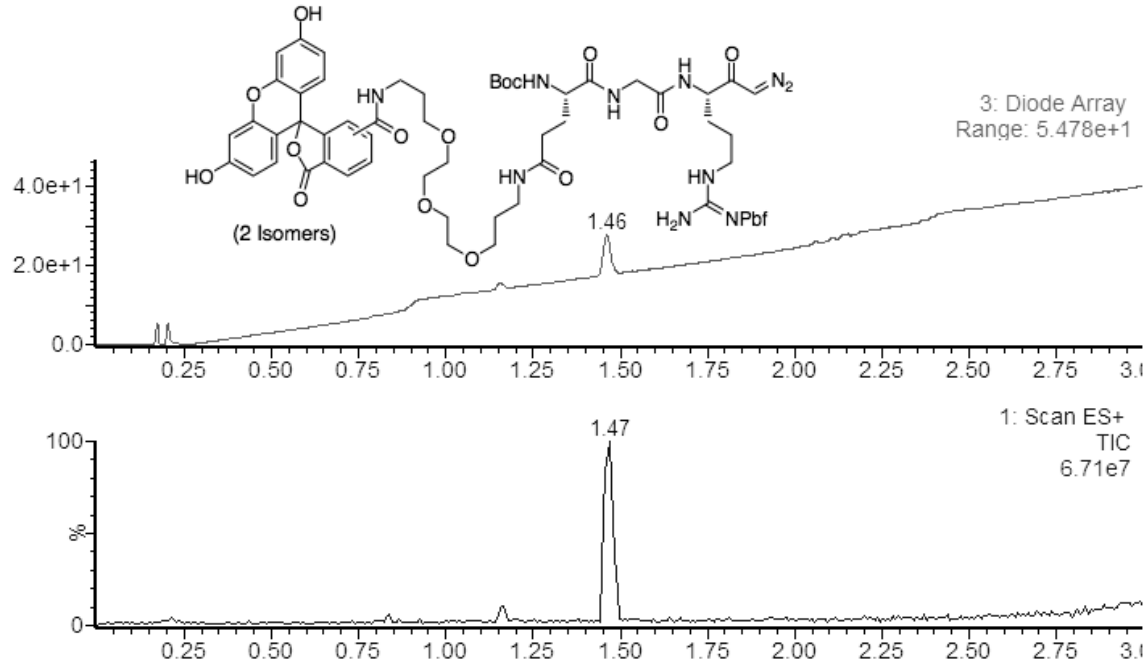
LC/MS Trace of Crude 1p



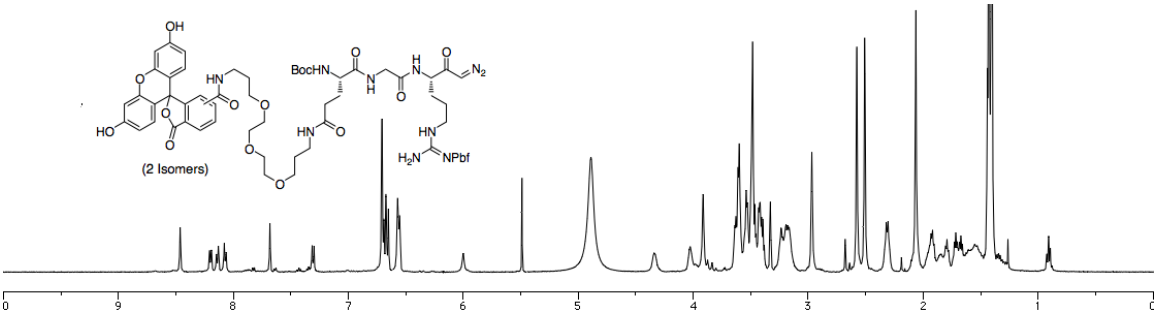
LC/MS Trace of Crude 1t



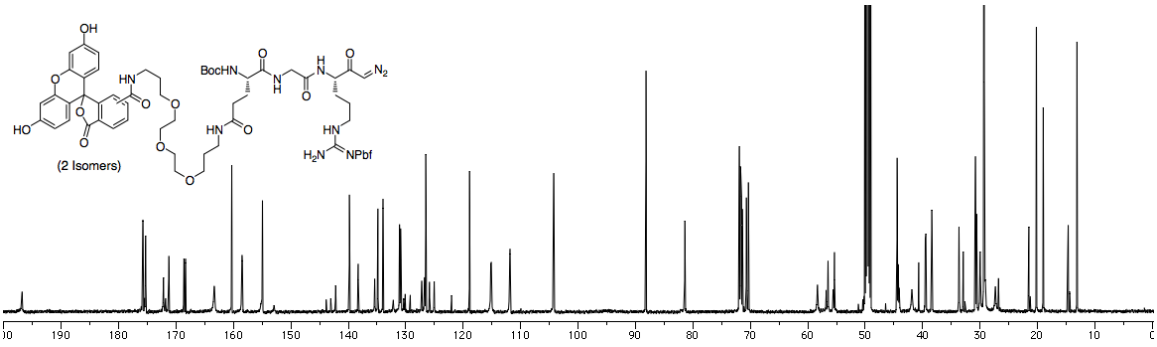
LC/MS Trace of Mostly-Pure 1u



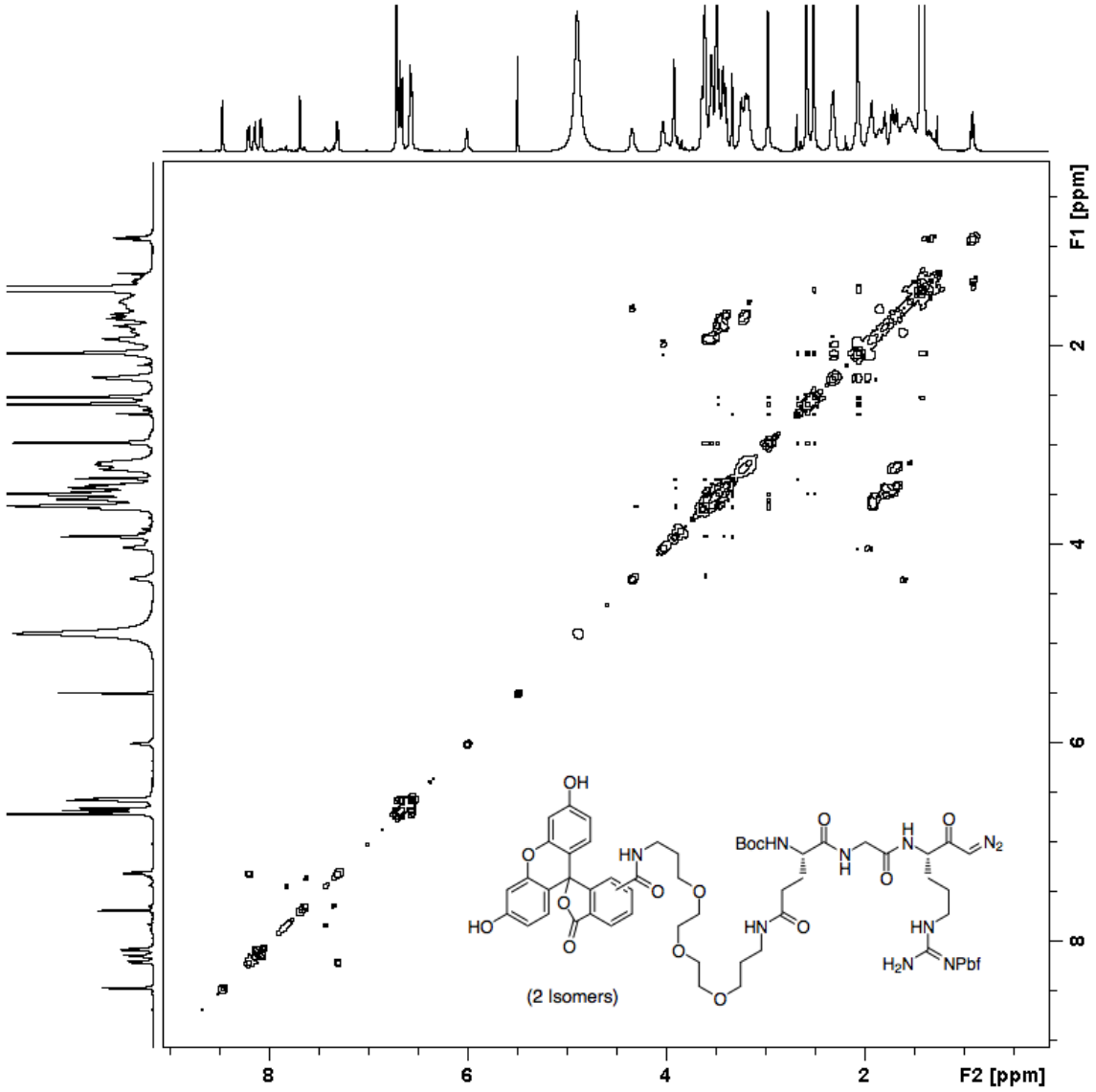
^{1}H NMR Spectrum of Mostly-Pure 1u (CD_3OD)



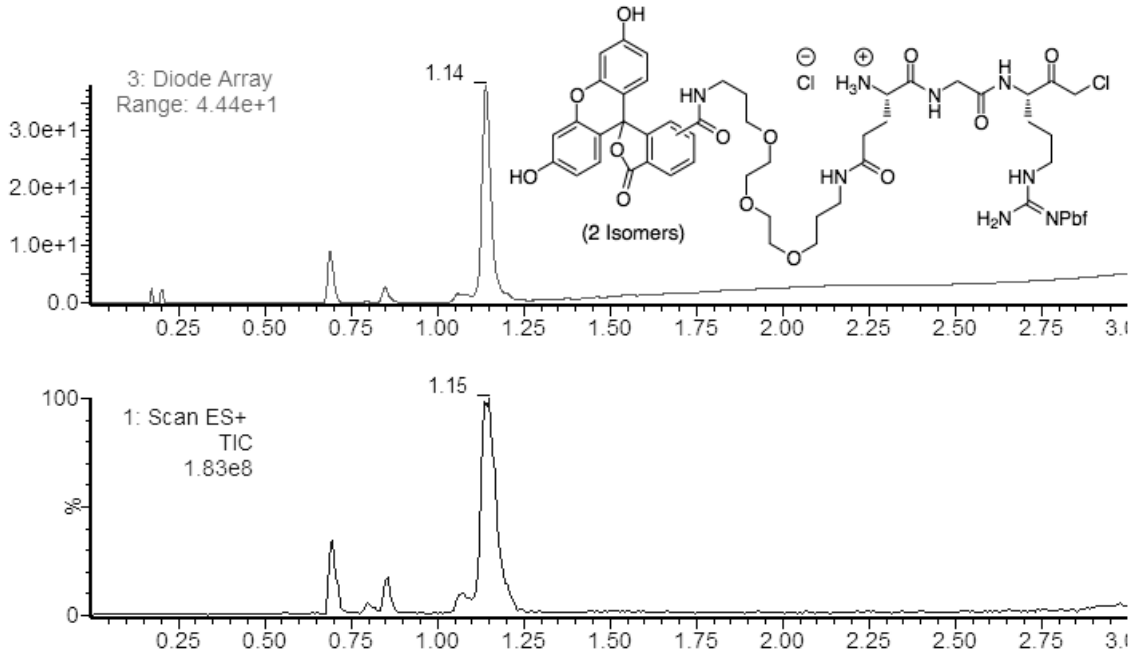
^{13}C NMR Spectrum of Mostly-Pure 1u (CD_3OD)



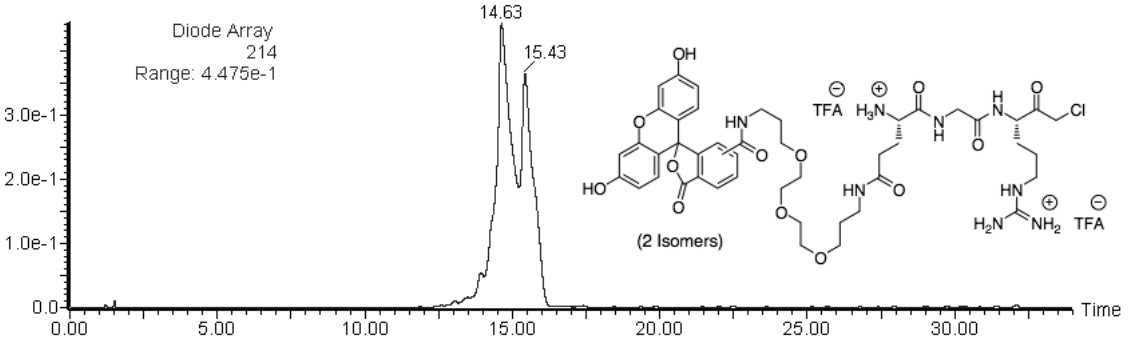
$^{1}H–^{1}H$ COSY Spectrum of Mostly-Pure 1u (CD_3OD)



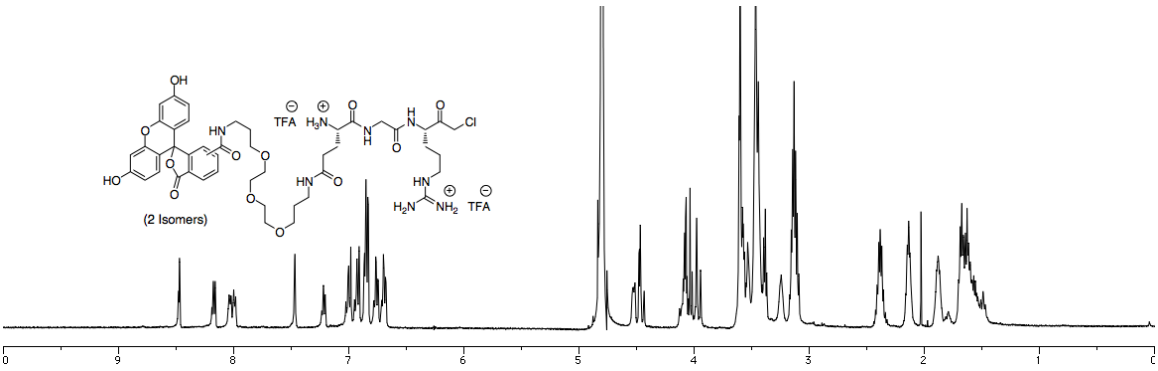
LC/MS Trace of Crude 1v



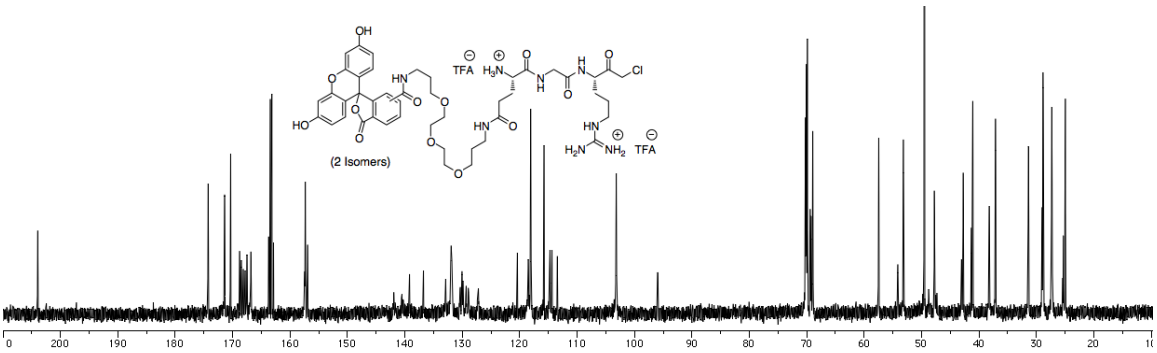
RP-HPLC Spectrum of 1



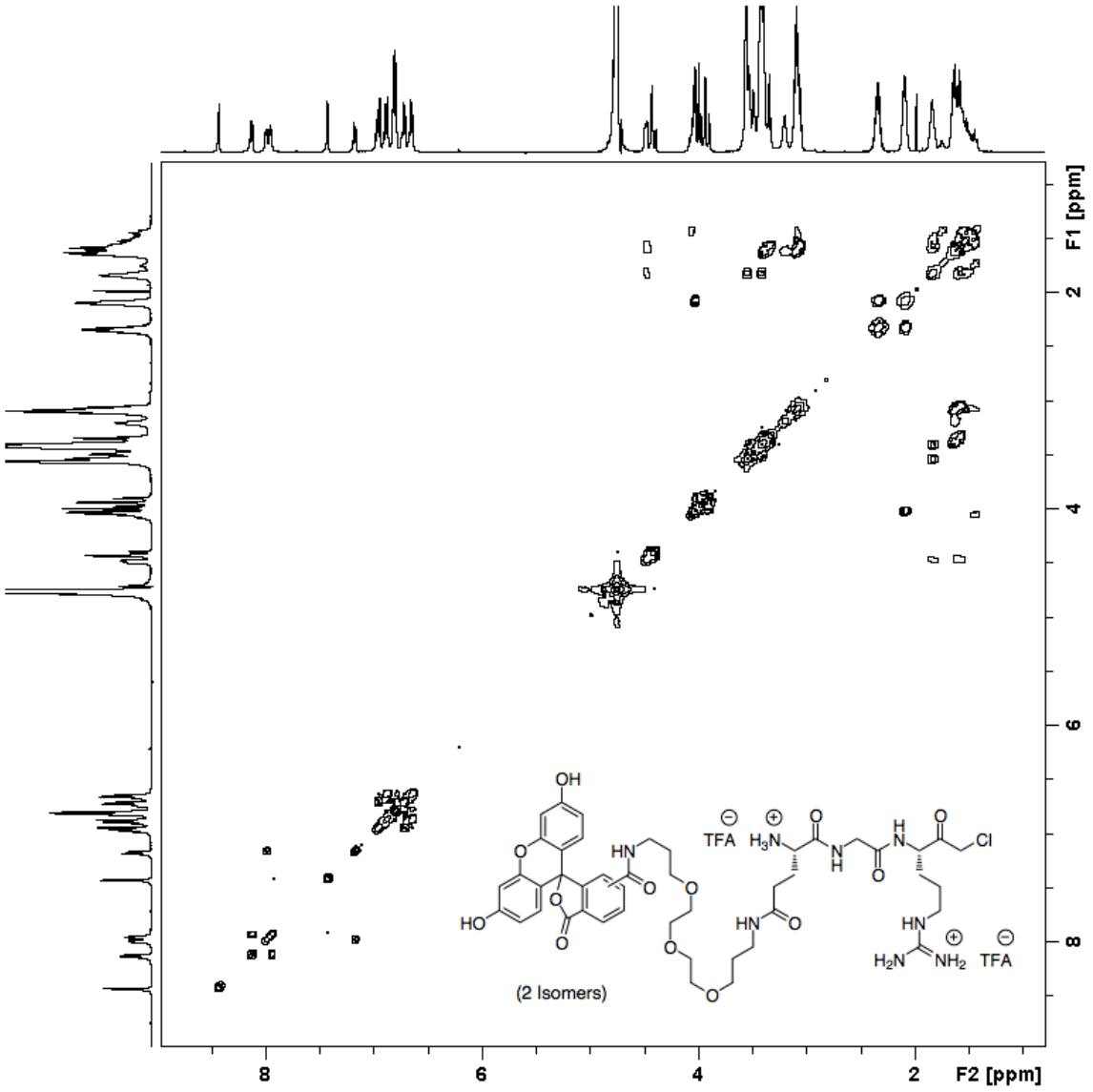
^{1}H NMR Spectrum of 1 (D_2O)



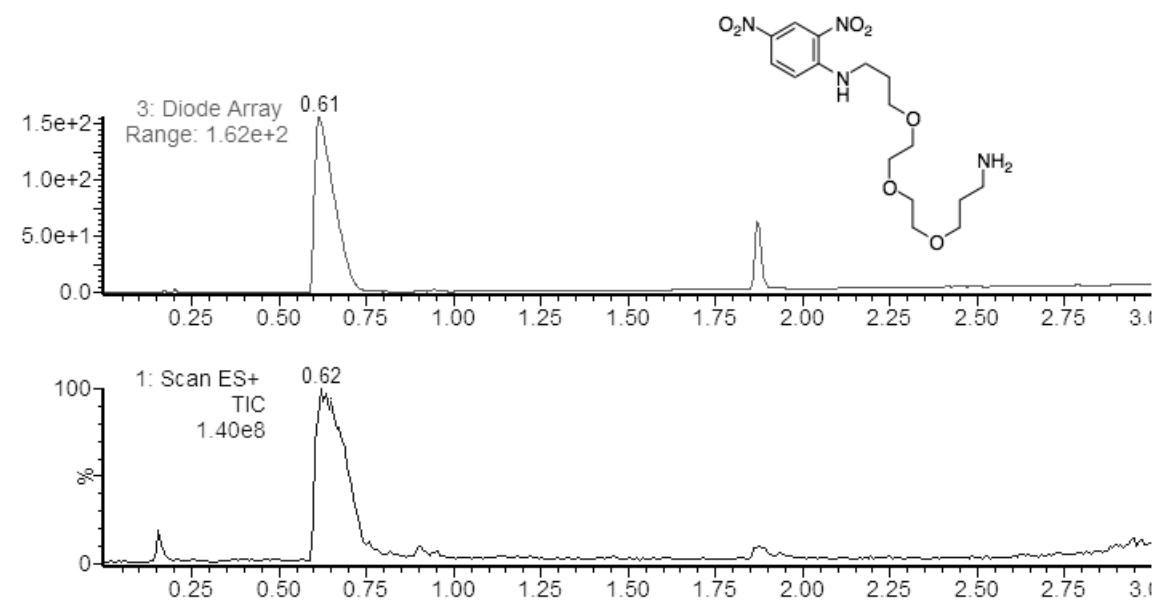
^{13}C NMR Spectrum of 1 (D_2O + 1.5% MeOH)



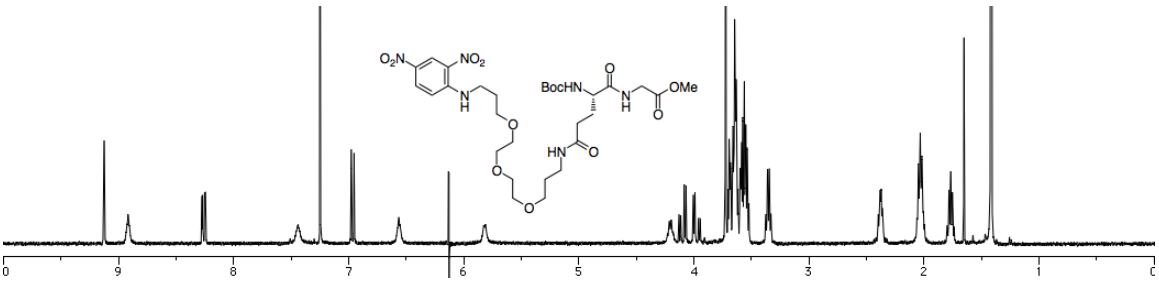
^{1}H–^{1}H COSY Spectrum of 1 (D_2O)



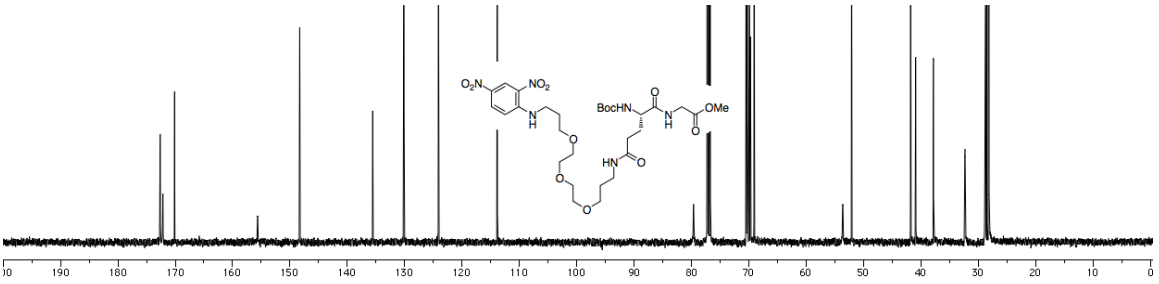
LC/MS Trace of Crude 2b



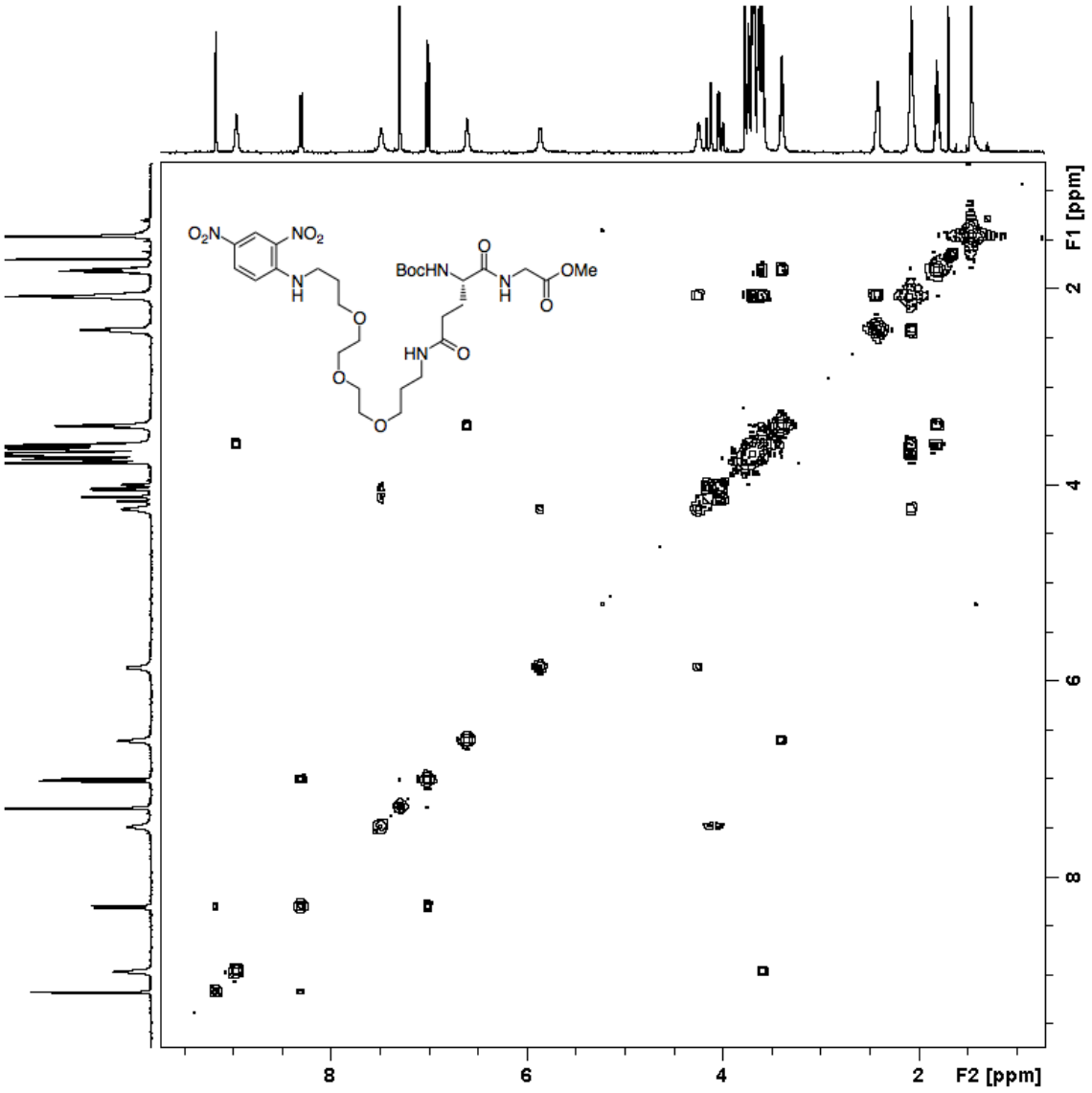
^{1}H NMR Spectrum of 2c



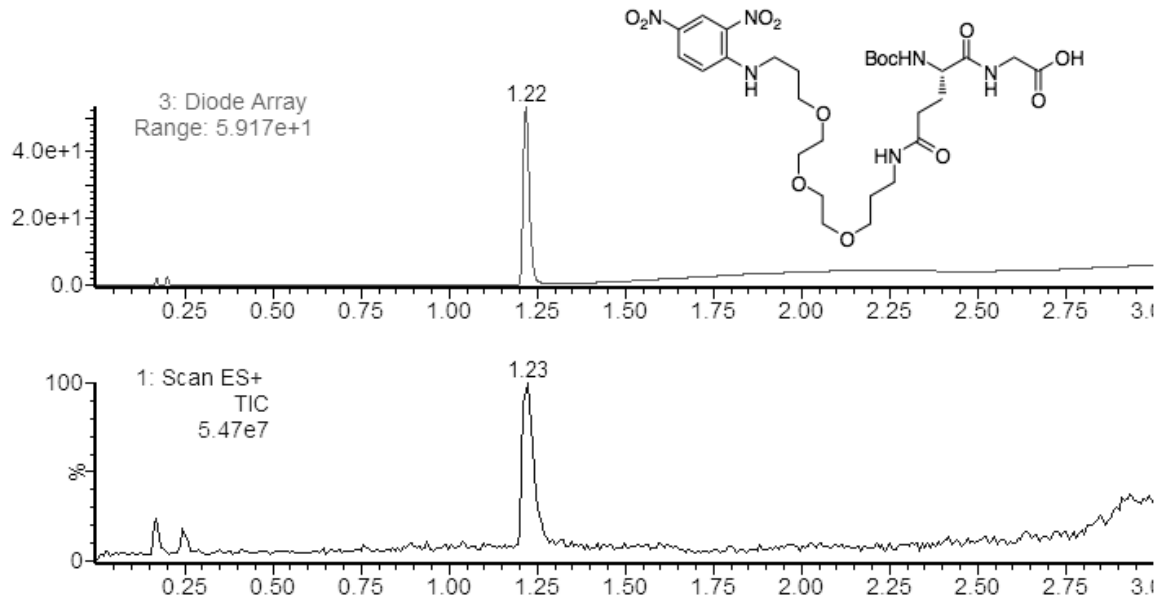
^{13}C NMR Spectrum of 2c



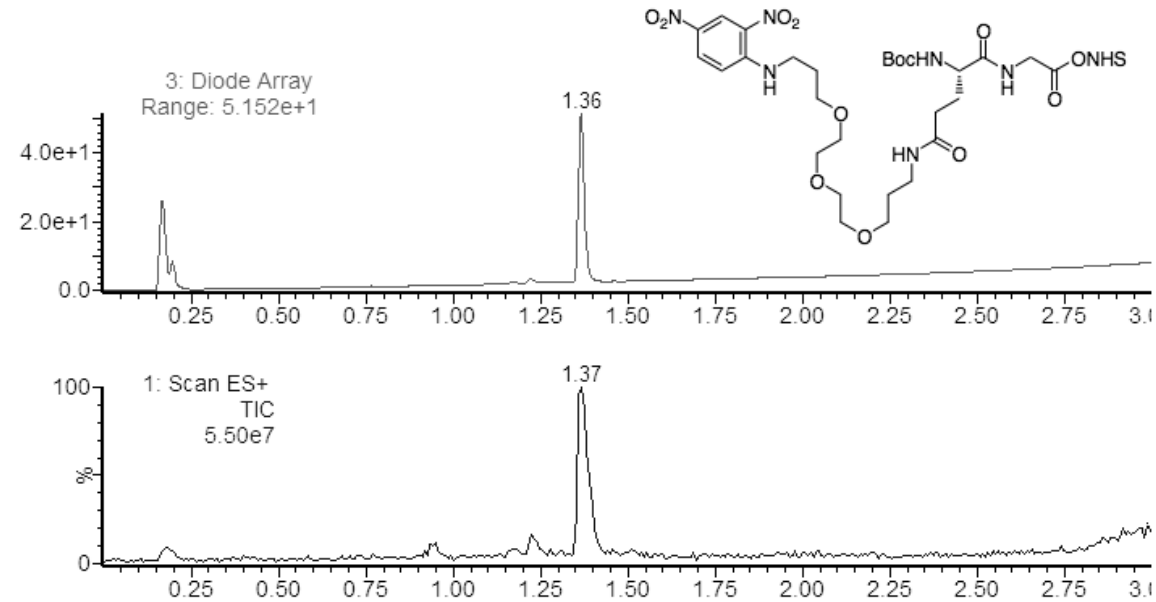
$^{1}H–^{1}H$ COSY Spectrum of 2c



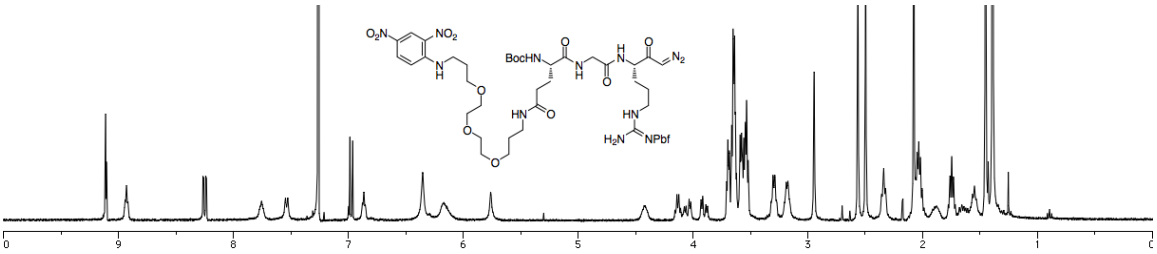
LC/MS Trace of Crude 2d



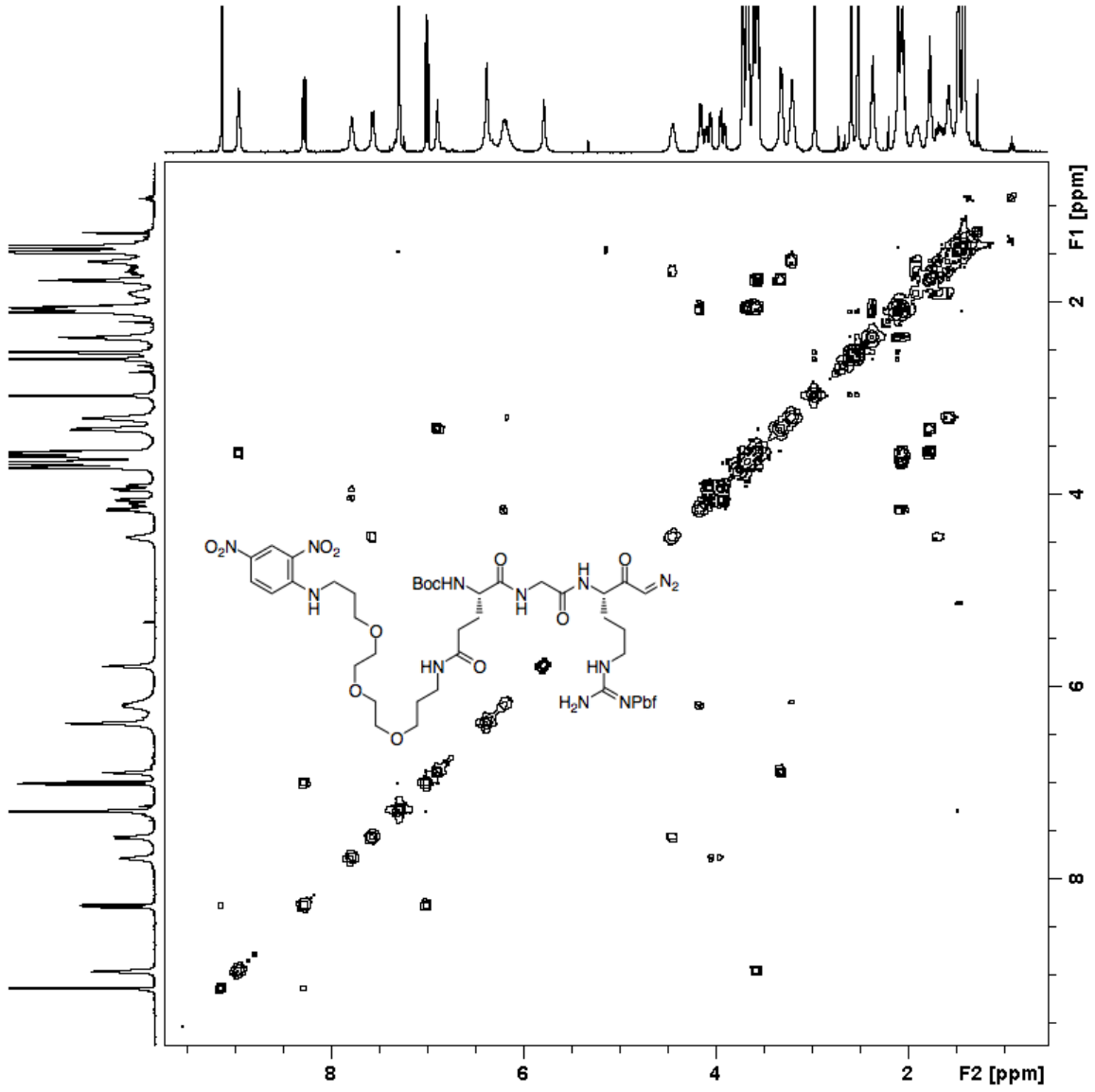
LC/MS Trace of Crude 2e



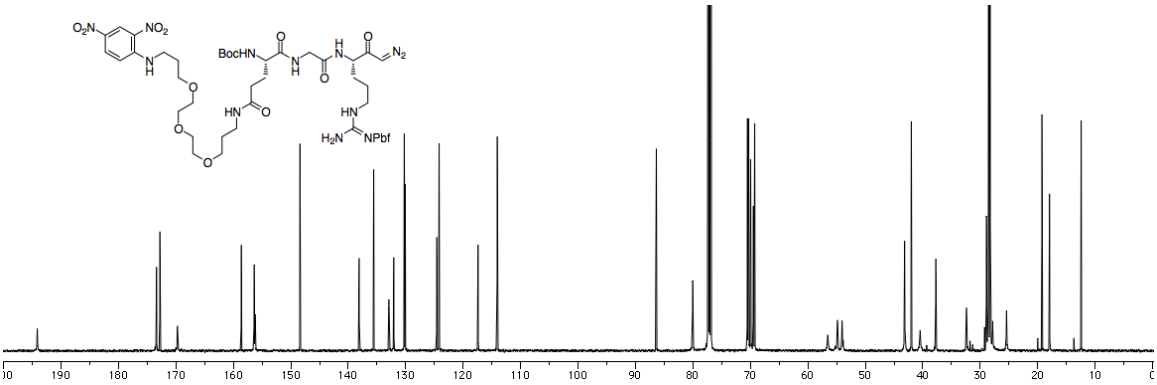
^{1}H NMR Spectrum of 2f



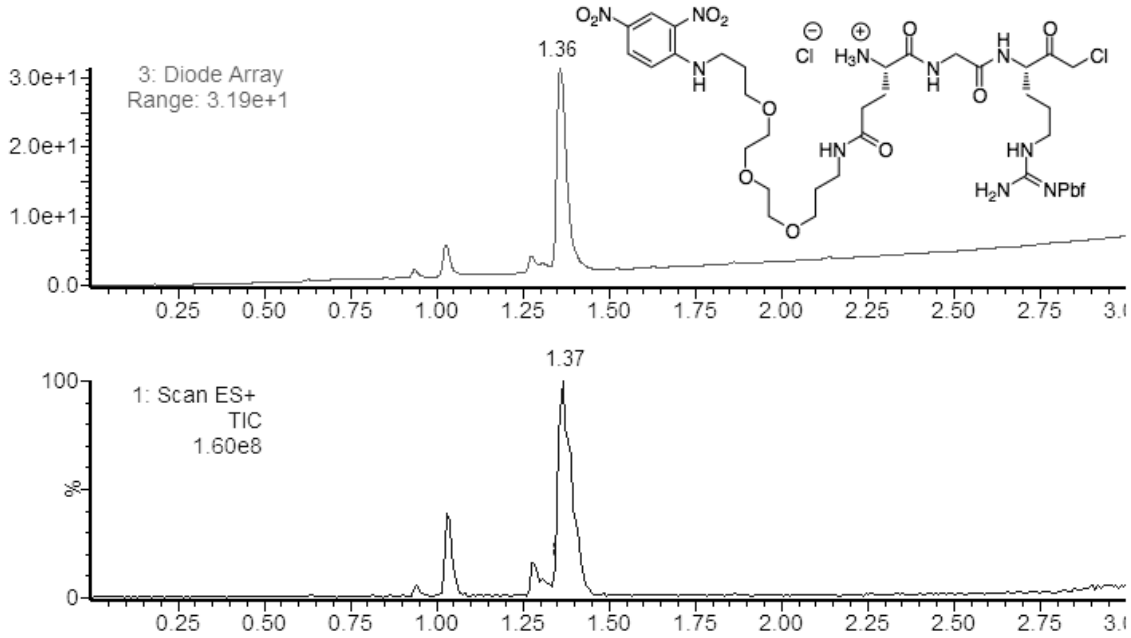
^{1}H–^{1}H COSY Spectrum of 2f



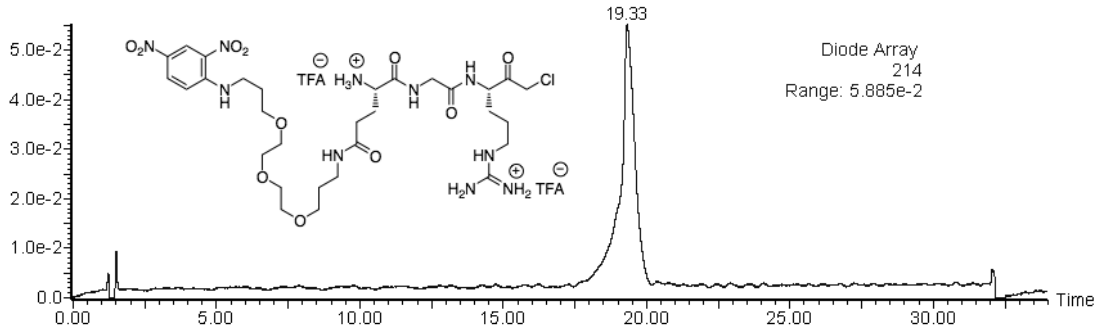
^{13}C NMR Spectrum of 2f



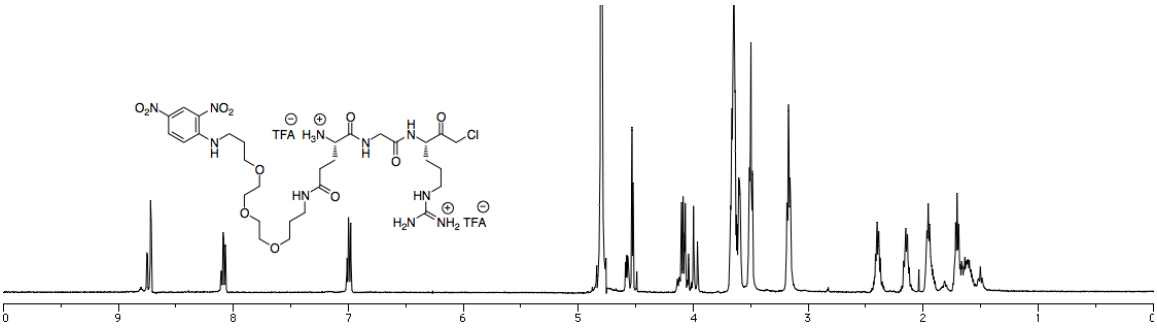
LC/MS Trace of Crude 2g



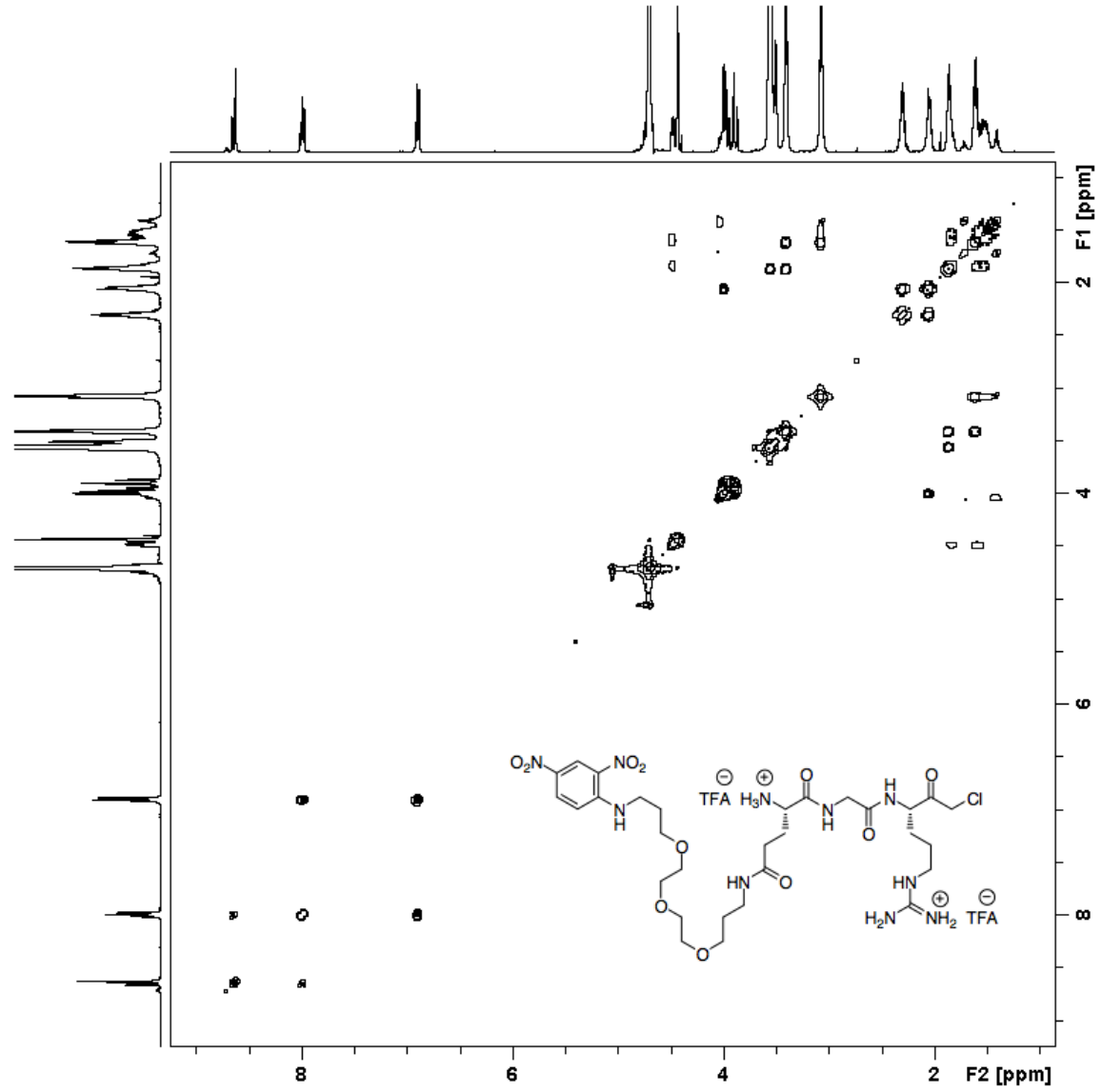
RP-HPLC Spectrum of 2



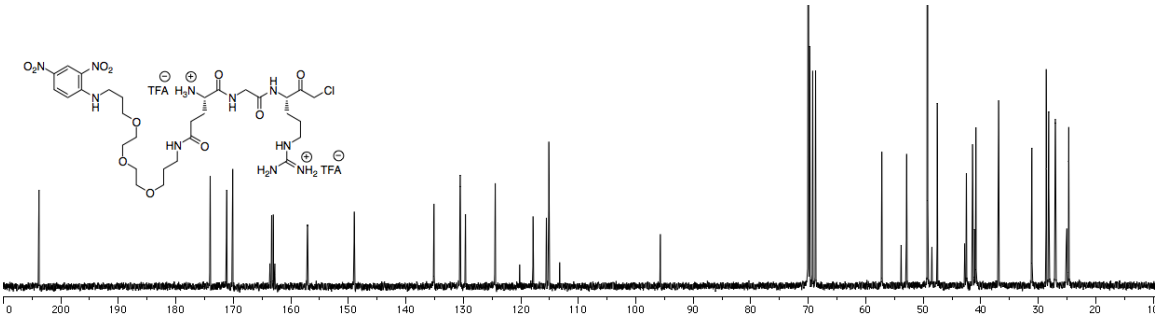
^{1}H NMR Spectrum of 2 (D_2O)



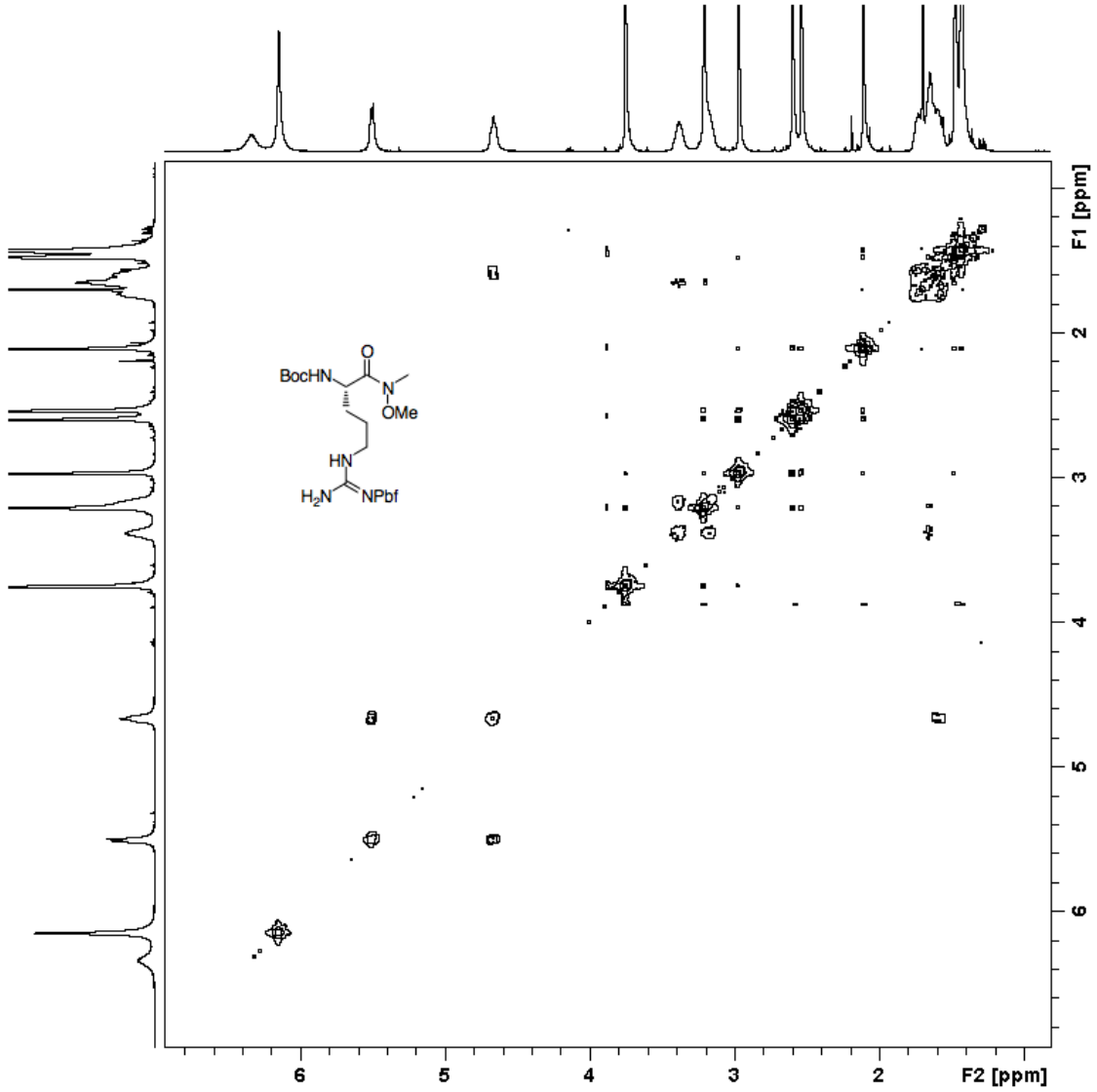
^{1}H–^{1}H COSY Spectrum of 2 (D_2O)



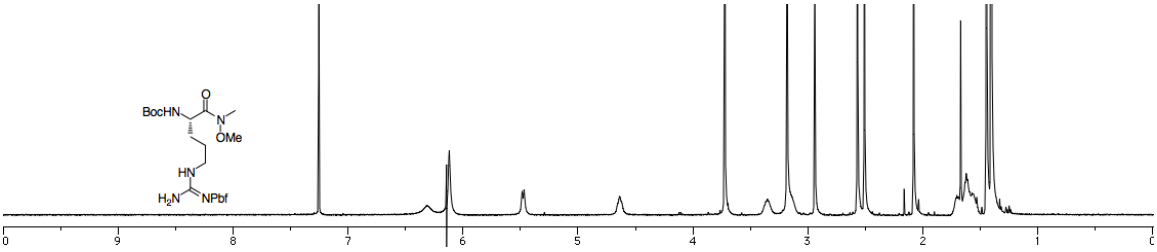
^{13}C NMR Spectrum of 2 (D_2O + 1.5% MeOH)



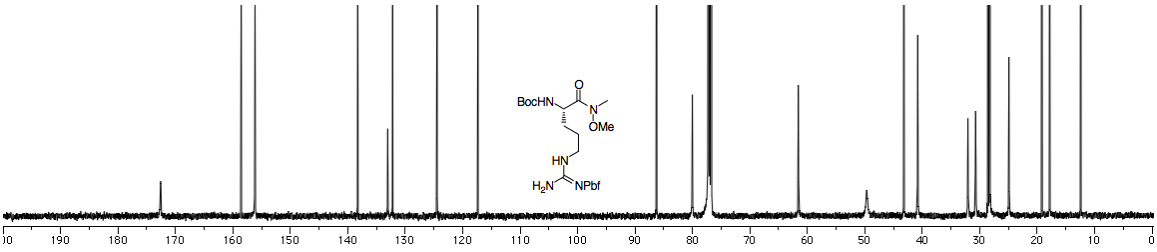
^{1}H–^{1}H COSY Spectrum of 3d



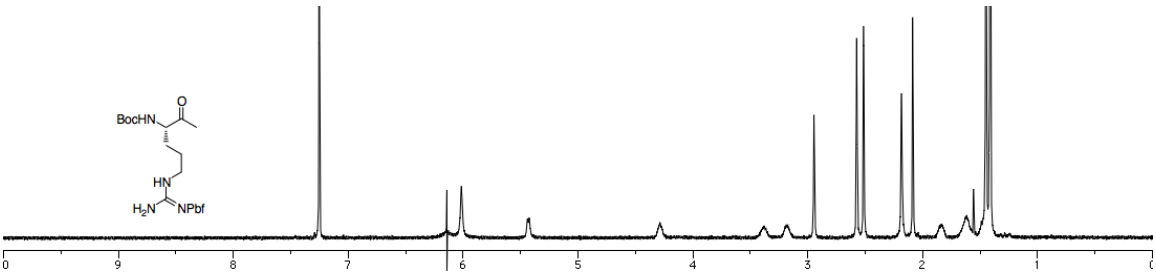
^{1}H NMR Spectrum of 3d



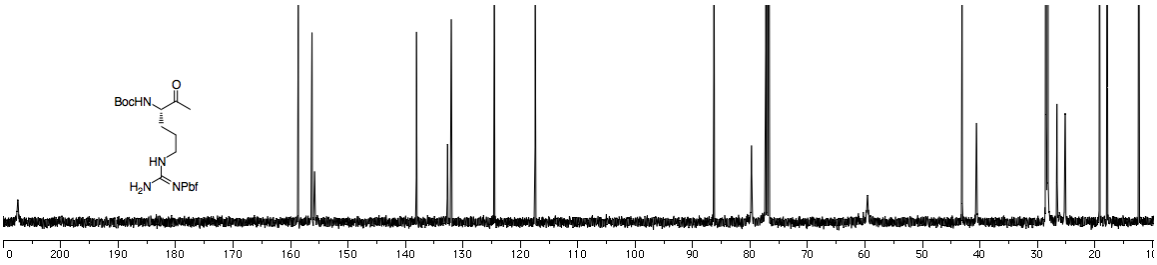
^{13}C NMR Spectrum of 3d



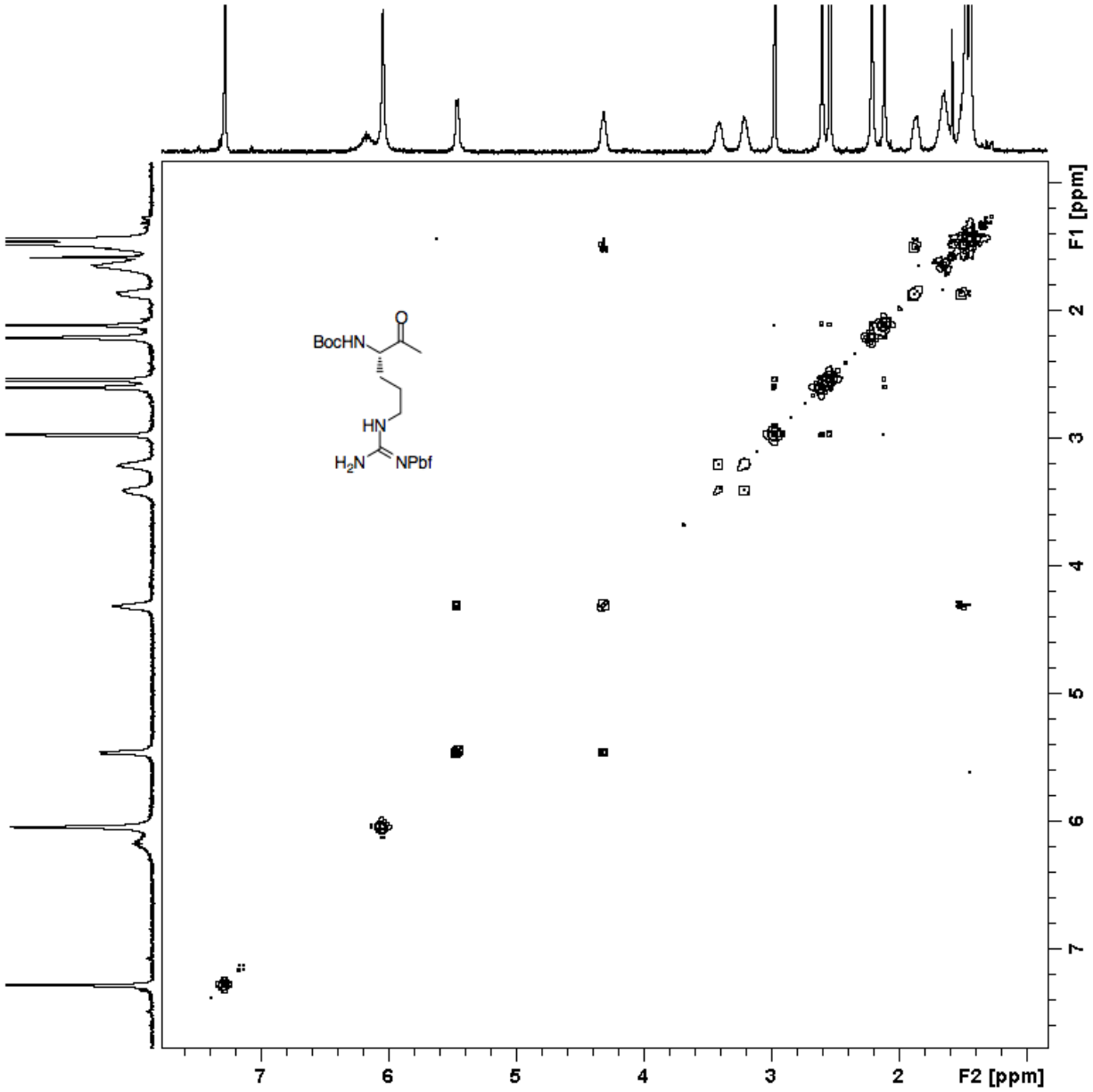
^{1}H NMR Spectrum of 3e



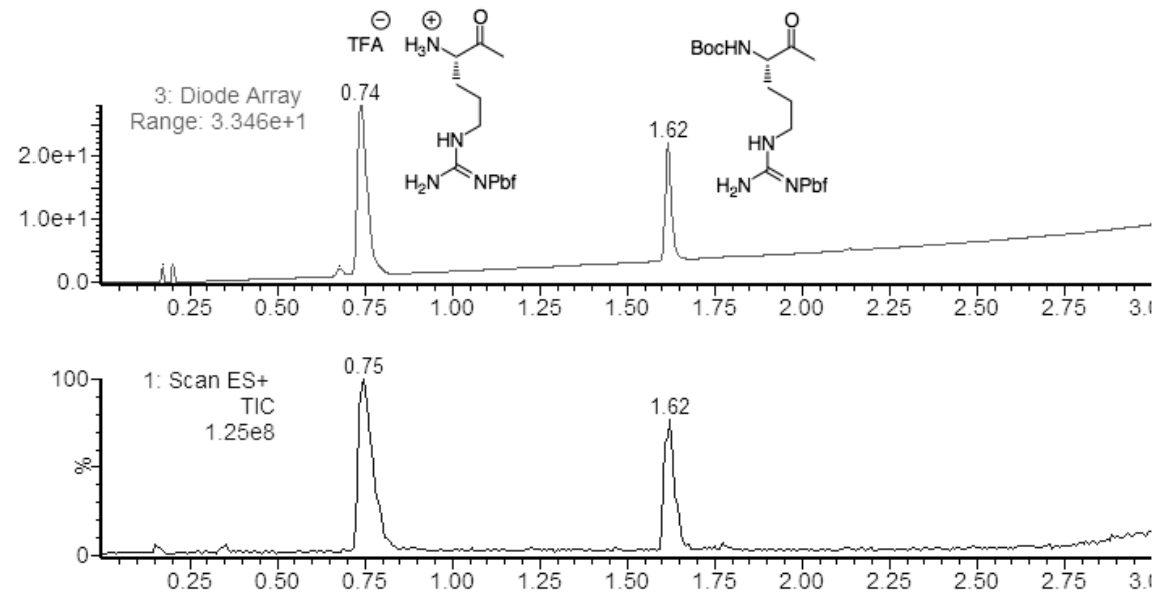
^{13}C NMR Spectrum of 3e



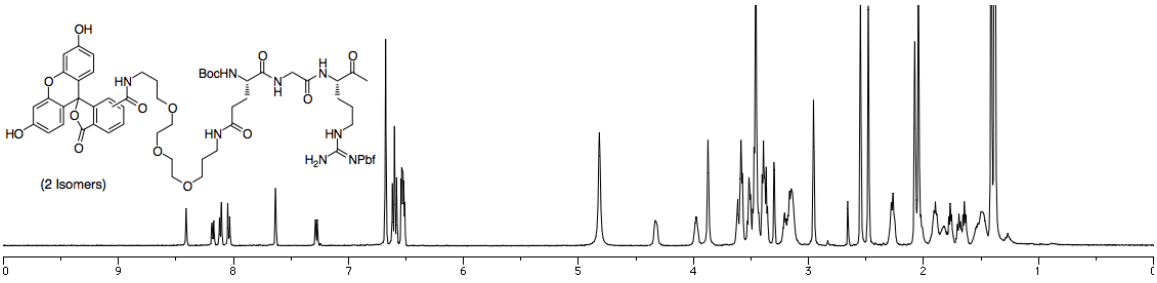
$^{1}H–^{1}H$ COSY Spectrum of 3e



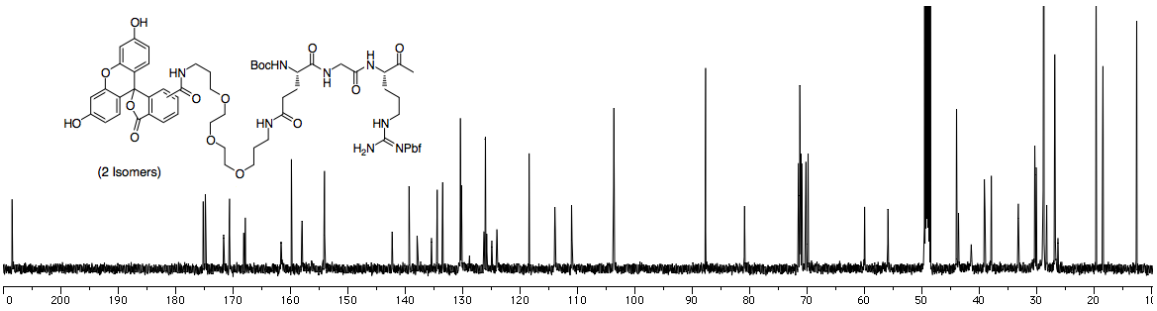
LC/MS Trace of Crude 3f



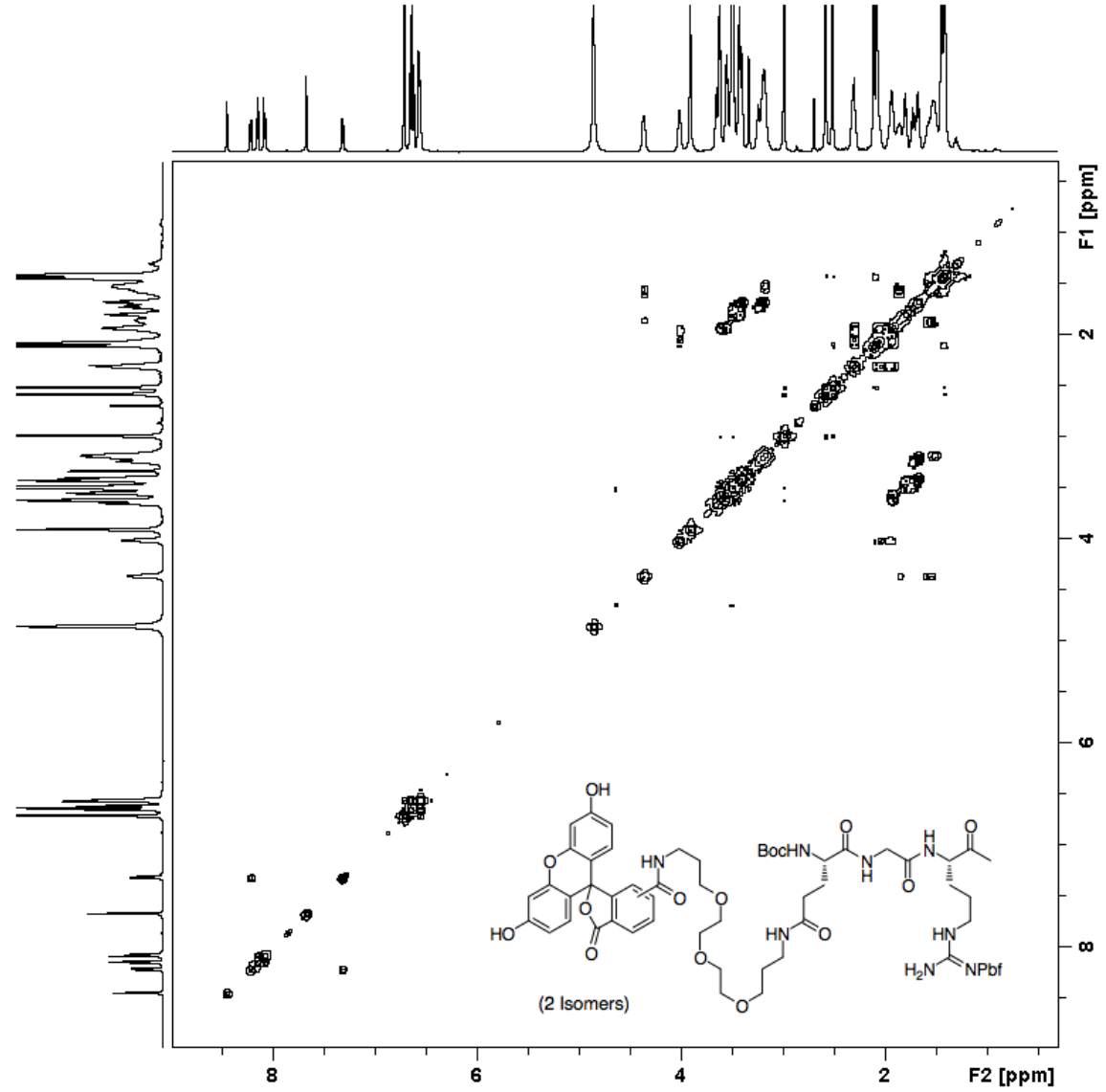
^{1}H NMR Spectrum of 3g (CD_3OD)



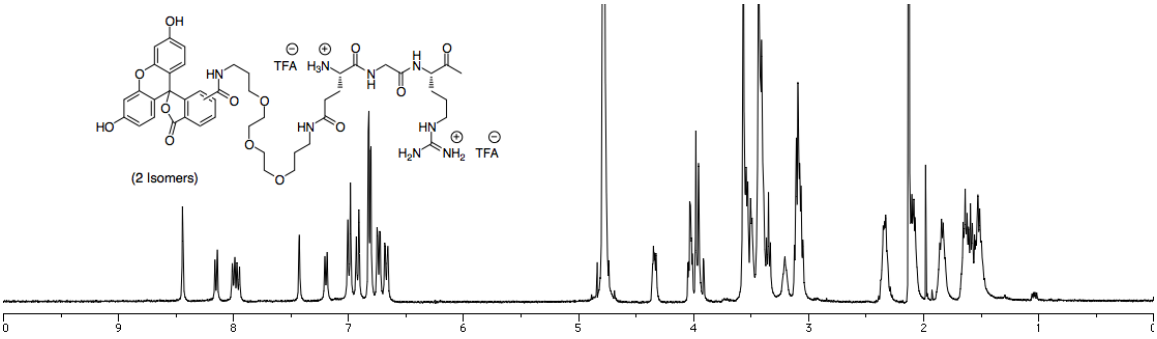
^{13}C NMR Spectrum of 3g (CD_3OD)



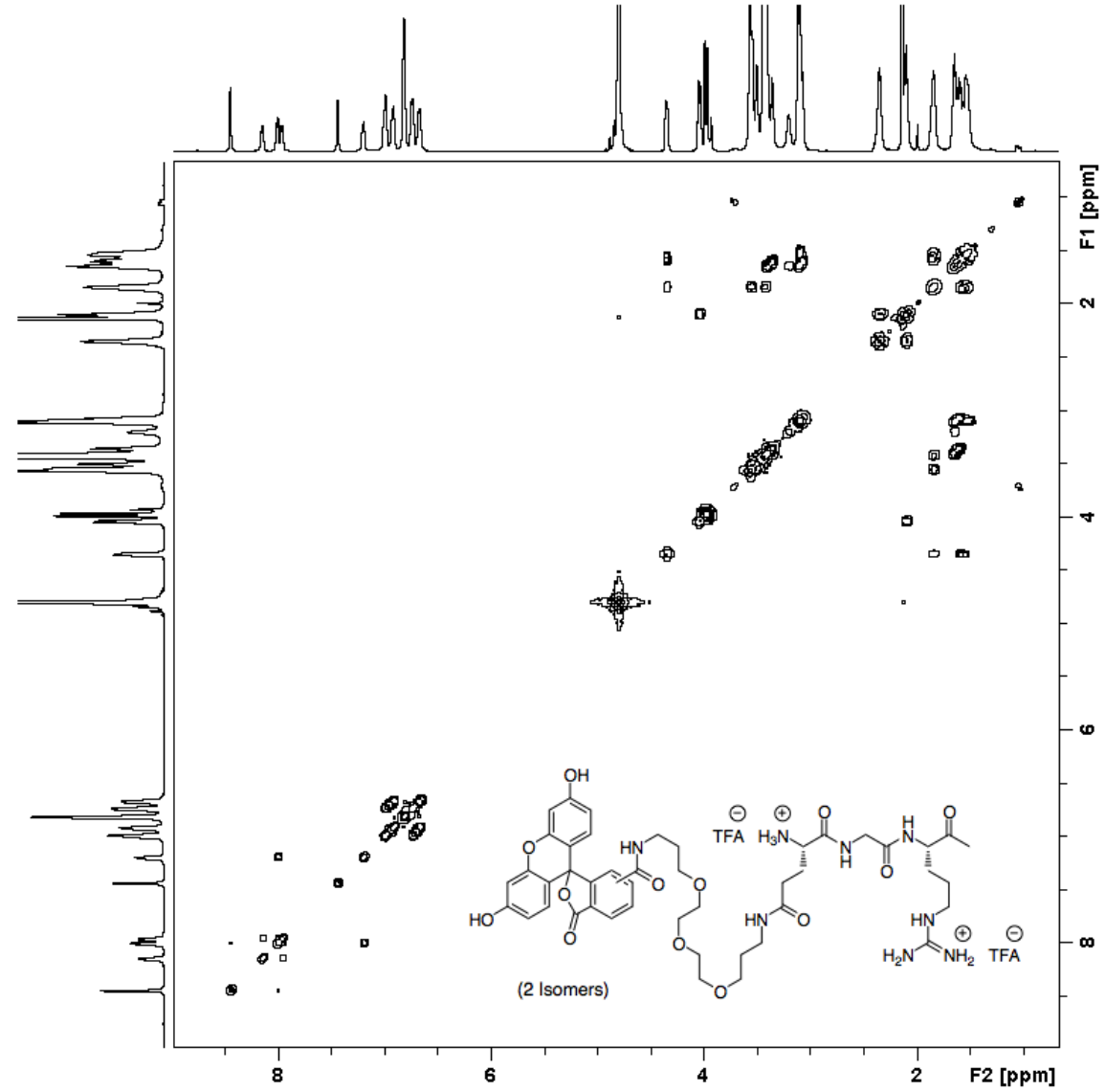
$^{1}H–^{1}H$ COSY Spectrum of 3g (CD_3OD)



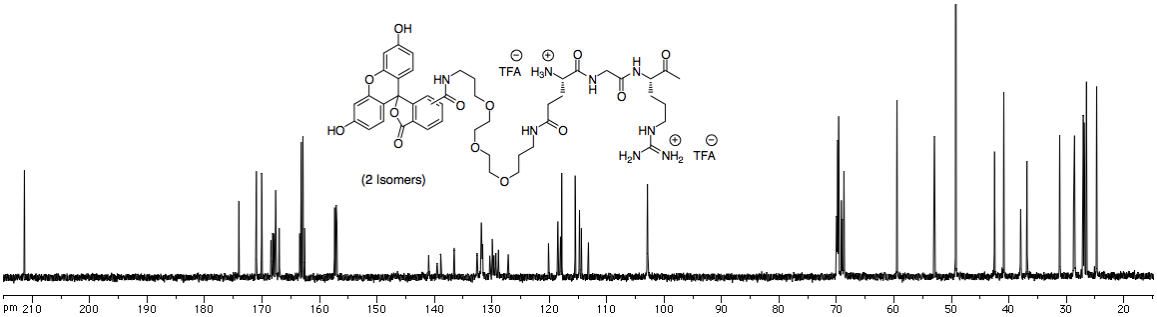
^{1}H NMR Spectrum of 3 (D_2O)



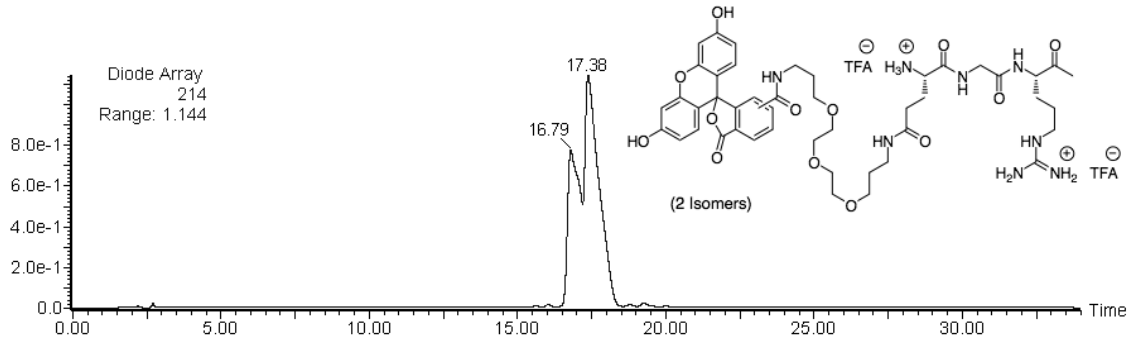
^{1}H–^{1}H COSY Spectrum of 3 (D_2O)



^{13}C NMR Spectrum of 3 (D_2O + 1.5% MeOH)



RP-HPLC Spectrum of 3



4. Biological Evaluation

Buffers and Solutions

•pH were adjusted with 1 M NaOH or 2 M HCl.

•Antibody-Loading Buffer
50 mL PBS Tween
50 mg BSA (1 mg/mL)
stored at 4 ºC

•Coating Buffer
100 mL water
530 mg Na_2CO_3 (50 mM)
to pH 9.6

•Color-Free ADCP Media
RPMI Medium 1640, liquid
ATCC #11835-030
without phenol red
supplemented with 5% HI-FBS
and 1% penicillin-streptomycin

•Colored ADCP Media
RPMI Medium 1640, liquid
Invitrogen #11875-093
supplemented with 5% HI-FBS
and 1% penicillin-streptomycin

•Destaining Solution
50 mL water
40 mL methanol
10 mL acetic acid

•DPBS Solution
Invitrogen #14190-144

•Dulbecco's modified Eagle's medium
ATCC #30-2002

•EDTA Detachment Solution
210 mL DPBS
392 mg EDTA disodium salt (5.0 mM)
84 mg EGTA (1.0 mM)
to pH 7.4
0.22 μM sterile filter

•FBS ADCC Media
RPMI Medium 1640, liquid
ATCC #11835-030
without phenol red
supplemented with 10% HI-FBS
and 1% penicillin-streptomycin
stored at 4 ºC

•FBS-free ADCC Media
RPMI Medium 1640, liquid
ATCC #11835-030
without phenol red
supplemented with 1% penicillin-streptomycin
stored at 4 ºC

•Flow Incubation Buffer
100 mL DPBS
12 mg NaN_3 (2 mM)
180 mg EDTA (5 mM)
to pH 7.4
100 mg BSA (1 mg / mL)
stored at 4 ºC

•Hanks Balanced Salt Solution
Invitrogen #14175-095

•HEPES/saline (2X)
100 mL water
1.6 g NaCl (270 mM)
1.0 g HEPES (42 mM)
0.20 g dextrose (11 mM)
74 mg KCl (10 mM)
27 mg Na_2HPO_4 (2 mM)
to pH 7.05

•McCoy's 5A medium
ATCC #30-2007

•Non-Reducing Loading Buffer
3.8 mL water
1 mL 0.5 M tris HCl at pH 6.8
1.6 mL 10% sodium dodecyl sulfate
0.4 mL 1% bromophenol blue
0.8 mL glycerol

•PBS
1 L water
8.0 g NaCl (137 mM)
0.20 g KCl (2.7 mM)
1.44 g Na_2HPO_4 (10 mM)
0.24 g KH_2PO_4 (1.8 mM)
to pH 7.4

•PBS–Tween
300 mL PBS
60 mg NaN_3 (0.02%)
30 μL tween-80 (0.01%)

•Protein-Loading Buffer
70 mL PBS Tween
130 mg EDTA (5 mM)
pH to 7.4
70 mg BSA (1 mg/mL)
stored at 4 ºC

•Reporter Buffer
45 mL water
5 g diethanolamine (10%)
5 mg $MgCl_2$ (0.1 mg/mL)
10 mg NaN_3 (0.02%)
to pH 9.6

•RPMI-1640 medium
Invitrogen #11875-093
stored at 4 ºC

•Running buffer
15.1 g tris base
94 g glycine
5 g sodium dodecyl sulfate
5 L water
to pH 8.3

•Staining Solution
100 mL destaining solution
100 mg Coomassie blue

•Substrate Solution
20 mL reporter buffer
20 mg *p*-nitrophenyl phosphate (1 mg/mL)
stored at 4 ºC

•Tris buffer
100 mL water
585 mg NaCl (100 mM)
158 mg Tris•HCl (10 mM)
to pH 7.5

•uPA Buffer
30 mL water
204 mg $NaOAc•3H_2O$ (50 mM)
176 mg NaCl (100 mM)
11 mg EDTA disodium salt (1 mM)
to pH 5.0
0.22 μM sterile filter

Proteins, Antibodies, and Reagents

•Alexa Fluor 488 donkey anti-rabbit IgG
Invitrogen #A21206 (Lot #439378)
The purchased solution was stored at 4 ºC.

•Anti-DNP Antibody Produced in Rabbit Whole Antiserum
Sigma #D9656 (Lot #089K4764)
The purchased frozen solution was thawed, split into aliquots, and stored frozen at -20 ºC.

•Anti-Dinitrophenyl-KLH Rabbit IgG Fraction
Invitrogen #A6430 (Lot 807872)
The purchased solution was stored at 4 ºC.

•Anti Fluorescein/Oregon Green goat IgG fraction
Invitrogen #A-11095 (Lot #675497)
The purchased solution was split into aliquots, each of which was frozen at -20 ºC. The aliquot being used was stored at 4 ºC for up to 3 months.

•Anti-Fluorescein/Oregon Green rabbit IgG fraction
Invitrogen #A889 (Lot #645149)
The purchased solution was stored at 4 ºC.

•Anti-Green Fluorescent Protein Rabbit IgG Fraction
Invitrogen #A11122 (Lot 743636)
The purchased solution was stored at 4 ºC.

•Anti-Goat IgG (H&L) (Rabbit) Antibody Alkaline Phosphatase Conjugated
Rockland #605-4502 (Lot #22999)
The purchased solution was stored at 4 ºC.

•Cbz–Gly–Gly–Arg–AMC
Bachem,#1140

•Fetal Bovine Serum, Qualified, Heat-Inactivated
Invitrogen #16140-071
The purchased solution split into aliquots,
each of which was stored at -20 ºC

•Fluorescein Isothiocyanate Isomer 1
90% pure
Acros #119250010

•Human HMW Urokinase (isolated from human urine)
Innovative Research #IUP-HTC (Lot #310)
The purchased solution (in the uPA buffer) was split into aliquots, each of which was frozen at -80 ºC.

•Human LMW Urokinase
Innovative Research #IUPA-LMW (Lot #210)
The purchased solution was split into aliquots, each of which was frozen at -80 ºC.

•Human uPAR Antibody: Polyclonal goat IgG
R&D Systems #AF807 (lot #BBS0208011)
The purchased lyophilized solid was stored at -80 ºC, reconstituted with 500 μL of sterile DPBS (producing a 200 μg/mL solution), and stored at 4 ºC

•Lympholyte-Poly
Cedarlane #CL5070

•Penicillin-Streptomycin, Liquid (10,000 units penicillin; 10,000 μg Streptomycin / mL)
Invitrogen #15140-163

•Rabbit anti human uPA IgG fraction
Innovative Research #IASHUPA-GF (lot #706)
The purchased solution was split into aliquots, each of which was frozen at -80 ºC.

•Recombinant Human IFN-γ
R&D Systems #285-IF
1 μg/mL

•Seeblue Prestained Standard
Invitrogen #LC5625 (Lot #670628)
The purchased solution was stored at 4 ºC.

•Trinitrobenzene sulfonic acid
Thermo Scientific #28997
5% w/v in methanol

•Trypan Blue Stain 0.4%
Invitrogen #15250

•Urokinase, Human
American Research Products #12-4306 Lot #920901
The purchased lyophilized solid was stored at -80 ºC, reconstituted with 67 μL of the uPA buffer (producing a 1.5 μg/μL solution), and split into aliquots, each of which was frozen at -80 ºC

•Vybrant DiD Cell Labeling Solution
Invitrogen #V-22887
1 mM in ethanol
FL-4 fluorophore

•Vybrant DiO Cell Labeling Solution
Invitrogen #V-22886
1 mM in DMF
FL-1 fluorophore

Equipment, Materials, and Software

•16-well E-plate-16
Roche #05469830001

•96 Well Flat Bottom Immuno Plate
MaxiSorp, Non Sterile, PS
Nunc #442404

•C6 Flow Cytometer
Accuri
with CFlow Plus software

•FlowJo software

•Mini-PROTEAN Tetra Cell for Ready Precast Gels
BioRad #165-8004

•Petri Dishes
BD Falcon # 351029
100 x 15 mm

•PowerPac Basic Power Supply
BioRad #164-5050

•Ready Gel Tris-HCl gel 15% crosslinking
8.6 x 6.8 cm, 10 wells (30 uL)
BioRad #161-1103

•Synergy 2 Multimode Microplate Reader
BioTek
with Gen 5 software

•T-Flasks
BD Falcon #353136
75 cm^2 tissue culture treated

•Typhoon Trio Variable Mode Imager
with ImageQuant software•Prism software

•xCelligence System model RTCA-DP
Roche
RTCA software v1.2

Catalytic Inhibition

Stock solutions were prepared of compound **1** (4 μg, 104 μL total volume 2X hepes/saline buffer), uPA (4.6 μg, diluted with water to 4.6 μL total volume), and Cbz–Gly–Gly–Arg–AMC (400 μg, 7 mL total volume 20:1 tris buffer/*N*-methlypyrrolidinone). Into microcentrifuge tubes were added 2 μL of the uPA solution (2 μg, 37 pmol) and 1.7 μL of either the compound **1** stock solution (66 ng, 56 pmol) or 2X hepes/saline buffer to give a final volume of 3.7 μL, final uPA concentration of 10 μM, and final compound **1** concentration of 15 μM. The mixtures were allowed to stand in the dark at ambient temperature (1 h) and diluted with water 25.9 μL. An aliquot of each mixture (12 μL, 15 pmol uPA) was diluted into 738 μL of the AMC solution to give a final volume of 750 μL, a final uPA concentration of 20 nM, and a final AMC concentration of 96 μM. Triplicate aliquots of 200 μL of each solution were loaded into a 96-well plate, and the fluorescence (330 nm absorbance, 460 nm emission) was recorded at one-minute intervals for ten minutes on an automated plate-reader. Figure S1 shows these data.

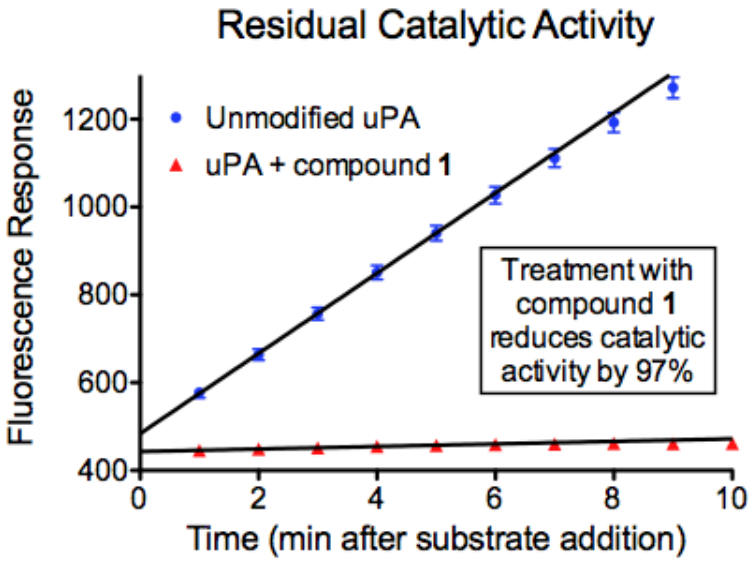


Figure S1. Residual catalytic activity of uPA with or without compound **1** treatment was measured by the increase of fluorescence over time corresponding to enzyme-mediated hydrolysis of Cbz–Gly–Gly–Arg–AMC. Fluorescence Response is measured with 330 nm absorbance and 460 nm emission in an automatic plate reader. Error bars represent the standard deviation of triplicate experiments.

uPA-mediated hydrolysis of Cbz–Gly–Gly–Arg–AMC liberates free AMC, whose fluorescence is substantially greater that that of the starting peptide. Thus, the initial rate of increase of fluorescence is proportional to the amount of active uPA remaining. Slopes for each line were calculated using linear regression (Prism software) of the first five data points. While unmodified uPA produces a slope of 91.3 ± 2.3 fluorescence units per minute, compound **1** treated uPA produces a slope of only 2.9 ± 0.5 fluorescence units per minute, which corresponds to a 97% reduction in catalytic ability.

In-Gel Fluorescence

Stock solutions were prepared of compound **1** (4 μg, 142 μL total volume 2X HEPES/saline buffer) and compound **3** (4 μg, 36.6 μL total volume 2X HEPES/saline buffer). Into microcentrifuge tubes were added 4 μL of either HMW uPA solution (1.5 μg/μL, 6 μg) or the uPA buffer and 7 μL of either the compound **1** stock solution (98 ng), the MeK–Fluor stock solution (382 ng), or 2X HEPES/saline buffer to give a final volume of 11 μL, final uPA concentration of 10 μM, final compound **1** concentration of 15 μM, and final compound **3** concentration of 60 μM. The mixtures were allowed to stand in the dark at ambient temperature (1 h) and diluted with the non-reducing loading buffer (12 μL). An aliquot of each mixture (15 μL) was loaded into a precast gel (15% Tris-HCl). The gel was run (200 mV, 30 min), analyzed by fluorescence (488 nm excitation, 532 nm emission), stained (1 h), and destained (2 h). In Figure S2, lane 3 shows that a substantial amount of compound **1** becomes bound to uPA after incubation, whereas lane 4 shows that compound **3** does not bind uPA.

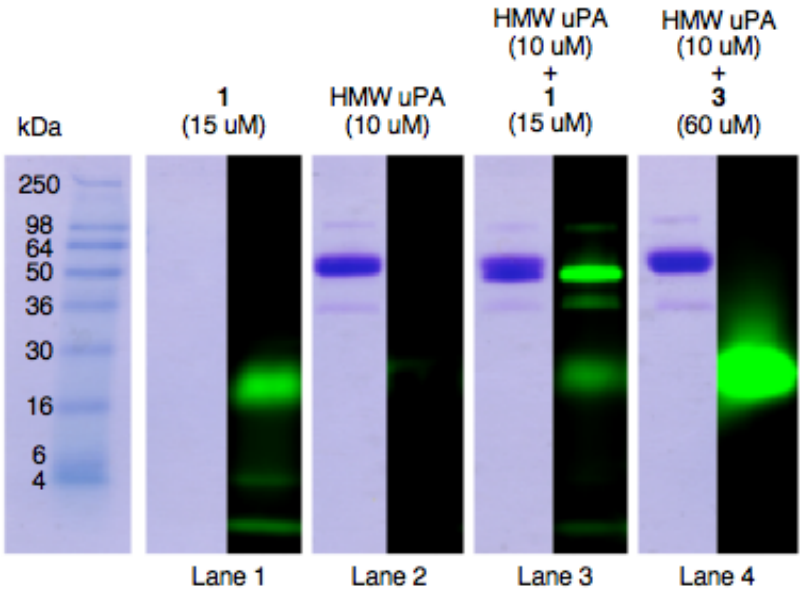


Figure S2. Coomassie (purple on white, left) and 488-to-532 nm fluorescence (green on black, right) analysis of uPA bound by compound **1**

A subsequent experiment was performed to assess the selectivity of the covalent modification of uPA. The following stock solutions were prepared.

Compound	Concentration	Solvent
HMW uPA	2.0 μg / 4.5 μL	2x HEPES/Saline Buffer
BSA	2.4 μg / 4.5 μL	2x HEPES/Saline Buffer
Rabbit anti-uPA antibody	5.5 μg / 4.5 μL	2x HEPES/Saline Buffer
compound **1**	524 ng / 4.5 μL	water
compound **1**	262 ng / 4.5 μL	water
compound **1**	131 ng / 4.5 μL	water
compound **1**	65 ng / 4.5 μL	water
compound **3**	509 ng / 4.5 μL	water

Into microcentrifuge tubes were added 4.5 μL of the appropriate protein solution (4 μM final concentration) and 4.5 μL of the appropriate ketone solution (6, 12, 24, 48 μM final concentrations). Mixtures were mixed, allowed to stand at ambient temperature (1 h, in the dark), and diluted with non-reducing loading buffer (15 μL). An aliquot of each mixture (15 μL) was loaded into a precast gel (15% Tris-HCl). The gel was run (200 mV, 30 min), analyzed by fluorescence (488 nm excitation, 532 nm emission), stained (1 h), and destained (2 h).

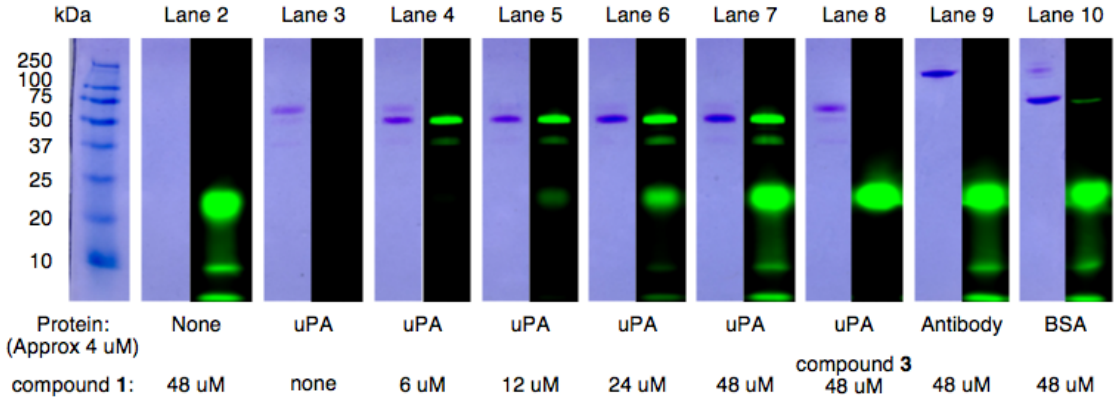


Figure S3. Coomassie (purple on white, left) and 488-to-532 nm fluorescence (green on black, right) analysis of various proteins bound by various concentrations to compound **1**

Comparing the amount of fluorescence incorporated into uPA by increasing concentrations of compound **1** (lanes 4–7) shows that increasing the equivalents of chloromethyl ketone beyond one equivalent does not substantially increase the amount of attachment to the protein, but rather just increases the amount of compound **1** that remains unreacted (as in Lane 2). Furthermore, treating other proteins with compound **1** (Lanes 9–10) does not cause substantial covalent attachment. These data are consistent with the hypothesis that one equivalent of compound **1** selectively attaches to the active site of uPA rather than all the compound **1** randomly attaching nonselectively to ubiquitous protein functional groups.

ELISA

General Procedures:

•Washing: The incubating solution was flicked out of the plate, and remaining liquid was removed by firmly pounding the plate upside-down five times onto a paper towel. Washing solutions (200 μL) were added to each well and removed by the same procedure. The subsequent incubation solution was added immediately.

ELISA Procedure:

•Coating: To each well was added a solution in the coating buffer of rabbit IgG anti human urokinase antibody (3 μg/mL, 200 μL). The plate was sealed with tape, allowed to stand (12 h), washed with PBS–tween (3 x 200 μL) and PBS (1 x 200 μL), loaded with PBS (200 μL), sealed, allowed to stand (2 h), and washed with PBS–tween (2 x 200 μL).

•ARM-U_{Fluor} Preincubation: A solution in 2X HEPES/saline (750 μL) of urokinase (3 μg, 55 pmol, 75 nM) was prepared. A solution in water (211 μL) of compound **1** (4 μg, 3.4 nmol, 16 μM) was prepared. Into four microcentrifuge tubes were added 100 μL of either the urokinase solution or buffer and 100 μL of either the compound **1** solution or water (giving a final uPA concentration of 37 nM and a final compound **1** concentration of 8 μM. The solutions were gently mixed, allowed to stand (1 h, in the dark), and diluted with the protein-loading buffer (30 mL).

•uPA Loading: To each well was added 150 μL of the preincubated urokinase solution (13 ng/mL final uPA concentration). The plate was sealed, allowed to stand (2 h), and washed with PBS–tween (4 x 200 μL).

•Primary Antibody: Several solutions in the antibody-loading buffer of goat IgG anti-fluorescein antibody were prepared by serial dilution (final concentrations from 15 μg/mL = 100 nM to 150 pg/mL = 1 pM). To each well was added 150 μL of the appropriate solution or of pure antibody-loading buffer as a negative control. The plate was sealed, allowed to stand (2 h), and washed with PBS–tween (4 x 200 μL).

•Secondary Antibody: A solutions in the antibody-loading buffer of rabbit anti-goat-IgG antibody conjugated with alkaline phosphatase was prepared (final concentration 0.4 μg/mL). To each well was added 150 μL of this solution. The plate was sealed, allowed to stand (2 h), and washed with PBS–tween (3 x 200 μL) and the reported buffer (3 x 200 μL).

•Reporting: To each well was added 150 μL of the substrate solution. The plate was allowed to stand for 10 min, and the 405 nm absorbance was measured at five-minute intervals (10, 15, 20, and 25 min) on an automated plate reader.

•Analysis: Each condition was run in triplicate. For each well, measurements showed a linear increase in the 405 nm absorbance over time (resulting from production of *para*-nitrophenol under substrate-saturation conditions). These data are graphed in Figure S4.

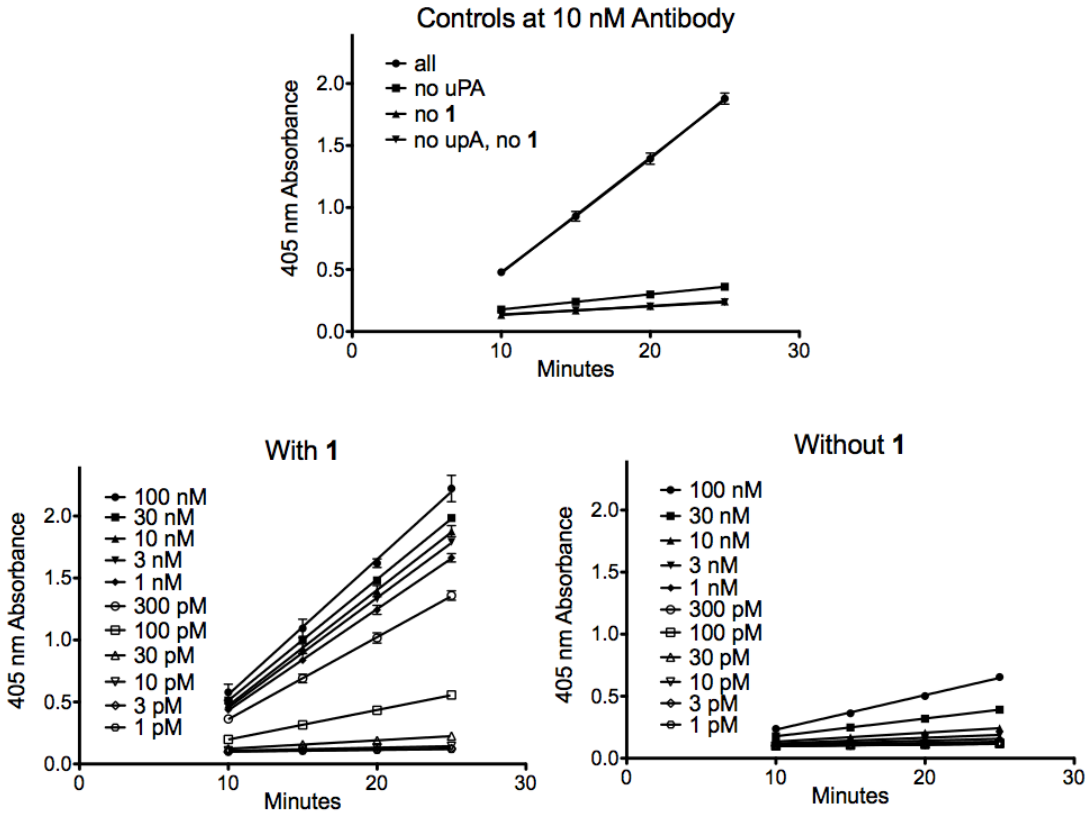


Figure S4. Crude data from ELISA analysis of compound **1**. Concentrations refer to anti-fluorescein antibody. Error bars represent standard deviations of triplicate experiments. Lines of best fit were calculated (Prism software) by linear regression.

The rate of increase of 405 nm absorbance in each well was calculated (Prism software), and mean slopes and standard deviations were calculated from the triplicate experiments at each concentration of anti fluorescein antibody. The slopes from the experiments without compound **1** (negative controls) were subtracted from the slopes from the experiments with compound **1** to remove the background binding, which was significant only at the highest antibody concentrations. From the resulting sigmoid-shaped curve, the dissociation constant was estimated graphically. Figures S5 and S6 show these analyses.

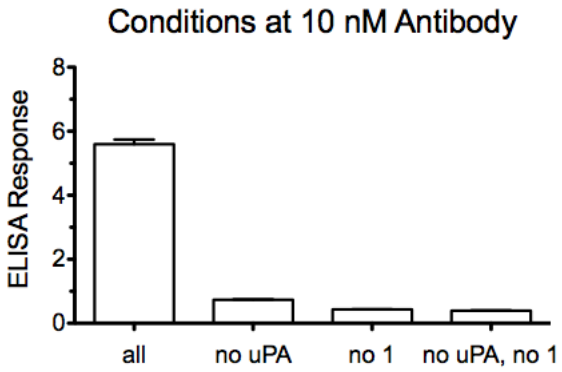


Figure S5. Control conditions. The ELISA response is described below.

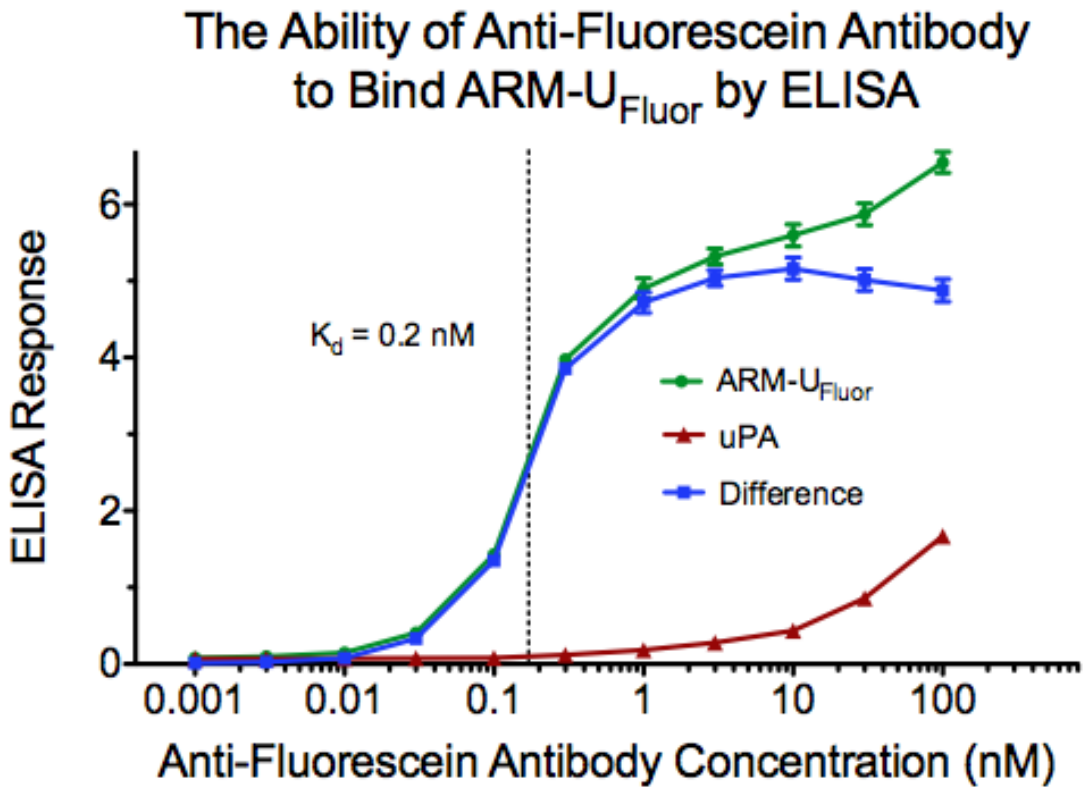


Figure S6. Concentration dependence of the binding between ARM-U_{Fluor} and anti-fluorescein antibody. The ELISA response (rate of change of 405 nm absorbance units per hour) is the slope calculated from linear regression analysis of the absorbance measured over four time points. Error bars represent standard deviations of triplicate experiments. The K_d value was approximated graphically from the background-subtracted curve.

CELL CULTURE

General Procedures:

•Cell Counting: A cell suspension (10 μL) was diluted in Trypan blue (0.4%, 90 μL). 10 μL of this mixture was loaded onto a hemocytometer. Live cells were counted visually under 10X magnification.

•EDTA Detachment: Adherent cells were aspirated and washed with DPBS (5 mL). To the flask was added the EDTA detachment solution (5 mL). The flask was incubated (15 min). Cells were fully detached by gently rinsing the solution over the bottom of the flask. The cell suspension was pelleted, aspirated, suspended in media, and split as desired into new flasks.

•Incubations were done at 37 ºC in a moist atmosphere supplemented with 5% CO_2.

•Pelleting was done by centrifuge for 5 minutes at 1100 rpm.

Cell Lines:

•All cell lines were grown in an incubator (37 ºC) supplemented with 5% CO_2. Media was changed approximately every 4 days. Cells were split approximately 4:1. Cells were not grown beyond approximately 30 passages.

•A172 human glioblastoma cells were purchased from ATCC (#CRL-1620), grown in T-flasks with Dulbecco's modified Eagle's medium supplemented with 10% HI-FBS, and detached by the EDTA detachment procedure.

•HT-29 human colon adenocarcinoma cells were purchased from ATCC (#HTB-38), grown in T-flasks with McCoy's 5A medium supplemented with 10% HI-FBS, and detached by the EDTA detachment procedure.

•U937 Cells were purchased from ATCC (#CRL-1593.2), grown in Petri dishes as a suspension with RPMI-1640 medium supplemented with 10% HI-FBS and 1% penicillin- streptomycin.

Flow Cytometry

General procedures:

•Washing: Beginning with aspirated cells in a Eppendorf or Falcon tube, the washing solution was added by pipette. Cells were mixed either by gently flicking the outside of the tube by hand or by mixing the suspension by pipette. Cells were pelleted and aspirated by pipette.

Flow cytometry procedure:

•Compound **1** incubation: 2X hepes/saline buffer (1 mL) was diluted to 1.6X by adding water (0.25 mL). The following stock solutions were prepared.

Compound	Concentration	Solvent
HMW uPA	6.0 μg / 4.0 μL	water
LMW uPA	3.7 μg / 4.0 μL	water
compound **1**	7 μg / 255 μL	1.6X HEPES/saline buffer

To each uPA solution was added 7.1 μL of the compound **1** solution (final volume = 11.1 μL, uPA final concentration = 10 μM, compound **1** final concentration = 15 μM). The solutions were mixed and maintained in the dark (1 h). Each uPA solution was diluted with the flow incubation buffer by serial dilution to make the following dilutions:

uPA Amount	Volume	uPA Concentration
10 pmol	1 mL	10 nM
3 pmol	1 mL	3 nM
1 pmol	1 mL	1 nM
240 fmol	1 mL	240 pM
240 fmol	3 mL	80 pM
240 fmol	10 mL	24 pM

•Detachment: HT-29 cells (approximately 80% confluent) were detached from their flask by the EDTA-detachment procedure. The cells were transferred into a Falcon tube and counted (approximately 10 million cells). Aliquots containing approximately 250,000 cells were transferred into Eppendorf or Falcon tubes (depending on the required volume for incubations), and cells were pelleted and aspirated.

•ARM-U Incubation: Cells were suspended in the appropriate uPA solution and allowed to stand at ambient temperature in the dark (1 h). Cells were pelleted, aspirated, and washed with DPBS (0.5 mL). Cells in Falcon tubes were transferred to Eppendorf tubes.

•Primary Antibody Incubation: Rabbit IgG anti-fluorescein antibody (1 μg/μL, 6 μL = 6 μg) was diluted in the flow incubation buffer (final volume = 6 mL, final antibody concentration = 1 μg/mL). 250 μL of this solution was added to each tube, the cells were suspended, and the suspensions were allowed to stand (4 ºC, 30 min) in the dark. Cells were pelleted, aspirated, and washed with cold DPBS (0.5 mL).

•Secondary Antibody Incubation: Alexa-Fluor-488-conjugated Donkey anti-rabbit-IgG antibody (2 μg/μL, 9 μL = 18 μg) was diluted in the flow incubation buffer (final volume = 6 mL, final antibody concentration = 3 μg/mL). 250 μL of this solution was added to each tube, the cells were suspended, and the suspensions were allowed to stand (4 ºC, 30 min) in the dark. Cells were pelleted, aspirated, and washed with cold DPBS (0.5 mL).

•Flow Cytometry: DPBS (0.25 mL) was added to each tube. Immediately before analysis, a solution of propidium iodide (500 μg/mL, 20 μL) was added, and the cells were suspended by pipette and analyzed by flow cytometry.

•Data analysis: For each experiment 10,000 events were counted. Forward and side scatter plots were optimally gated to remove debris particles and cellular aggregates. The FL-3 channel was gated to omit dead cells (whose FL-

3 signal is greater than approximately 10^6). Figure S7 shows the crude data distributions for some key conditions. Although fluorescein and Alexa Fluor 488 both fluoresce in the FL1 channel, the Alexa-Fluor-488-conjugated antibody significantly increases the signal relative to fluorescein. Control experiments without antibodies gave <10% of the corresponding increase in FL1 signals.

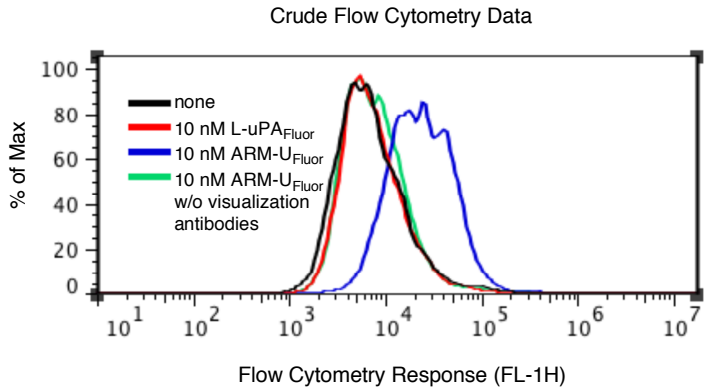


Figure S7. Crude flow cytometry data for some conditions.

The geometric means of the FL-1H signals were calculated (FlowJo software). Figure S8 shows these calculations.

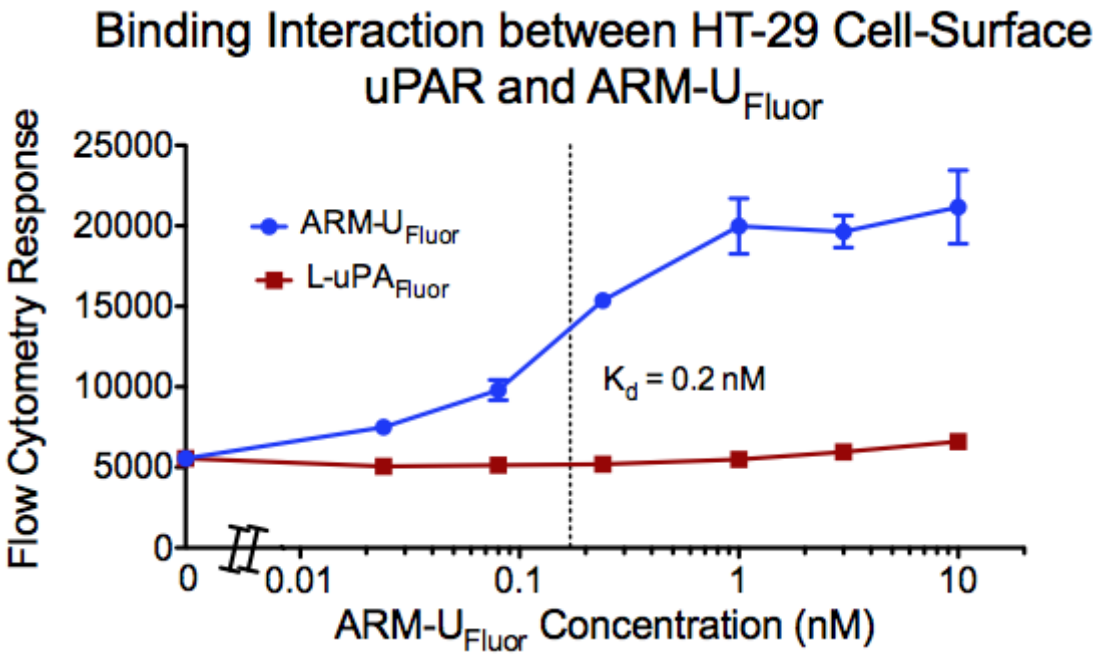


Figure S8. Concentration dependence of the interaction between HT-29 cell surface uPAR and ARM-U_{Fluor}. The flow cytometry response represents the geometric mean of the FL-1H channel, which is measured at 488 nm excitation and 530 / 30 nm emission. Error bars represent the standard deviation of triplicate experiments except those for L-uPA$_{Fluor}$ 0.024 nM through 1 nM. The K_d value was approximated graphically.

A subsequent experiment was performed to assess the specificity with which ARM-U_{Fluor} targets the cells via the uPAR receptor. The following conditions were tested.

Condition	Anti-uPAR Blocking	ARM-U_{Fluor}	Primary Antibody	Secondary Antibody
positive control	none	1 nM	rabbit anti-fluorscein	donkey anti-rabbit
no ARM-U_{Fluor}	none	none	rabbit anti-fluorscein	donkey anti-rabbit
isotype control	none	1 nM	rabbit anti-DNP	donkey anti-rabbit
low blocking	5 μg/mL goat anti-uPAR	1 nM	rabbit anti-fluorscein	donkey anti-rabbit
high blocking	20 μg/mL goat anti-uPAR	1 nM	rabbit anti-fluorscein	donkey anti-rabbit

•Compound **1** incubation: Compound **1** and HMW uPA were mixed as described above. After the 1-hour incubation, the mixture was diluted with the flow incubation buffer to a final concentration of 1 nM uPA and cooled on ice.

•Detachment: HT-29 cells (approximately 70% confluent) were detached from their flask by the EDTA-detachment procedure. The cells were transferred into a Falcon tube and counted. Five aliquots containing approximately 750,000 cells were transferred into Falcon tubes, and cells were pelleted and aspirated.

•Anti-uPAR Blocking: Goat anti-uPAR antibody (0.2 μg/μL, 10 or 40 μL = 2 or 8 μg) was diluted in the flow incubation buffer (400 μL, final antibody concentration = 5 or 20 μg/mL). Cells were suspended in 400 μL of either the high or the low concentration of the anti-uPAR solution or buffer. Cell suspensions were maintained on ice (30 min), pelleted, aspirated, and washed with DPBS (1 mL).

•ARM-U Incubation: Cells were suspended in the uPA solution (800 μL) or buffer. Cells were and maintained on ice in the dark (30 min), pelleted, aspirated, and washed with DPBS (1 mL).

•Primary Antibody Incubation: Rabbit IgG anti-fluorescein antibody (1 μg/μL, 10 μL = 10 μg) was diluted in the flow incubation buffer (final volume = 4 mL, final antibody concentration = 2.5 μg/mL). Rabbit IgG anti-DNP antibody (2 μg/μL, 2.5 μL = 5 μg) was diluted in the flow incubation buffer (final volume = 2 mL, final antibody concentration = 2.5 μg/mL). Cells were suspended in on of these solutions (790 μL), aliquots (250 μL) were transferred into Eppendorf tubes, and the suspensions were maintained on ice (30 min) in the dark. Cells were pelleted, aspirated, and washed with DPBS (0.3 mL).

•Secondary Antibody Incubation: Alexa-Fluor-488-conjugated donkey anti-rabbit-IgG antibody (2 μg/μL, 10 μL = 20 μg) was diluted in the flow incubation buffer (final volume = 5 mL, final antibody concentration = 4 μg/mL). 250 μL of this solution was added to each tube, the cells were suspended, and the suspensions were maintained on ice (30 min) in the dark. Cells were pelleted, aspirated, and washed with cold DPBS (0.3 mL).

•Flow Cytometry: DPBS (0.25 mL) was added to each tube. Immediately before analysis, a solution of propidium iodide (500 μg/mL, 20 μL) was added, and the cells were suspended by pipette and analyzed by flow cytometry.

•Data analysis: For each experiment 10,000 events were counted. Forward and side scatter plots were optimally gated to remove debris particles and cellular aggregates. The FL-3 channel was gated to omit dead cells (whose FL-3 signal is greater than approximately 10^6). The geometric means of the FL-1H signals were calculated (FlowJo software). Figure S9 shows these calculations.

The data show that using either the high or the low concentration of anti-uPAR antibody to block the uPAR on the surface of the cells results in a reduction of the flow cytometry signal, causing the output signal to be essentially equal to the signal observed when ARM-U_{Fluor} is omitted. These results suggest that all the output signal in the positive control condition results from ARM-U_{Fluor} specifically binding to uPAR on the cellular surface, rather than from non-specific binding. Furthermore, substituting an isotype-matched anti-DNP antibody for the anti-fluorescein antibody also reduces the signal to near the background level, which further supports that the observed signal is due to a specific interaction between ARM-U_{Fluor} and the anti-fluorescein antibody.

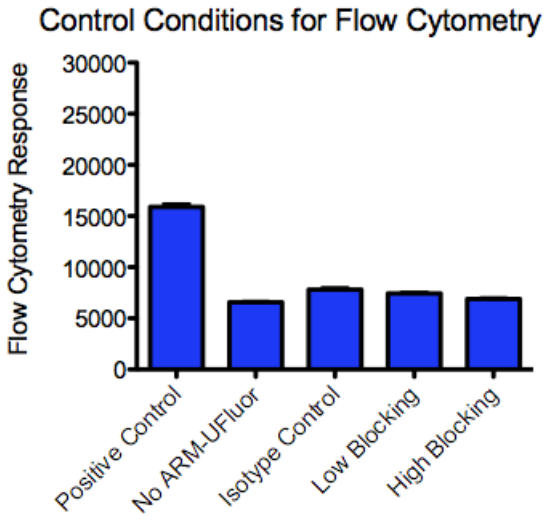


Figure S9. Control conditions that show the specificity of ARM-U_{Fluor} binding to surface uPAR. The flow cytometry response represents the geometric mean of the FL-1H channel, which is measured at 488 nm excitation and 530 / 30 nm emission. Error bars represent the standard deviation of triplicate experiments.

Condition	Anti-uPAR Blocking	ARM-U_{Fluor}	Primary Antibody	Secondary Antibody Alexa Fluor 488 Conjugate
positive control	none	1 nM	rabbit anti-fluorscein	donkey anti-rabbit
no ARM-U_{Fluor}	none	none	rabbit anti-fluorscein	donkey anti-rabbit
isotype control	none	1 nM	rabbit anti-DNP	donkey anti-rabbit
low blocking	5 μg/mL goat anti-uPAR	1 nM	rabbit anti-fluorscein	donkey anti-rabbit
high blocking	20 μg/mL goat anti-uPAR	1 nM	rabbit anti-fluorscein	donkey anti-rabbit

Phagocytosis Assay (ADCP)

•Target cell passing: Two days before the experiments, a plate of target cells (A172 or HT-29, 60–80% confluent) was passed into a new flask.

•Effector cell priming: Two days before the experiments, a plate of U937 cells (approximately 60% confluent) was passed into a new Petri dish (10 mL colored ADCP media total volume). IFN-γ (5 μL, 1 μg/mL in DPBS) was added, and the cells were maintained in an incubator (37 ºC, 24 h). The cells were transferred into a Falcon tube, and DiD (19 uL, final concentration = 1.9 μM) was added. The cells were maintained in an incubator (37 ºC, 30 min), pelleted, aspirated, resuspended in colored ADCP media (10 mL), and split equally into two Petri dishes. To each dish were added additional colored ADCP media (5 mL) and IFN-γ (5 μL, 1 μg/mL in DPBS), and the cells were maintained in an incubator (37 ºC, 24 h).

•ARM-U formation: 2X HEPES/saline buffer (1 mL) was diluted to 1.6X by adding water (0.25 mL). The following stock solutions were prepared. (In each day of experiments either compound **1** or compound **2** was used.)

Compound	Concentration	Solvent
HMW uPA	6.0 μg / 4.0 μL	water
LMW uPA	3.7 μg / 4.0 μL	water
compound **1**	4 μg / 145 μL	1.6X HEPES/saline buffer
compound **2**	4 μg / 172 μL	1.6X HEPES/saline buffer

To each uPA solution was added 7.1 μL of the chloromethyl ketone solution (final volume = 11.1 μL, uPA final concentration = 10 μM, final chloromethyl ketone concentration = 15 μM). The solutions were mixed and maintained in the dark (1 h). Each uPA solution was diluted with the flow incubation buffer by serial dilution to give the following final uPA concentrations: 100 nM, 10 nM, 1 nM, 0.1 nM.

•Target cell preparation: To a plate of target cells (60–80% confluent, either HT-29 or A172, 10 mL total media volume) was added DiO (20 μL, final concentration = 2 μM). The cells were maintained in an incubator (37 ºC, 30 min), aspirated, and washed with colored ADCP media (3 x 10 mL). colored ADCP media (10 mL) was added. The cells were maintained in an incubator (37 ºC, 2 h), detached, split in to the required number of Falcon tubes, pelleted, aspirated, and resuspended in 1 mL of either one of the stock solutions of the ARM-U complexes (described above) or a stock solution of 2,4,6-trinitrophenylsulfonic acid (2 μL of a 5% solution in methanol, diluted in 1 mL colored ADCP media, final concentration = 340 μM) or fluorescein isothiocyanate (10 μL of 1 μg/mL in DPBS, plus 10 mL DPBS, final concentration = 2.6 nM) as a positive control or the flow incubation buffer as a negative control. The cells were allowed to stand at ambient temperature (30 min), pelleted, aspirated, washed with color-free ADCP media (3 x 10 mL), pelleted, resuspended in color-free ADCP media (1 mL), counted, and diluted with color-free ADCP media to give a final concentration of 250,000 cells per mL.

•Effector cell preparation: Both dishes of primed U937 cells were transferred into a Falcon tube, pelleted, aspirated, resuspended in color-free ADCP media (10 mL), counted, and diluted with color-free ADCP media to give a final concentration of 4 million cells per mL.

•Phagocytosis: All conditions were run in triplicate. For each experiment, into a sterile 2 mL Eppendorf tube were added color-free ADCP media (20 μL), either rabbit IgG anti-DNP antibody (0.625 μL, 2 mg/mL) or rabbit IgG anti-fluorescein antibody (1.25 μL, 1 mg/mL) or neither, target cells (50 μL = 12,500 cells), and effector cells (50 μL = 200,000 cells), to give an effector-to-target ratio of 16:1 and a final antibody concentration of 10 μg/mL. Tubes were gently agitated by hand, maintained at 4 ºC (30 min), and gently agitated again. The cells were pelleted (2 min, 1100 rpm). The tubes were opened, covered with parafilm, pierced three times each with a 22 gauge needle, maintained in an incubator (37 ºC, 1 h), resuspended by briefly agitating with a vortex, and analyzed by flow cytometry.

•Data analysis: For each experiment 100,000 events were counted. Forward and side scatter plots were optimally gated to remove debris particles and cellular aggregates. On a plot of FL-1 vs FL-4, the following populations were counted.

Population	Approximate FL-1 Signal	Approximate FL-4 Signal
Effector Cells	10^3–10^4	10^5–$10^{6.5}$
Target Cells	10^5–10^7	$10^{2.5}$–10^4
Double Positive Cells	10^5–10^7	10^5–$10^{6.5}$

Phagocytosis was calculated by the following formula. Figure S10 shows these data.

$$\% \text{ phagocytosis} = \frac{(\text{double positive cells})}{(\text{remaining target cells}) + (\text{double positive cells})} \times 100$$

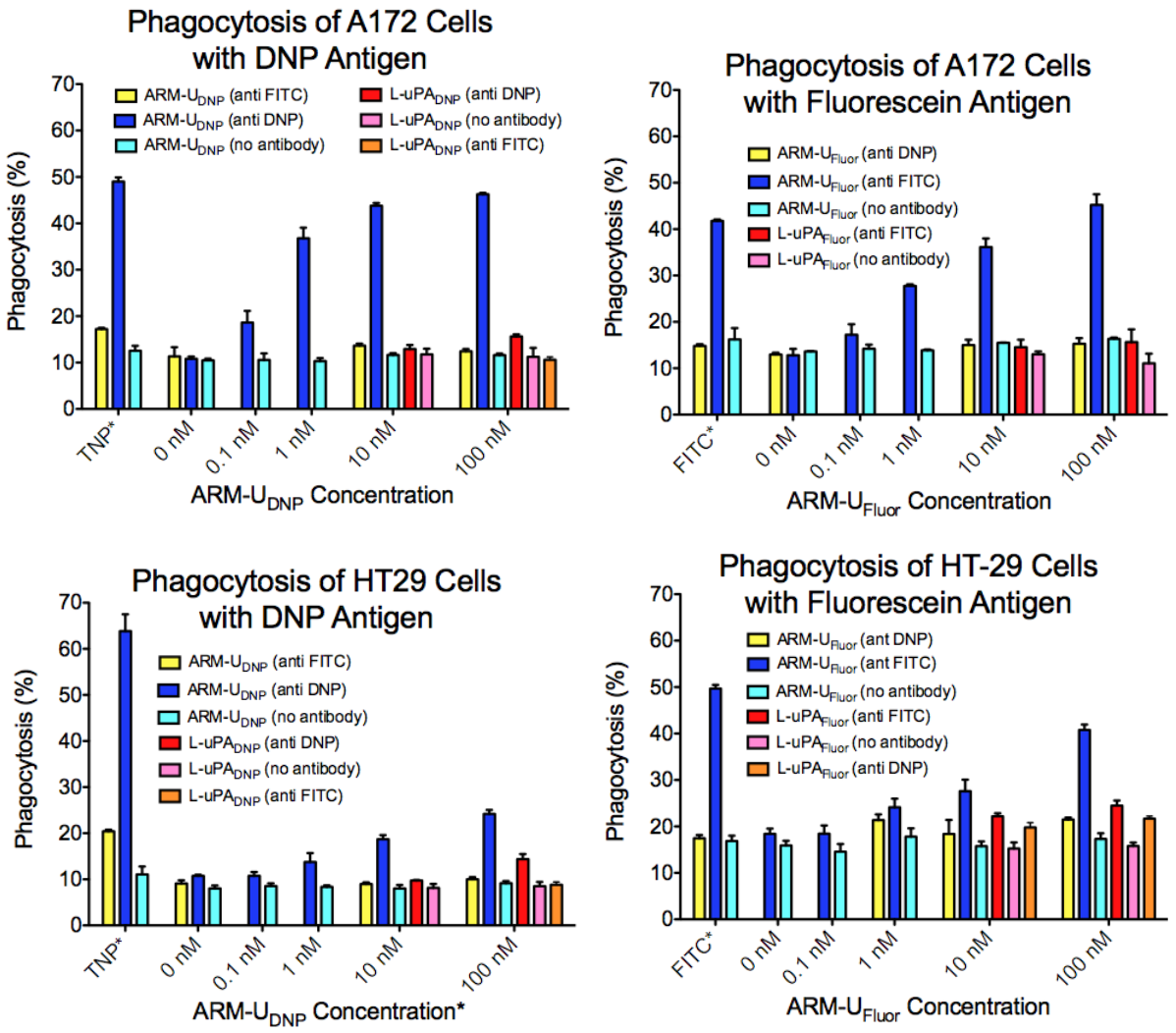


Figure S10. Antibody-dependent cellular phagocytosis of cancer cells directed by ARM-U_{Fluor} or ARM-U_{DNP}. Error bars represent standard deviations of triplicate experiments. *TNP and FITC conditions represents treating the target cells with either 2,4,6-trinitrophenylsulfonic acid (340 μM) or fluorescein isothiocyanate (2.6 nM). *1.0 rather than 1.5 equiv of compound **2** was used in the experiments with HT-29 cells.

A subsequent experiment was performed by the same procedure, except using commercially available serum from rabbits immunized against DNP–KLH (final concentration = 0.5%) instead of 10 μg/mL rabbit IgG anti-DNP. Figure S11 shows these data.

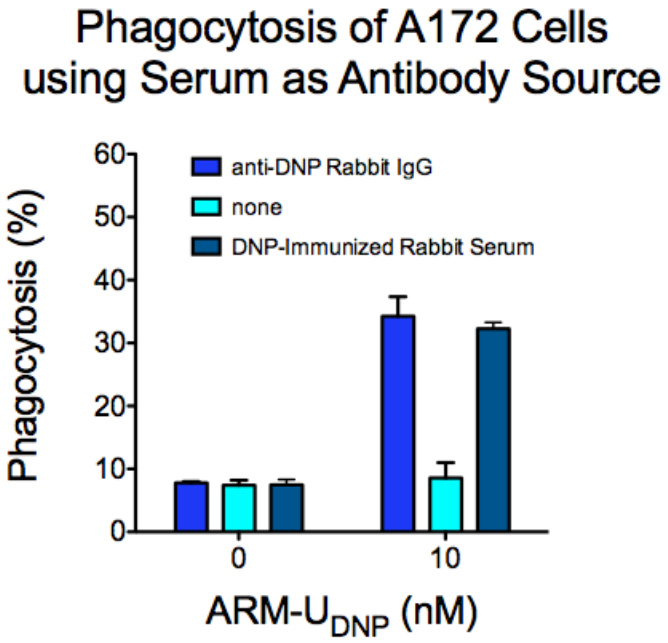


Figure S11. Antibody-dependent cellular phagocytosis of A172 cells directed by ARM-U_{DNP}. Final serum concentration = 0.5%. Final anti-DNP concentration = 10 μg/mL. Error bars represent standard deviations of triplicate experiments.

Cytotoxicity Assay (ADCC)

•Target cell seeding: One day before the experiment, A172 cells were detached, counted, aspirated, and diluted in FBS ADCC media (to a final concentration of 25,000 cells/mL). Into each well of an E-plate was added 200 μL of the cell suspension (5,000 cells). The plate was allowed to stand at ambient temperature (30 min) and maintained in an incubator (37 ºC, 12 h).

•Growth curves: The xCelligence system was maintained inside the incubator (37 ºC). An E-plate containing 100 μL FBS ADCC media per well was used for obtaining background measurements. The seeded E-plate was placed in the port, and cell index readings were obtained (every 2 min for 30 min) to confirm that the cells had adhered properly.

•ARM-U formation: 2X HEPES/saline buffer (1 mL) was diluted to 1.6X by adding water (0.25 mL). The following stock solutions were prepared.

Compound	Concentration	Solvent
HMW uPA	6.0 μg / 4.0 μL	water
compound **2**	4 μg / 259 μL	1.6X HEPES/saline buffer

To each uPA solution or 64 μL of the uPA buffer was added 7.1 μL of either water or the chloromethyl ketone solution (final volume = 11.1 μL, uPA final concentration = 10 μM, final chloromethyl ketone concentration = 10 μM). The solutions were mixed, maintained in the dark (1 h), and diluted with FBS-free ADCC media (544 μL, to a final uPA concentration of 200 nM). For the 5 nM experiment, these solutions were diluted another 10-fold in FBS-free ADCC media (to a final concentration of 20 nM)

•Experimental setup: Either rabbit IgG anti-DNP (2 mg/mL stock) or rabbit IgG anti-GFP (2 mg/mL stock) were added to the ARM-U solutions to give final concentrations of 108 μg/mL antibody. Supernatant (100 μL) was removed from each well of the E-plate to make room for reagent addition. Solutions of ARM-U and antibody (50 μL) were added to each well (giving final concentrations of 50 or 5 nM uPA or ARM-U and 27 μg/mL antibody). The E-plate was returned to the port, and cell index readings were obtained (every 2 min for 90 min)

•PBMC isolation: Fresh blood (50 mL, stabilized with sodium heparin) was obtained from a healthy volunteer on the day of the experiment. Into each of two Falcon tubes were added first Lympholyte Poly (25 mL) and then blood (25 mL) gently on top. The tubes were centrifuged (0.5 rcf, 35 min). The top (pale yellow) layer was removed, and the top layer of cells was isolated, diluted with Hank's balanced salt solution, counted, pelleted (0.2 rcf, 15 min), aspirated, and diluted in FBS-free ADCC media (to a final concentration of 6.25 million cells per mL). 50 μL of this suspension (312,500 cells, 62.5:1 effector:target) was added to each well of the E-plate. The E-plate was returned to the port, and cell index readings were obtained (every 2 min for 20 h, 37 ºC).

•Data analysis: Cell index readings were normalized (RTCA software) at the timepoint immediately after addition of the PBMCs. Figure S12 shows these data. Values from the 3-hour time point were used to calculate specific killing. Normal growth was defined as that shown by target A172 cells treated with effector cells and anti-DNP antibody, but no uPA or ARM-U constructs. Specific killing was calculated as the following formula. Figure S13 shows these data.

$$\%\ \text{specific killing} = 100 - \frac{(\text{cell index})}{(\text{normal growth cell index})} \times 100$$

Interestingly, increases in cell index were observed during the first 5 hours after treatment of A172 cells with negative control conditions including uPA plus anti-DNP, ARM-U_{DNP} plus isotype control anti-GFP, and ARM-U_{DNP} without antibody. We speculate that these observations reflect morphological changes in the adherent cells, which are known to be caused by the uPA–uPAR interaction[6] and are known to be measurable by the xCelligence System[7]. For the conditions with ARM-U_{DNP} plus anti-DNP (blue), on the other hand, a dramatic decrease in cell index is observed within the first 3 hours, and therefore this effect is specific to the combination of ARM-U_{DNP} and an isotope-matched antibody.

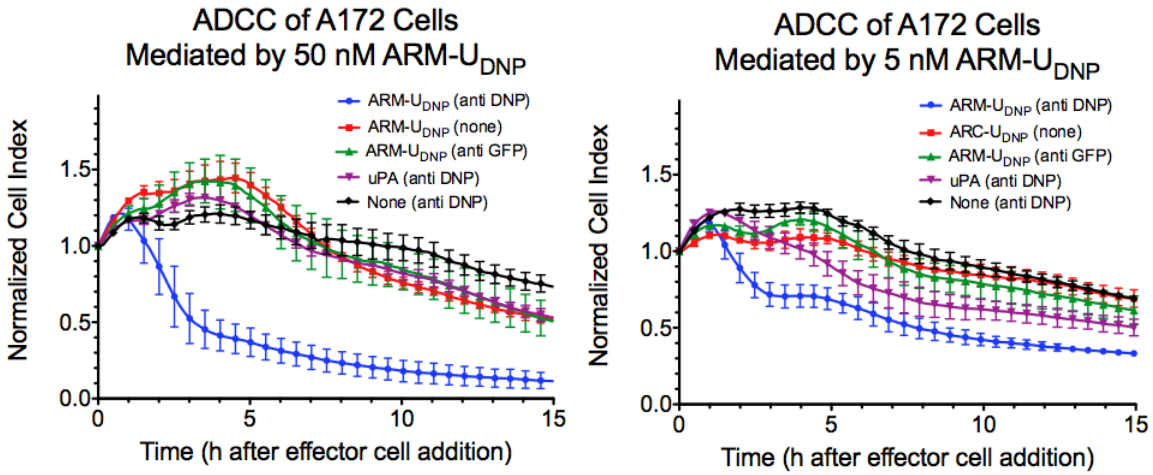


Figure S12. Antibody-dependent cellular cytotoxicity of A172 cancer cells directed by ARM-U_{DNP}. Cell index values were normalized immediately after effector cell addition. Error bars represent standard deviations of triplicate experiments.

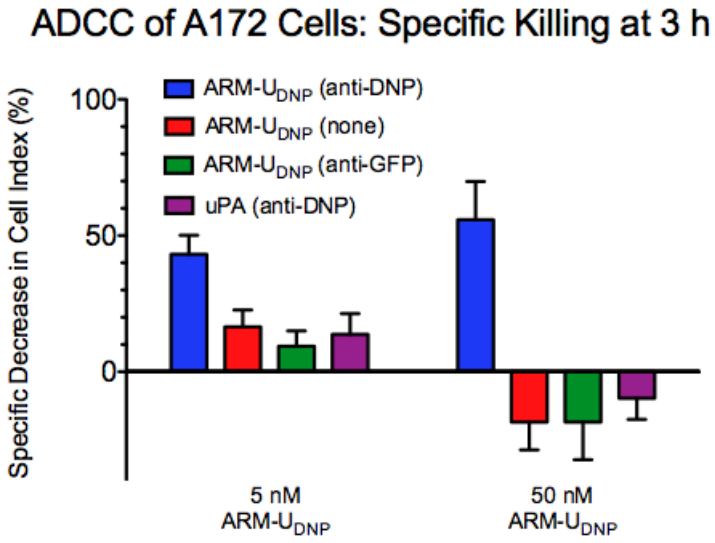


Figure S13. Antibody-dependent cellular cytotoxicity, as measured by the Roche xCelligence System, of A172 cells by freshly isolated PBMCs (60:1 effector:target). Antibody concentration = 27 μg/mL. At 3 h, the specific cytotixicity was calculated as the specific decrease in cell index compared to cells treated with only anti-DNP antibody and PBMCs. Data points represent average values of triplicate experiments ± standard deviation.

[6] Blasi, F., Carmeliet, P. (2002) *Nat. Rev. Mol. Cell. Biol. 3,* 932.
[7] Yu, N., Atienza, J. M., Bernard, J., Blanc, S., Zhu, J., Wang, X., Xu, X., Abassi, Y. A. (2006) *Anal. Chem. 78*, 35.

A subsequent experiment was performed using an analogous procedure, but omitting the PBMC effector cells. Cell index readings were normalized (RTCA software) at the timepoint immediately after addition of ARM-U. Figure S14 shows these data. Values from the 3-hour time point were used to calculate specific killing. Normal growth was defined as that shown by target A172 cells treated with anti-DNP antibody, but no uPA or ARM-U constructs. Specific killing was calculated in the same way. Figure S14 shows these data.

Consistent with the previous experiments, conditions with high ARM-U concentrations cause initial increases in the cell index over the first 4 hours, followed by gradual decreases in cell index. There is no significant difference between 50 nM ARM-U_{DNP} with or without antibody (dark blue vs red), which shows that in the absence of PBMCs there is no effect that is specific to the combination of ARM-U_{DNP} and matched antibody, and which further shows that the results observed in the experiments with PBMCs do require the presence of the PBMCs.

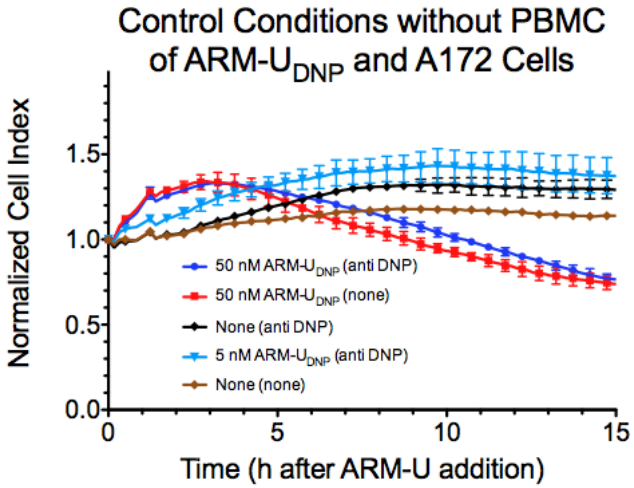


Figure S14. Control conditions for antibody-dependent cellular cytotoxicity of A172 cancer cells directed by ARM-U_{DNP}. Cell index values were normalized immediately after ARM-U addition. Error bars represent standard deviations of triplicate experiments.

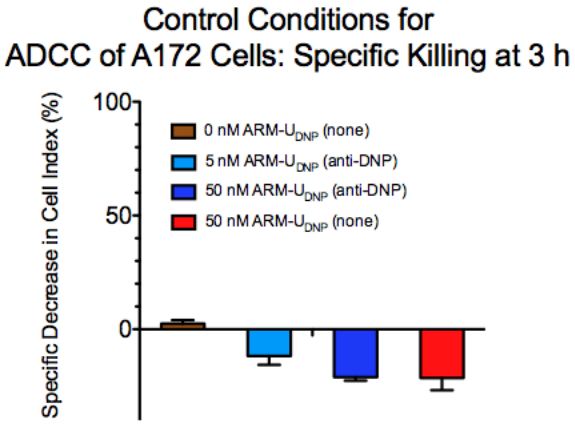


Figure S15. Controls for antibody-dependent cellular cytotoxicity, as measured by the Roche xCelligence System, of A172 cells without PBMCs. Antibody concentration = 27 μg/mL. At 3 h, the specific cytotixicity was calculated as the specific decrease in cell index compared to cells treated with only anti-DNP antibody. Data points represent average values of triplicate experiments ± standard deviation.

5. ADCP Flow Cytometry Data

The first gating, to omit small debris particles and any large cell aggregates, was performed on a plot of forward scatter versus side scatter. All conditions were gated in the same way. Figure S16 shows a representative example.

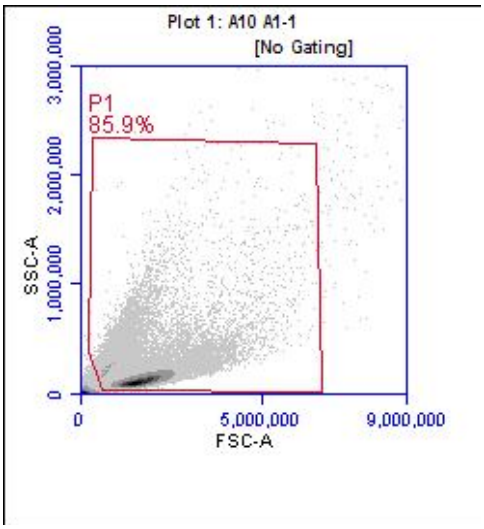


Figure S16. A representative example of the initial gating of flow cytometry data.

The second gating, used to identify phagocytosed target cells and remaining target cells, was performed on a plot of channel 1 fluorescence (FL1) versus channel 4 fluorescence (FL4). Figure S17 shows a representative example with the populations labeled for clarity.

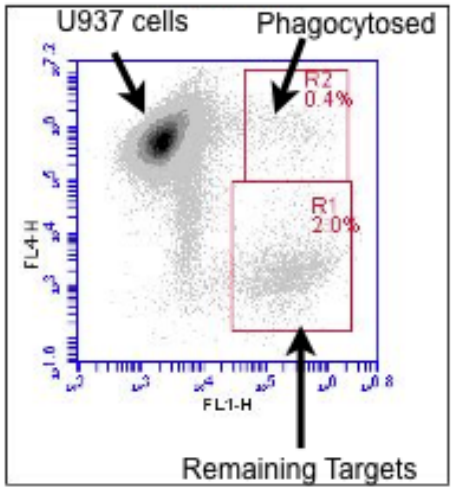
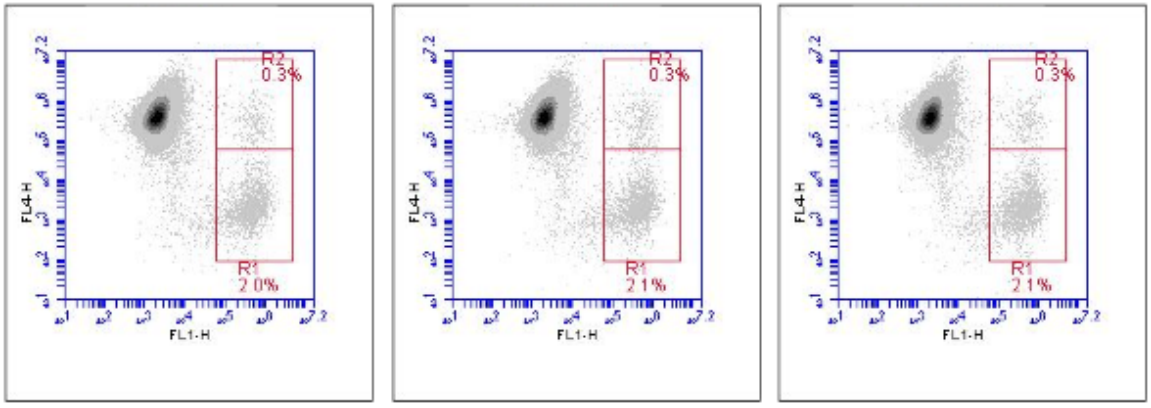


Figure S16. A representative example of the final gating of flow cytometry data.

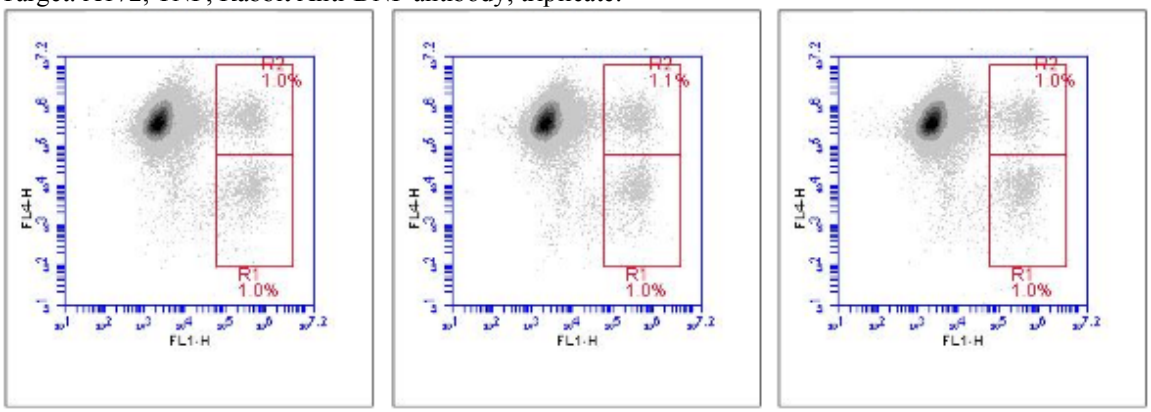
Triplicate data for all conditions are presented on the following pages.

ARM-U_{DNP} to Target A172 Cells

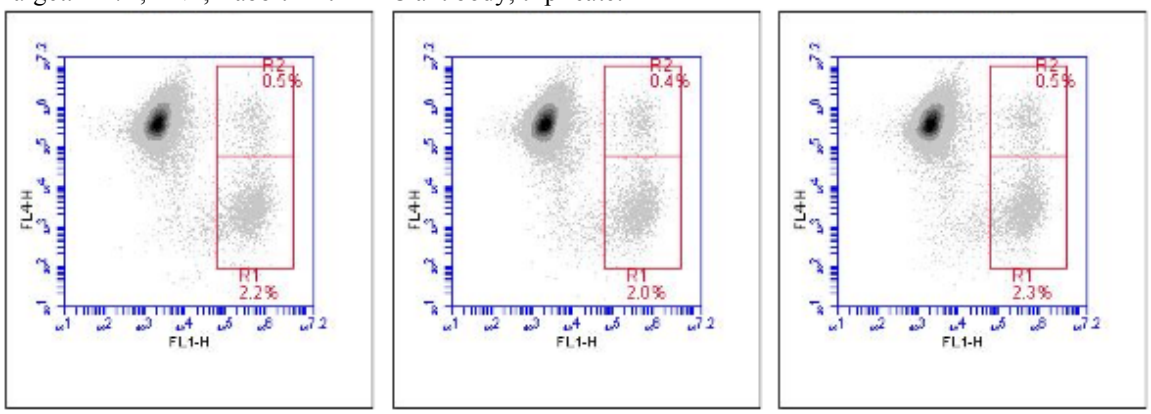
Target: A172, TNP, no antibody, triplicate:



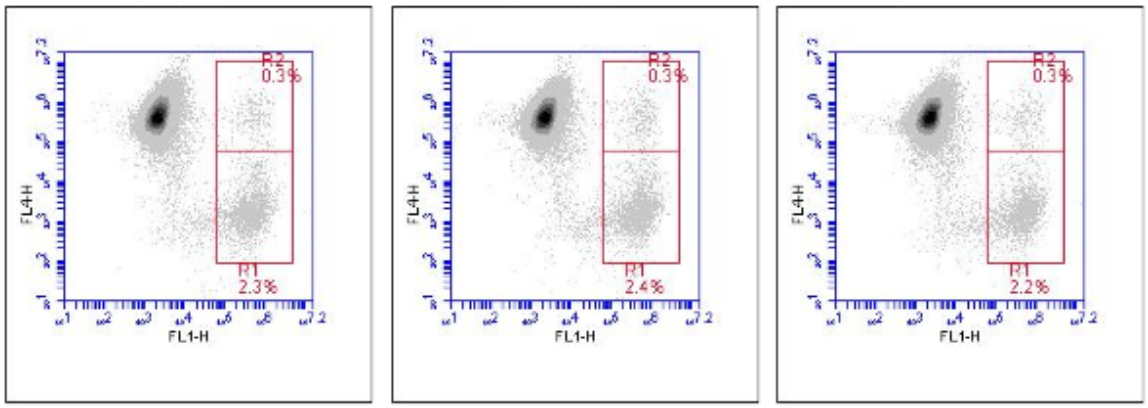
Target: A172, TNP, Rabbit Anti-DNP antibody, triplicate:



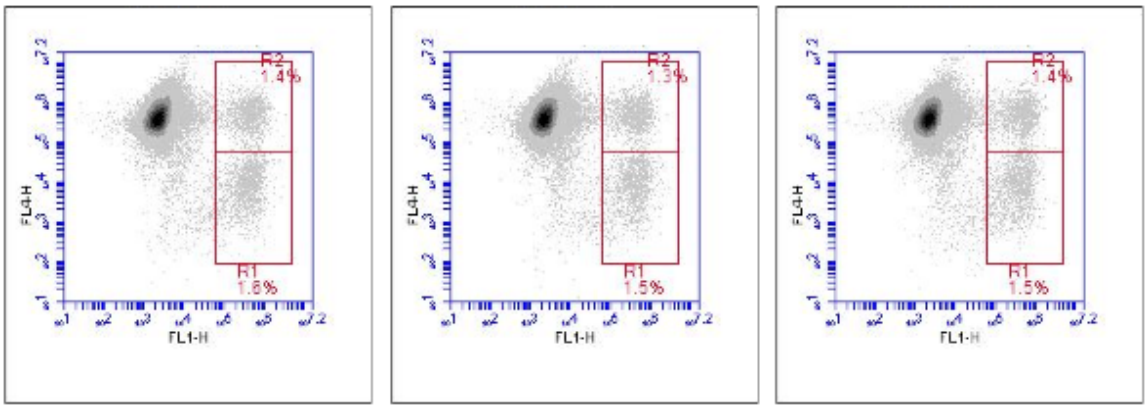
Target: A172, TNP, Rabbit Anti-FITC antibody, triplicate:



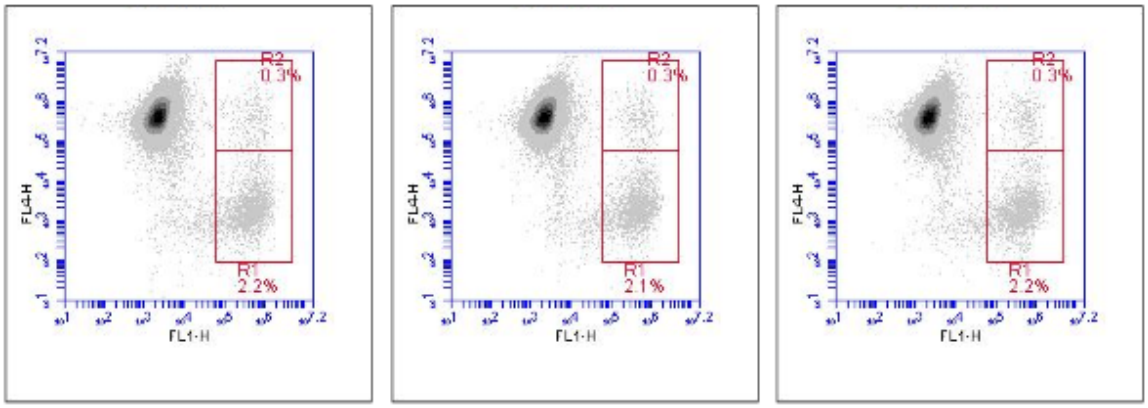
Target: A172, 100 nM ARM-U_{DNP}, no antibody, triplicate:



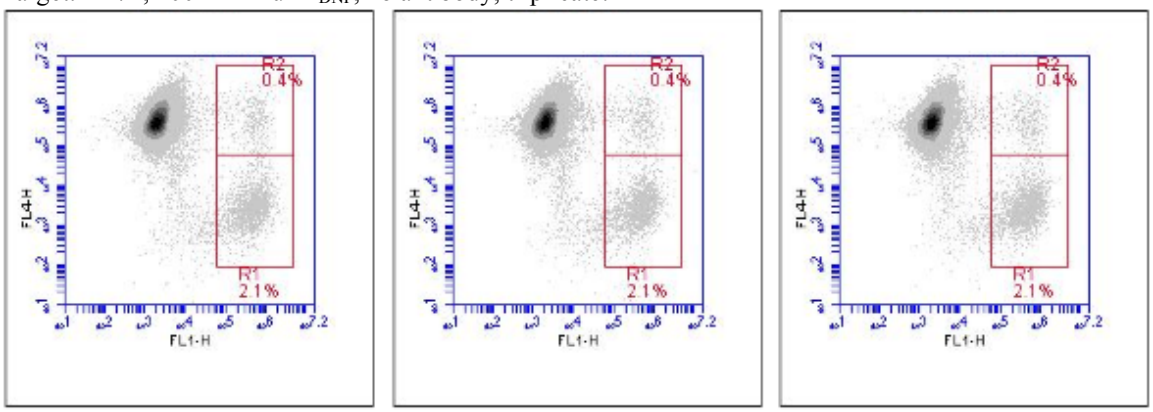
Target: A172, 100 nM ARM-U_{DNP}, Rabbit Anti-DNP antibody, triplicate:



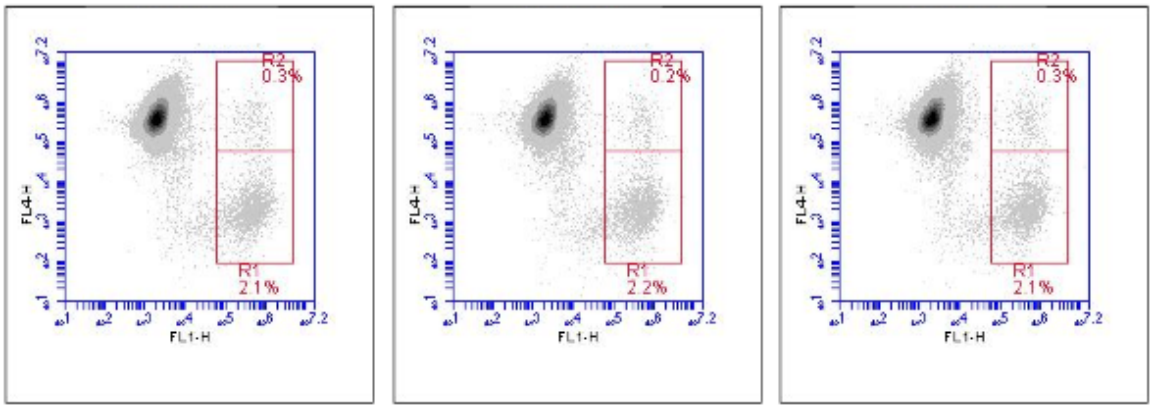
Target: A172, 100 nM ARM-U_{DNP}, Rabbit Anti-FITC antibody, triplicate:



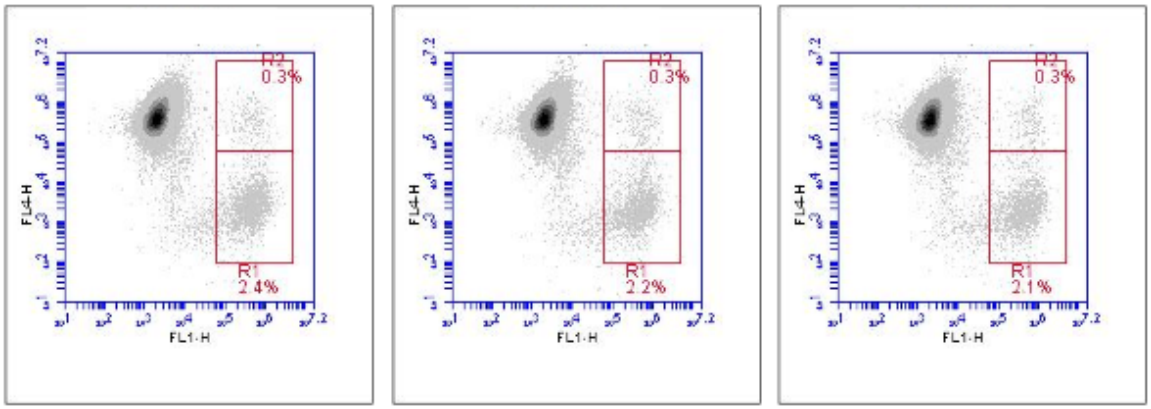
Target: A172, 100 nM L-uPA$_{DNP}$, no antibody, triplicate:



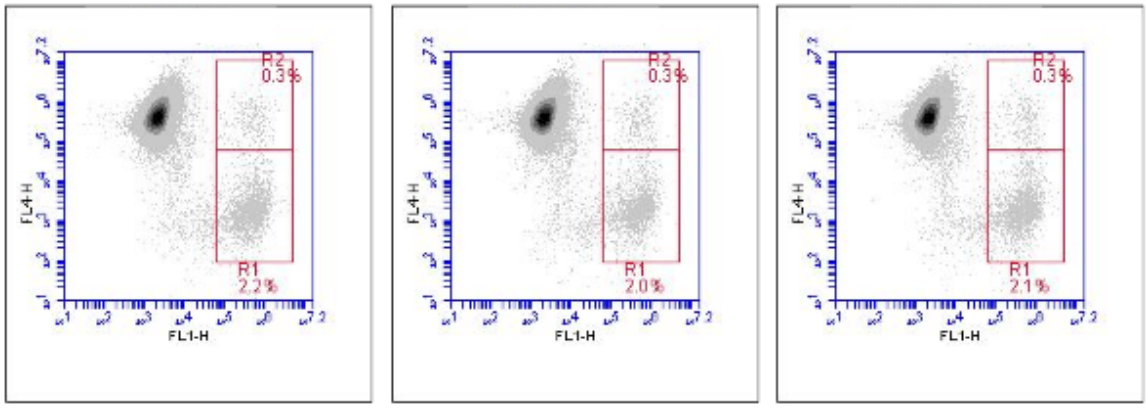
Target: A172, 100 nM L-uPA$_{DNP}$, Rabbit Anti-DNP antibody, triplicate:



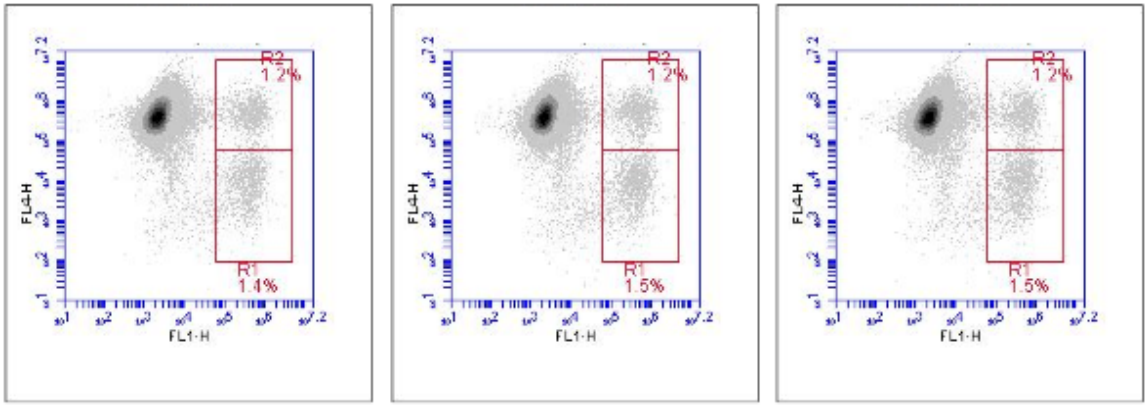
Target: A172, 100 nM L-uPA$_{DNP}$, Rabbit Anti-FITC antibody, triplicate:



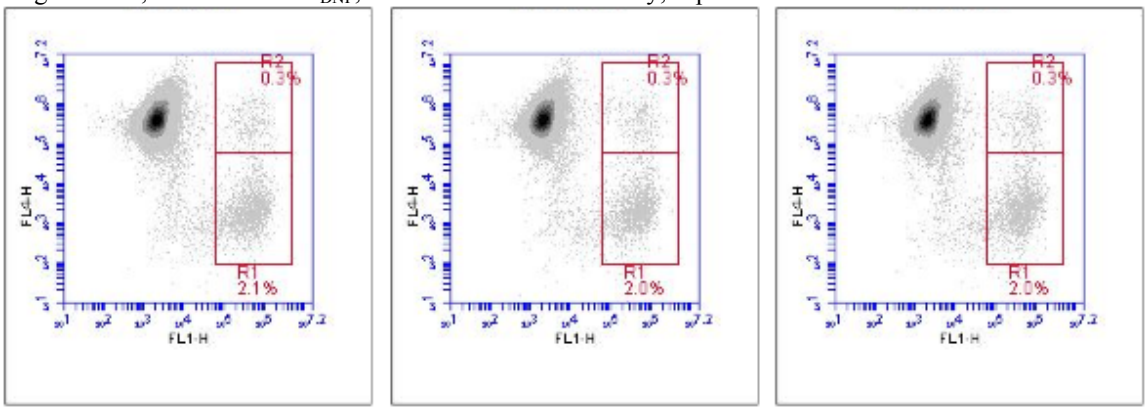
Target: A172, 10 nM ARM-U_{DNP}, no antibody, triplicate:



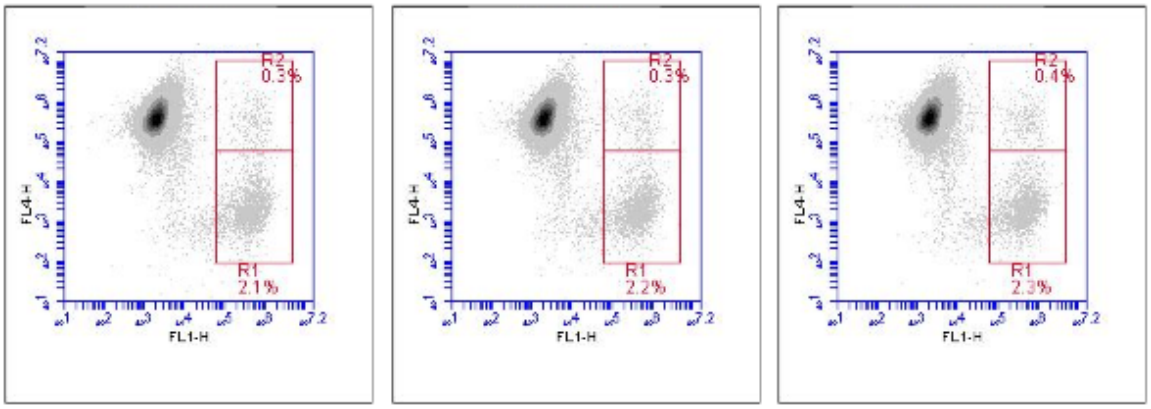
Target: A172, 10 nM ARM-U_{DNP}, Rabbit Anti-DNP antibody, triplicate:



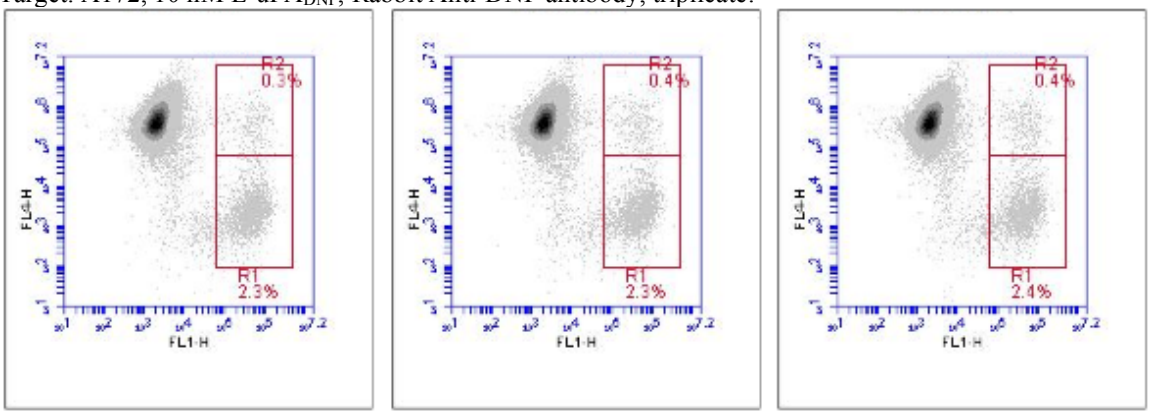
Target: A172, 10 nM ARM-U_{DNP}, Rabbit Anti-FITC antibody, triplicate:



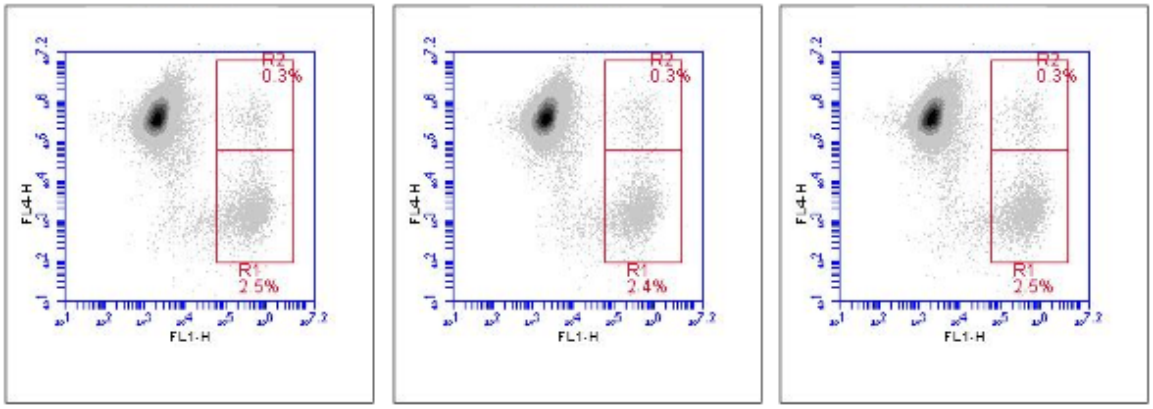
Target: A172, 10 nM L-uPA$_{DNP}$, no antibody, triplicate:



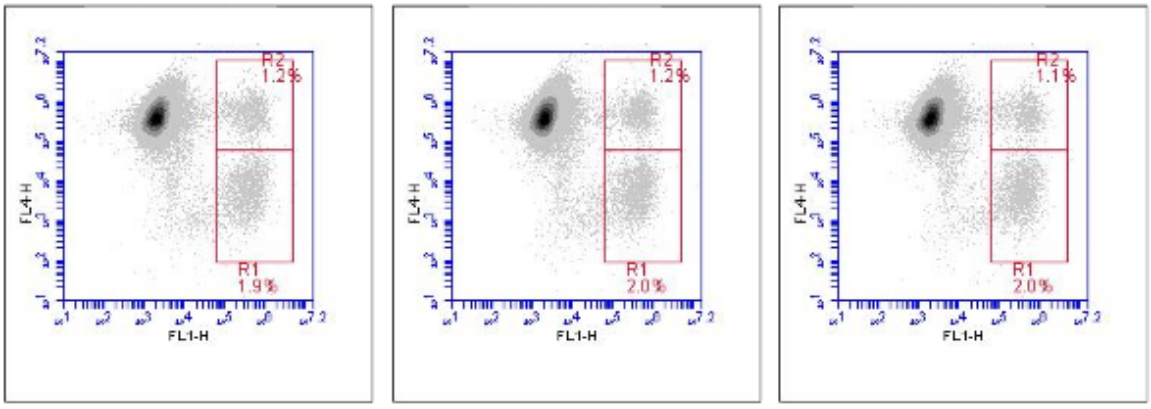
Target: A172, 10 nM L-uPA$_{DNP}$, Rabbit Anti-DNP antibody, triplicate:



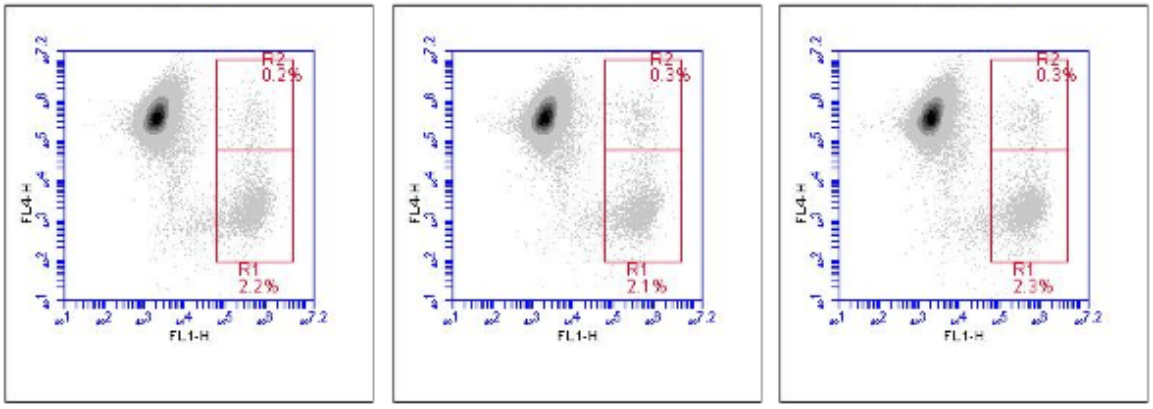
Target: A172, 1 nM ARM-U$_{DNP}$, no antibody, triplicate:



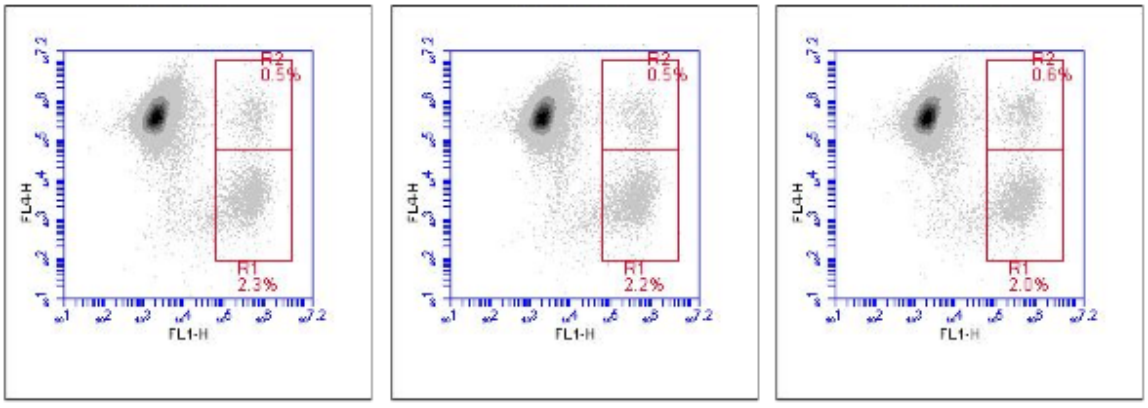
Target: A172, 1 nM ARM-U_{DNP}, Rabbit Anti-DNP antibody, triplicate:



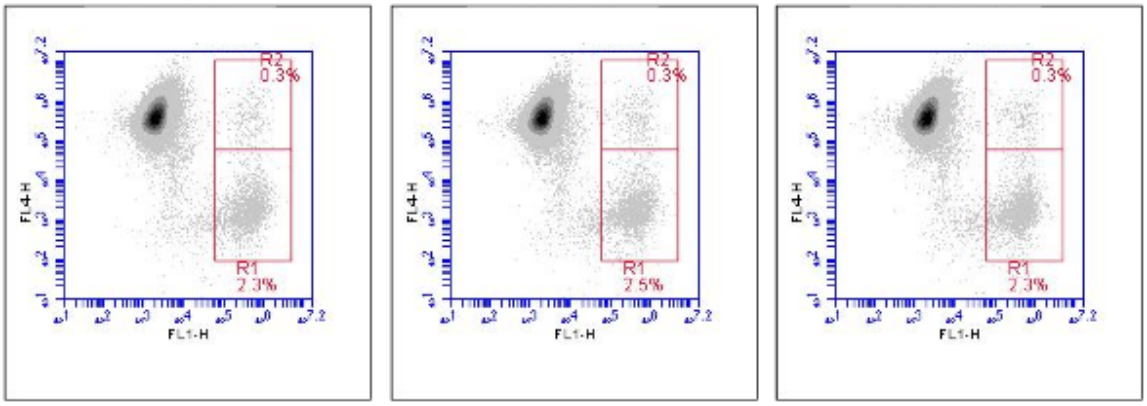
Target: A172, 0.1 nM ARM-U_{DNP}, no antibody, triplicate:



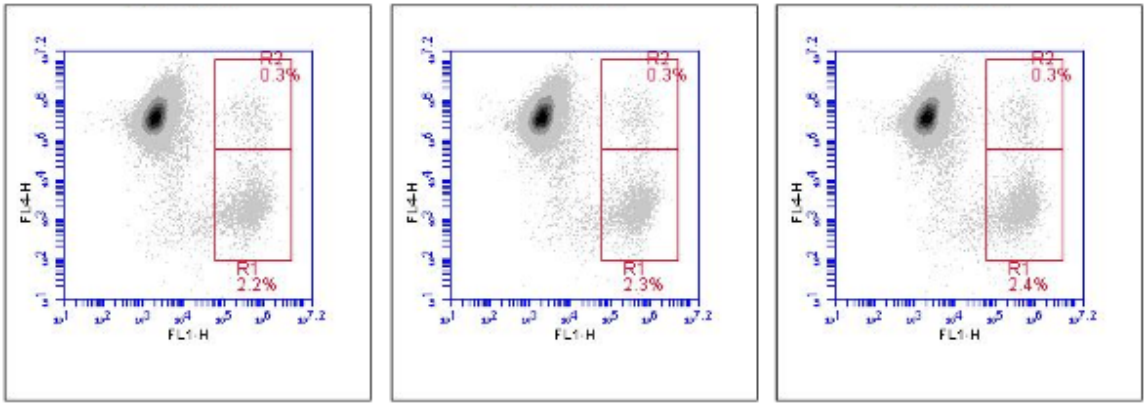
Target: A172, 0.1 nM ARM-U_{DNP}, Rabbit Anti-DNP antibody, triplicate:



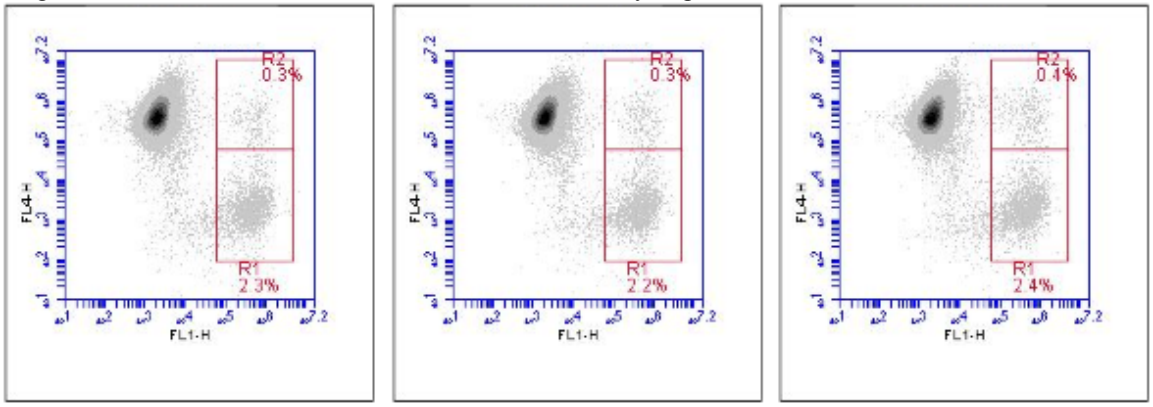
Target: A172, 0 nM ARM-U_{DNP}, no antibody, triplicate:



Target: A172, 0 nM ARM-U_{DNP}, Rabbit Anti-DNP antibody, triplicate:

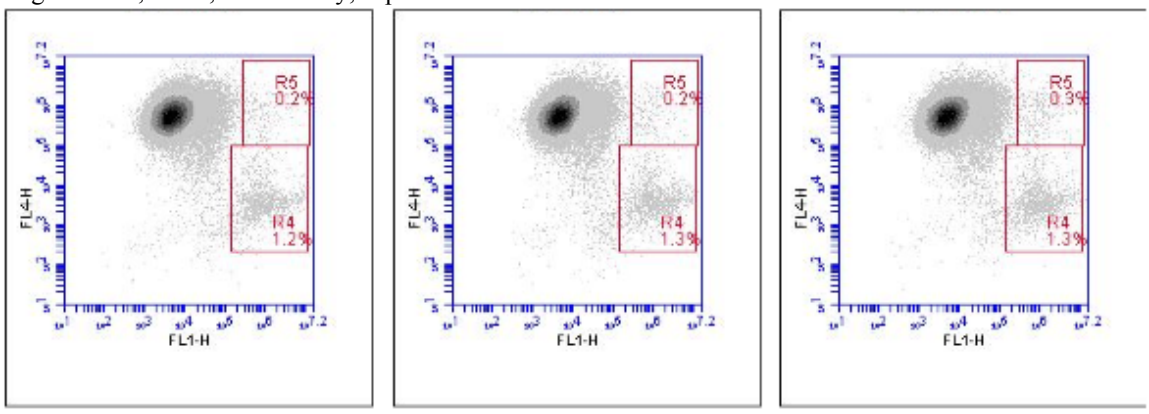


Target: A172, 0 nM ARM-U_{DNP}, Rabbit Anti-FITC antibody, triplicate:

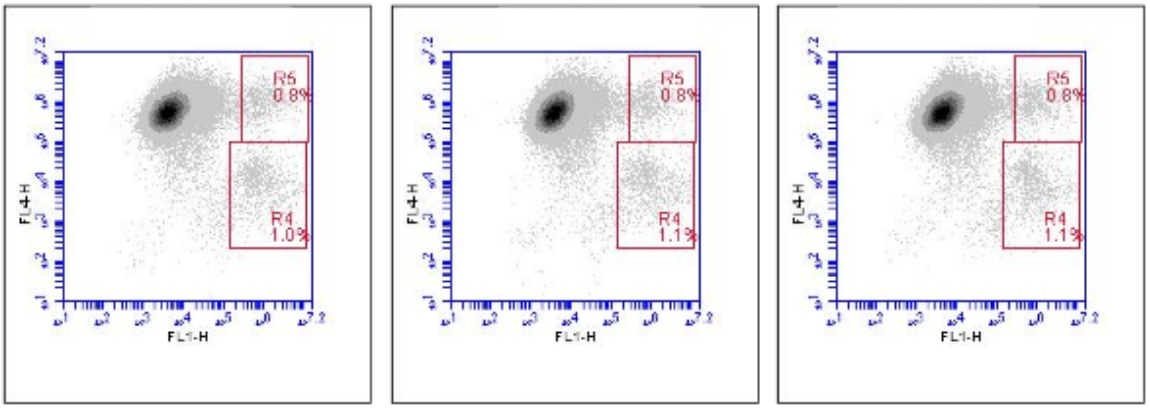


ARM-U$_{Fluor}$ to Target A172 Cells

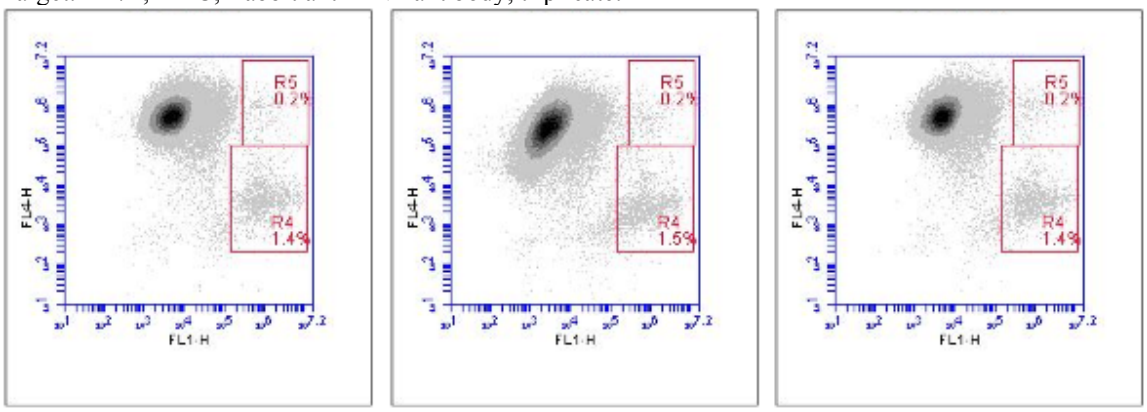
Target: A172, FITC, no antibody, triplicate:



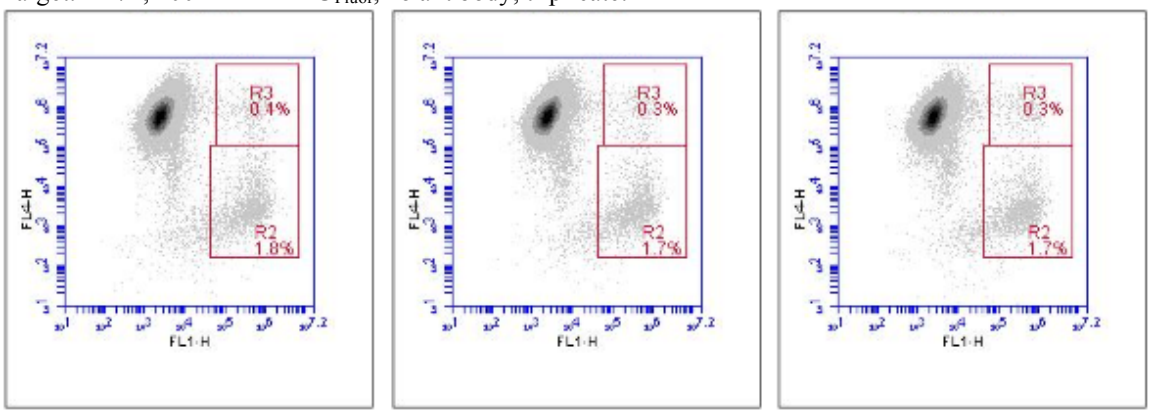
Target: A172, FITC, Rabbit anti-FITC antibody, triplicate:



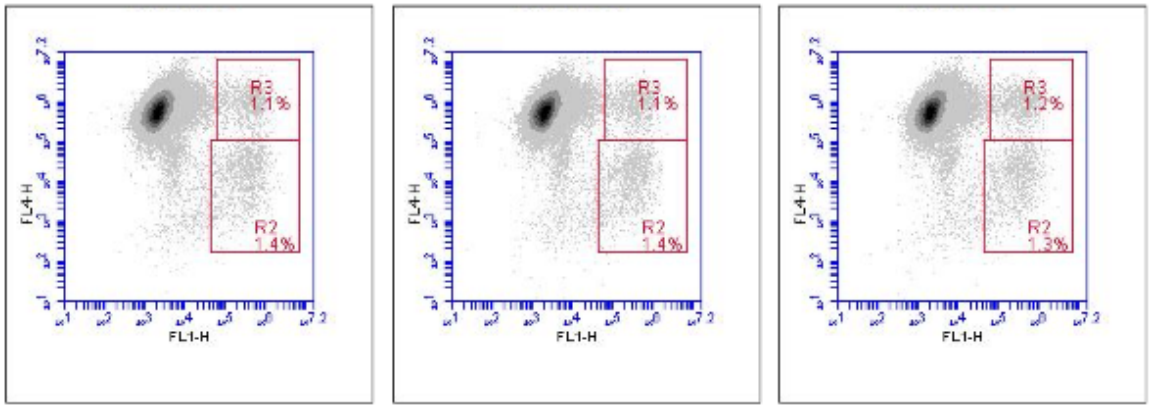
Target: A172, FITC, Rabbit anti-DNP antibody, triplicate:



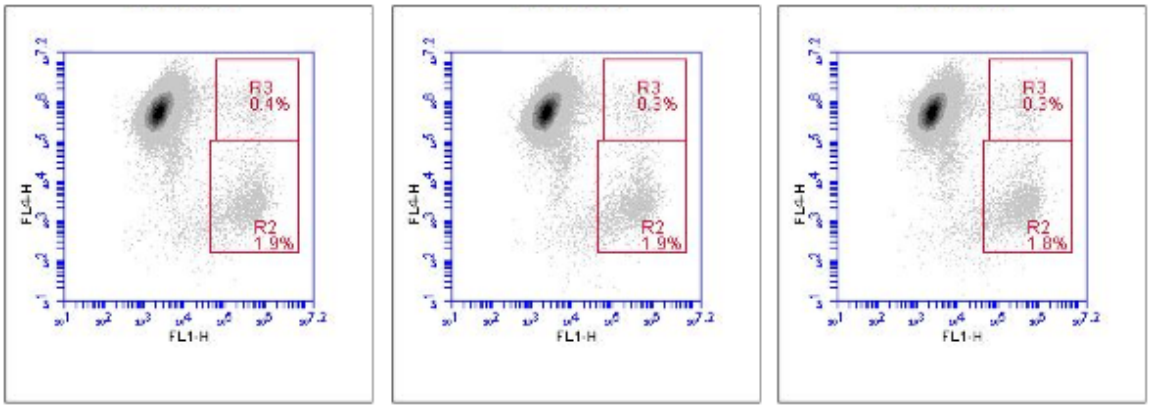
Target: A172, 100 nM ARM-U_{Fluor}, no antibody, triplicate:



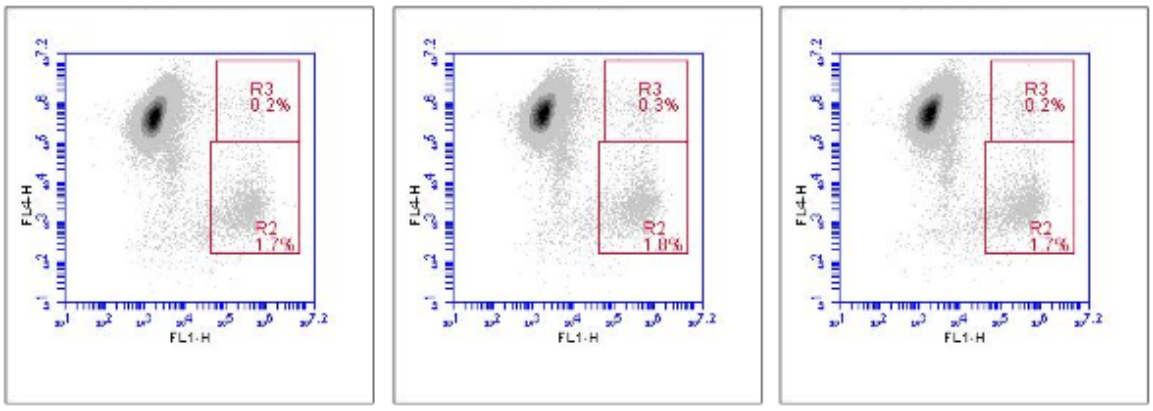
Target: A172, 100 nM ARM-U_{Fluor}, Rabbit anti-FITC antibody, triplicate:



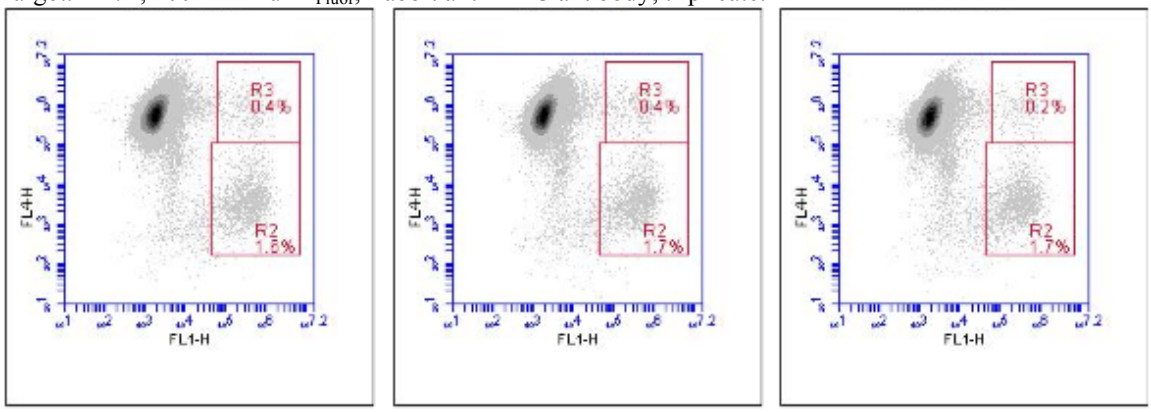
Target: A172, 100 nM ARM-U_{Fluor}, Rabbit anti-DNP antibody, triplicate:



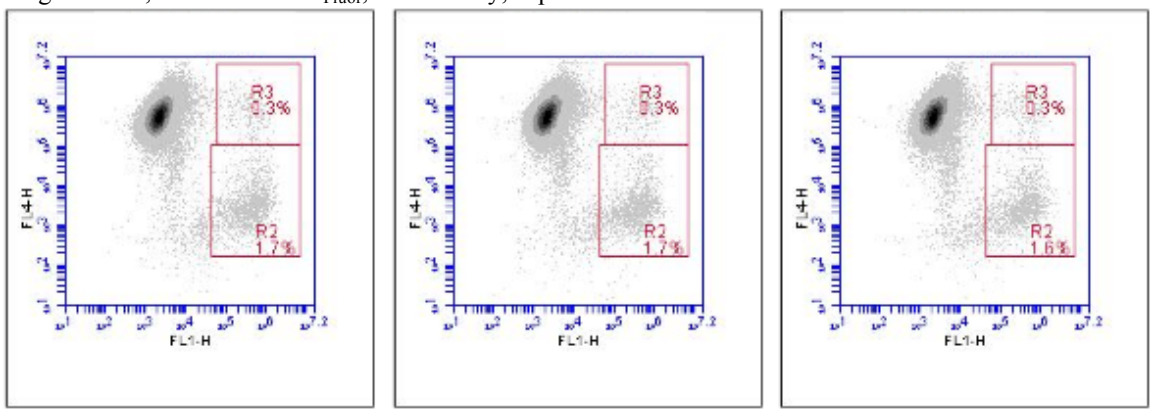
Target: A172, 100 nM L-uPA$_{Fluor}$, no antibody, triplicate:



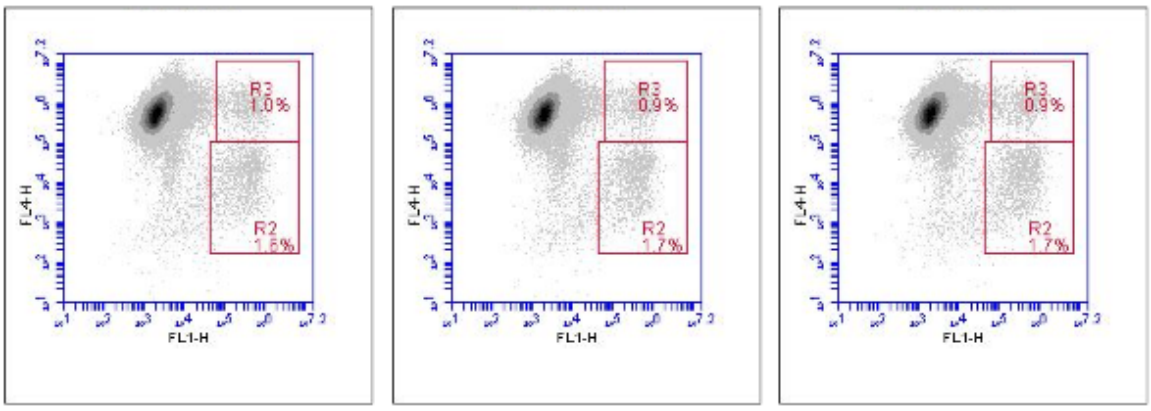
Target: A172, 100 nM L-uPA$_{Fluor}$, Rabbit anti-FITC antibody, triplicate:



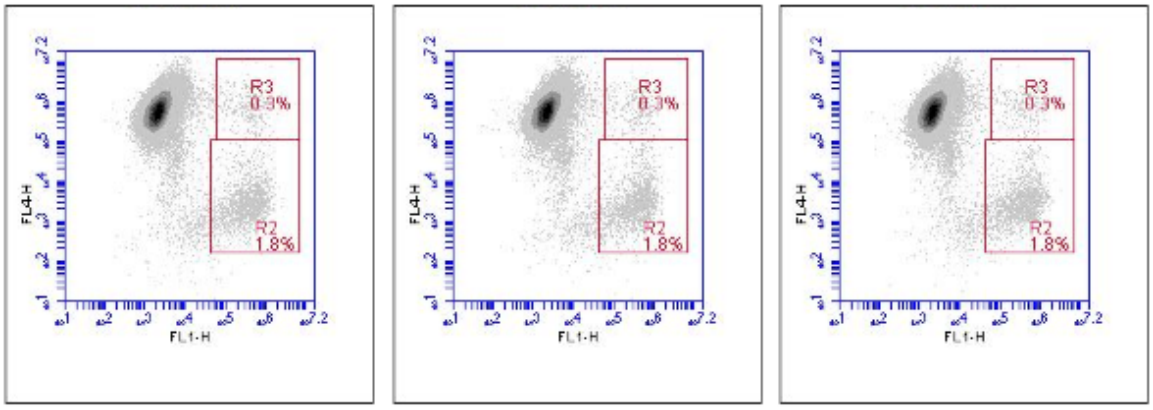
Target: A172, 10 nM ARM-U$_{Fluor}$, no antibody, triplicate:



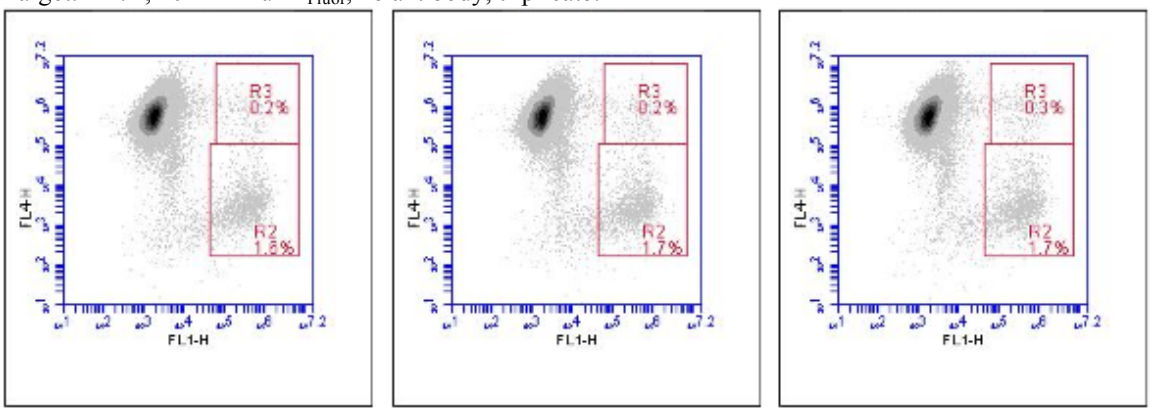
Target: A172, 10 nM ARM-U_{Fluor}, Rabbit anti-FITC antibody, triplicate:



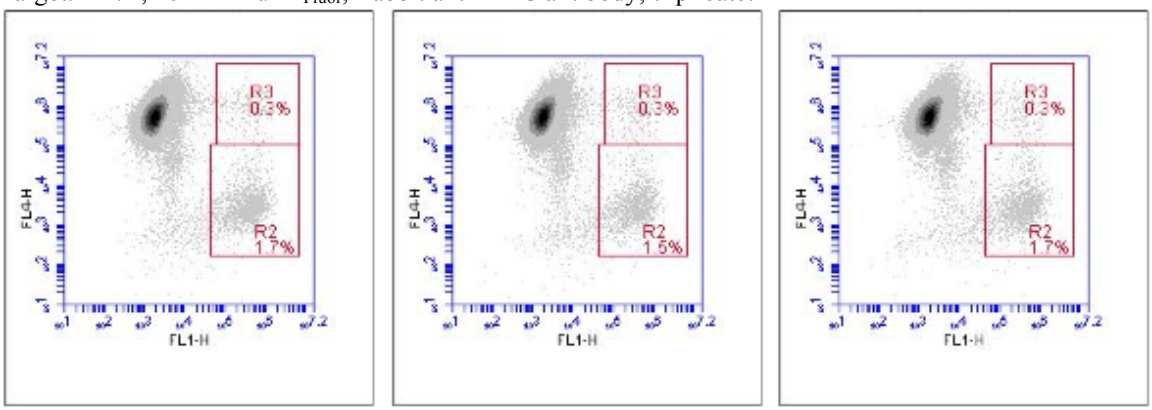
Target: A172, 10 nM ARM-U_{Fluor}, Rabbit anti-DNP antibody, triplicate:



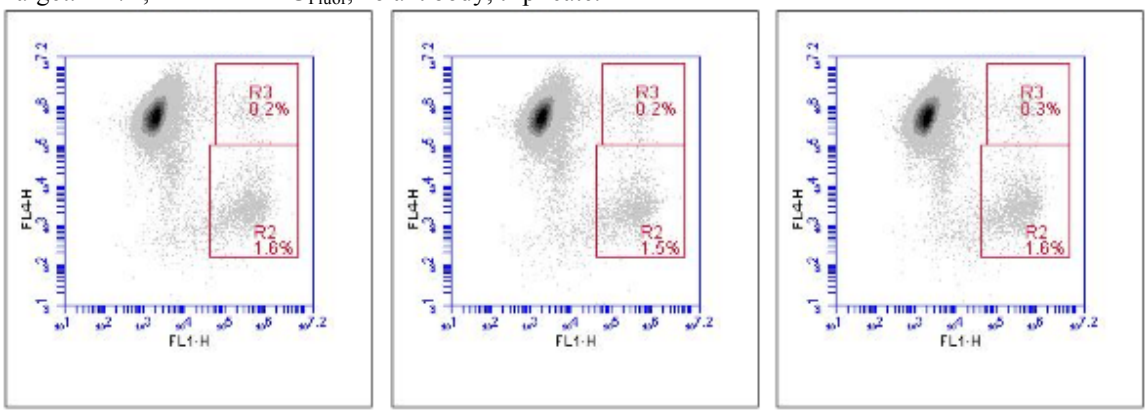
Target: A172, 10 nM L-uPA$_{Fluor}$, no antibody, triplicate:



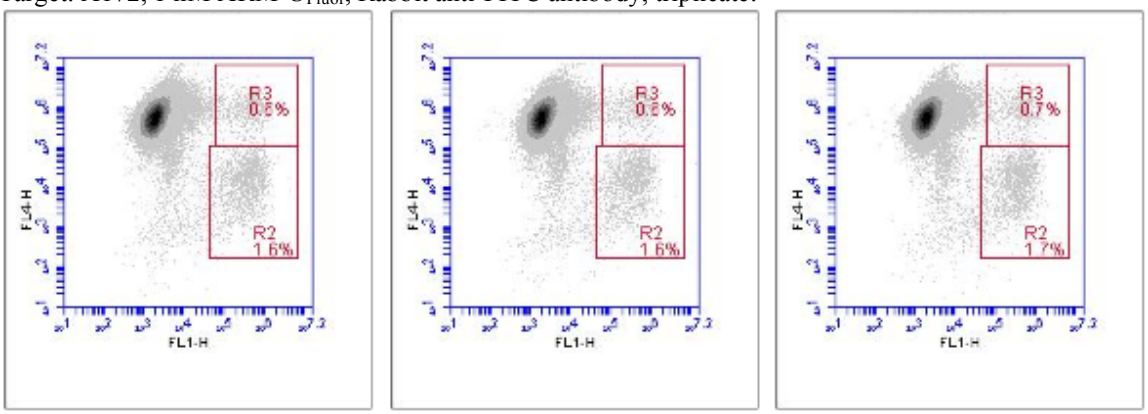
Target: A172, 10 nM L-uPA$_{Fluor}$, Rabbit anti-FITC antibody, triplicate:



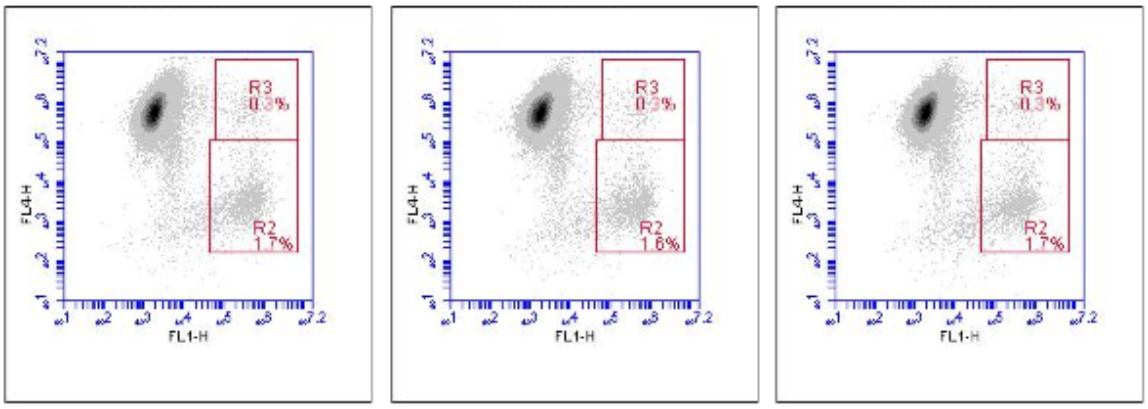
Target: A172, 1 nM ARM-U$_{Fluor}$, no antibody, triplicate:



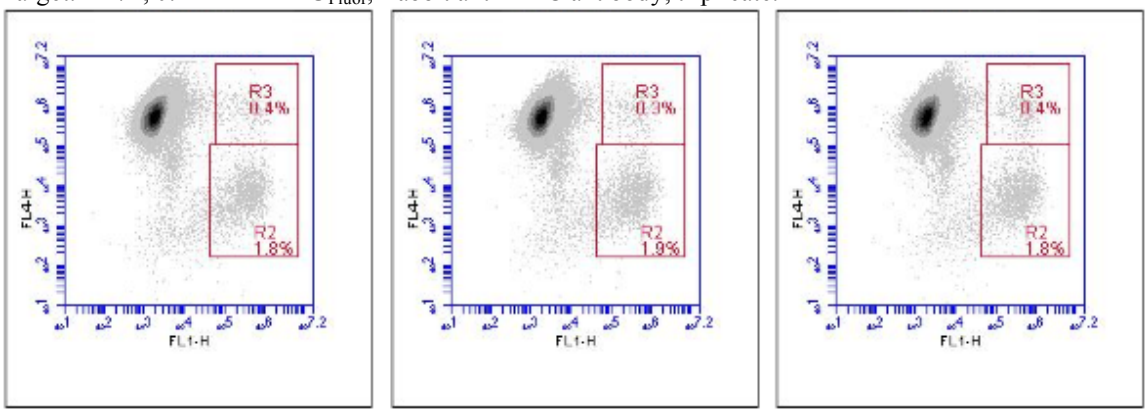
Target: A172, 1 nM ARM-U$_{Fluor}$, Rabbit anti-FITC antibody, triplicate:



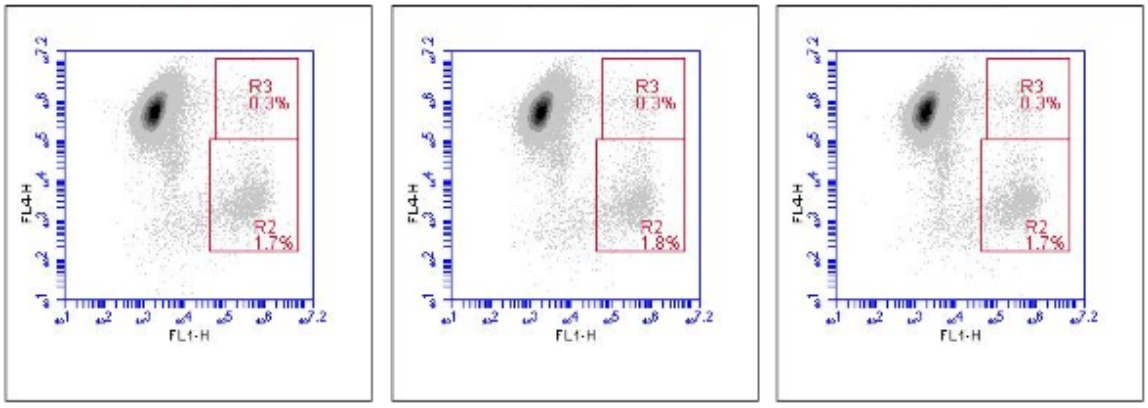
Target: A172, 0.1 nM ARM-U_{Fluor}, no antibody, triplicate:



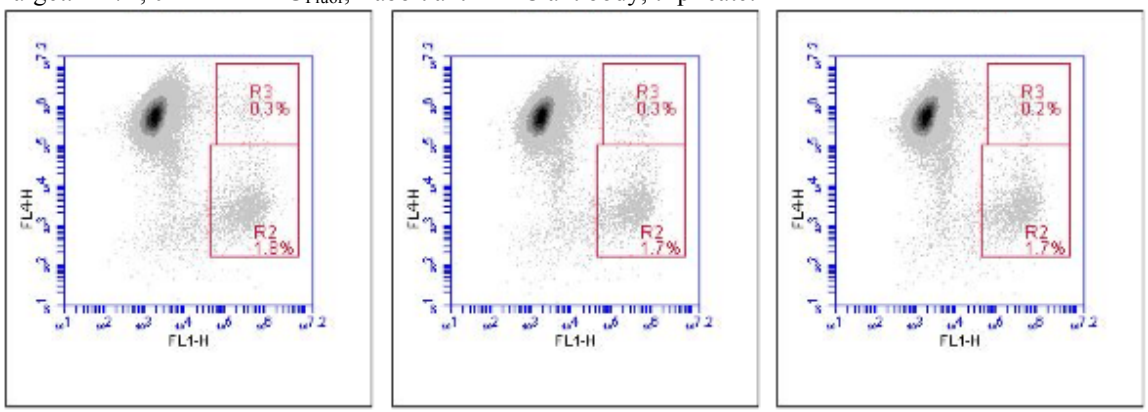
Target: A172, 0.1 nM ARM-U_{Fluor}, Rabbit anti-FITC antibody, triplicate:



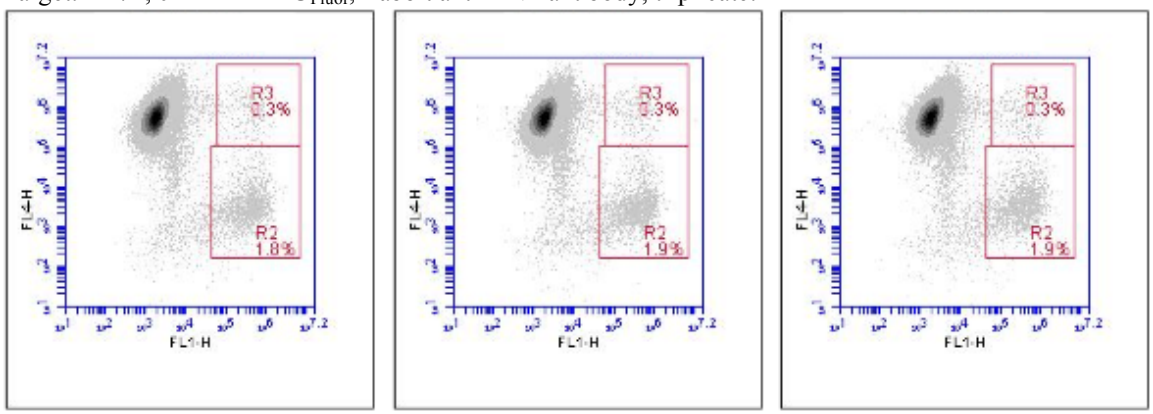
Target: A172, 0 nM ARM-U_{Fluor}, no antibody, triplicate:



Target: A172, 0 nM ARM-U_{Fluor}, Rabbit anti-FITC antibody, triplicate:

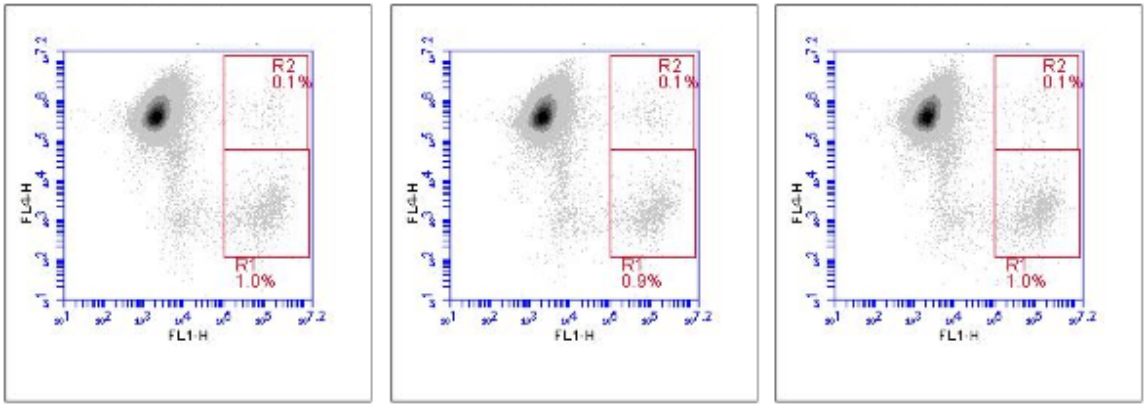


Target: A172, 0 nM ARM-U_{Fluor}, Rabbit anti-DNP antibody, triplicate:

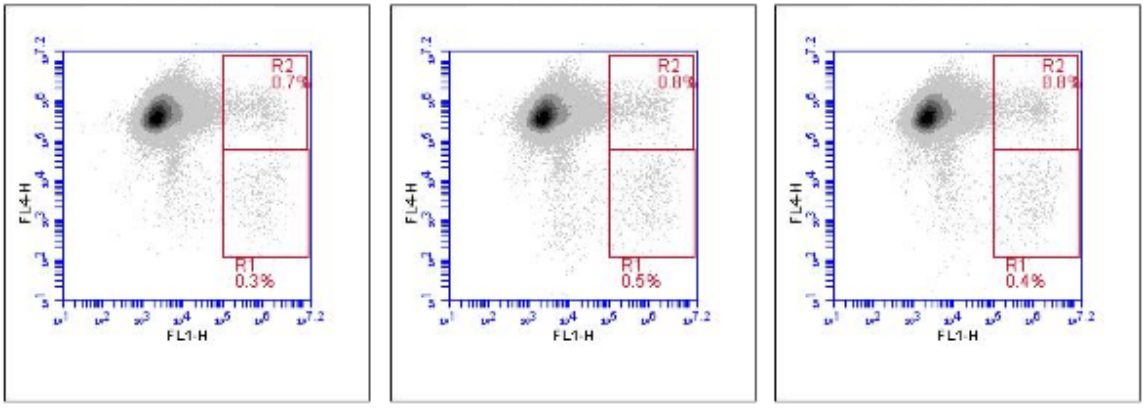


ARM-U_{DNP} to Target HT-29 Cells

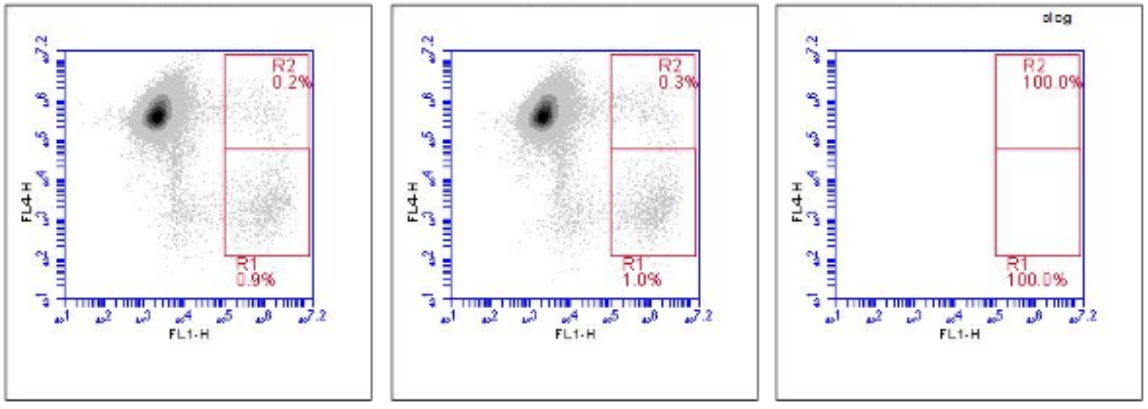
Target: HT-29, TNP, no antibody, triplicate:



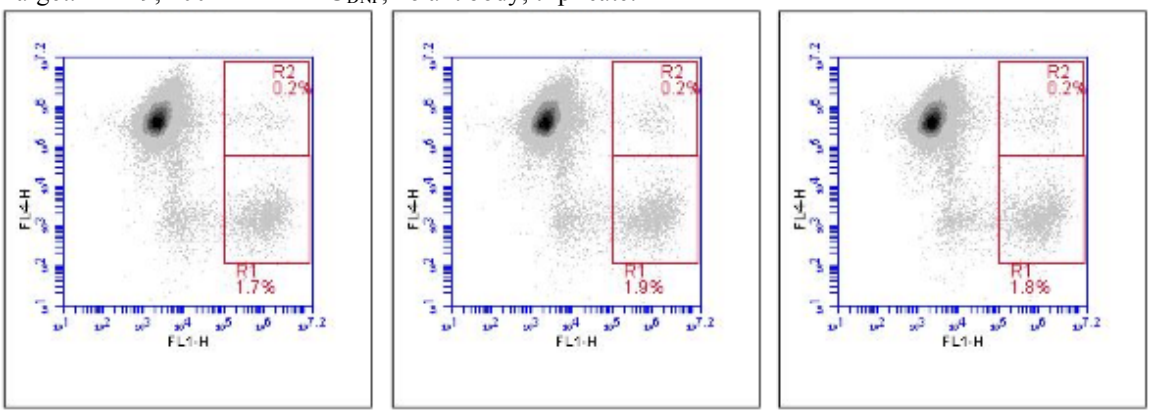
Target: HT-29, TNP, Rabbit Anti-DNP antibody, triplicate:



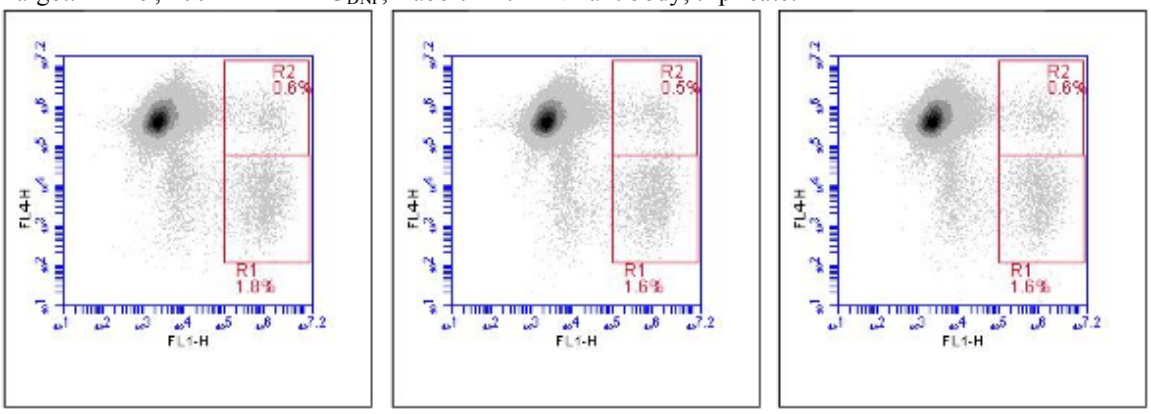
Target: HT-29, TNP, Rabbit Anti-FITC antibody, Duplicate due to machine error:



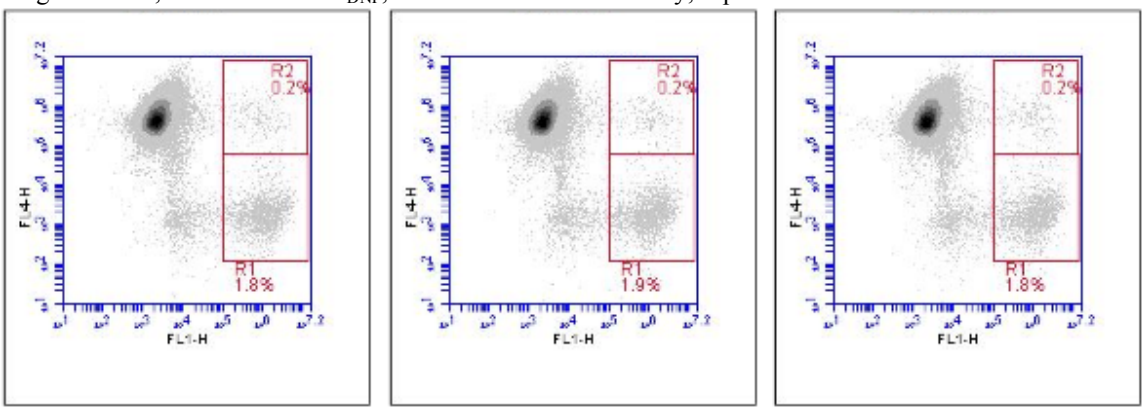
Target: HT-29, 100 nM ARM-U_{DNP}, no antibody, triplicate:



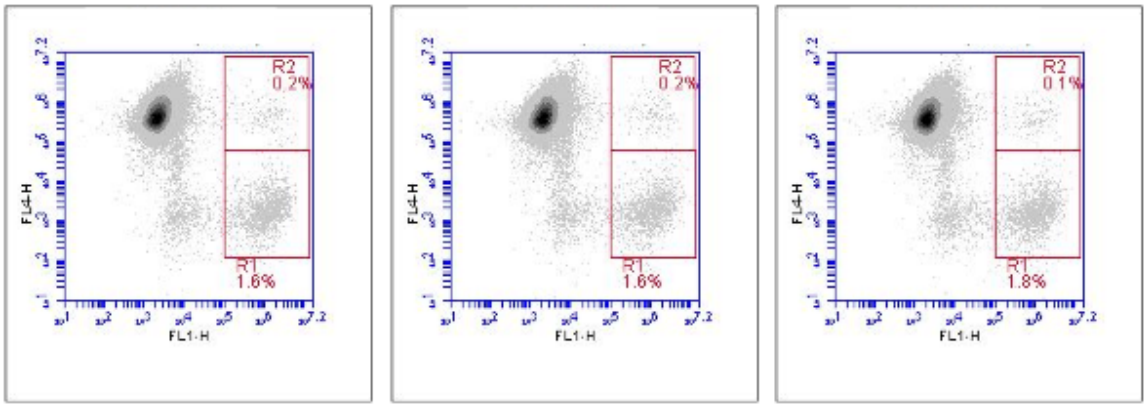
Target: HT-29, 100 nM ARM-U_{DNP}, Rabbit Anti-DNP antibody, triplicate:



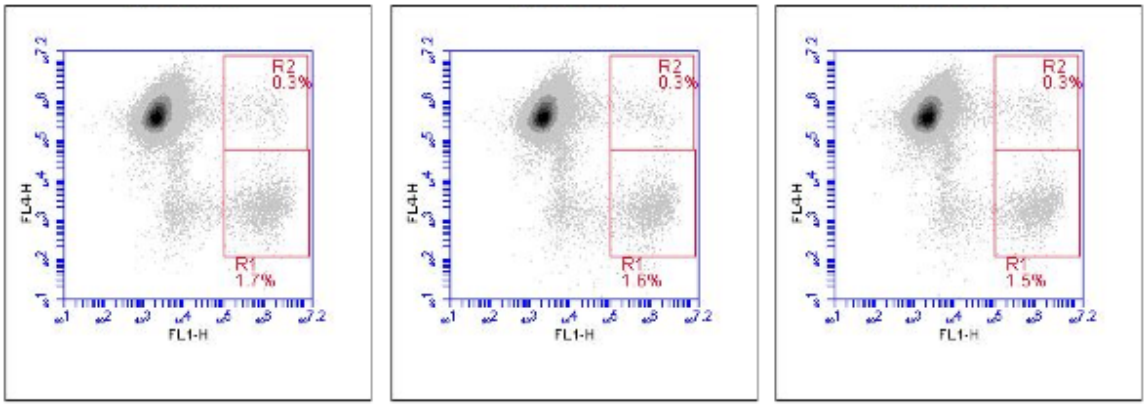
Target: HT-29, 100 nM ARM-U_{DNP}, Rabbit Anti-FITC antibody, triplicate:



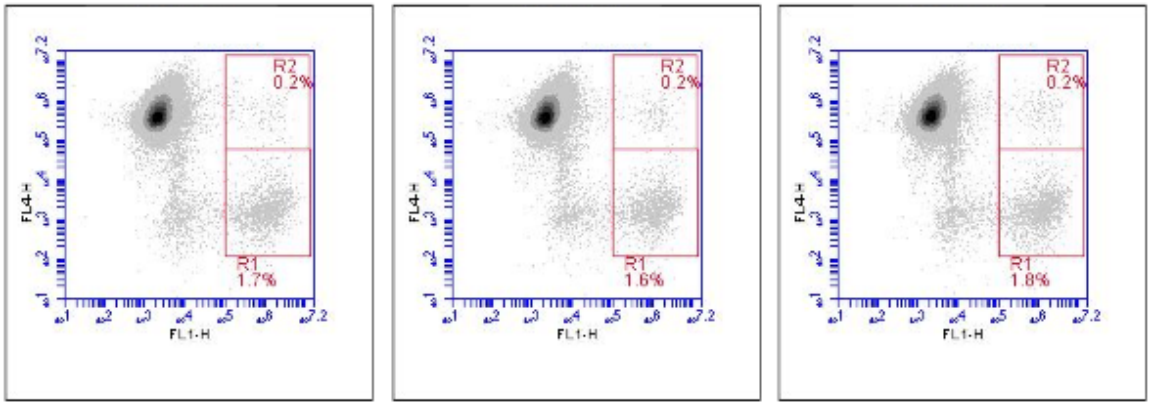
Target: HT-29, 100 nM L-uPA$_{DNP}$, no antibody, triplicate:



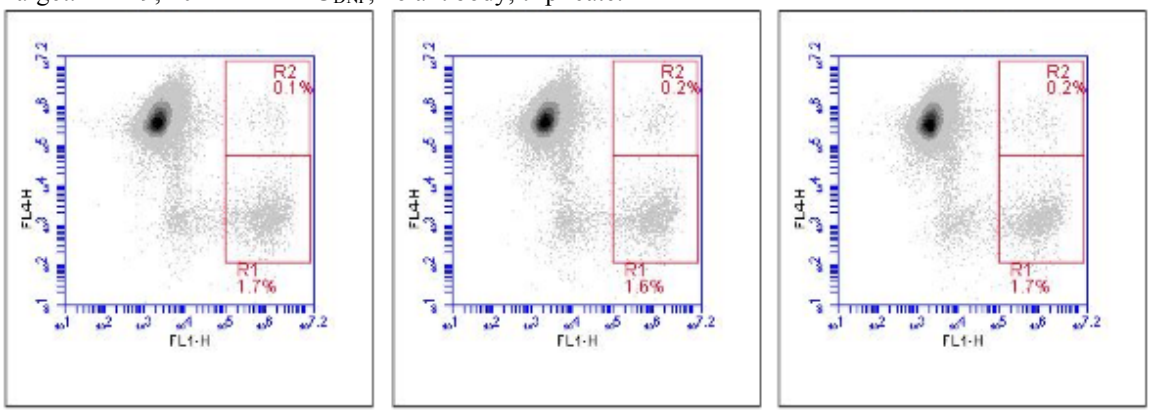
Target: HT-29, 100 nM L-uPA$_{DNP}$, Rabbit Anti-DNP antibody, triplicate:



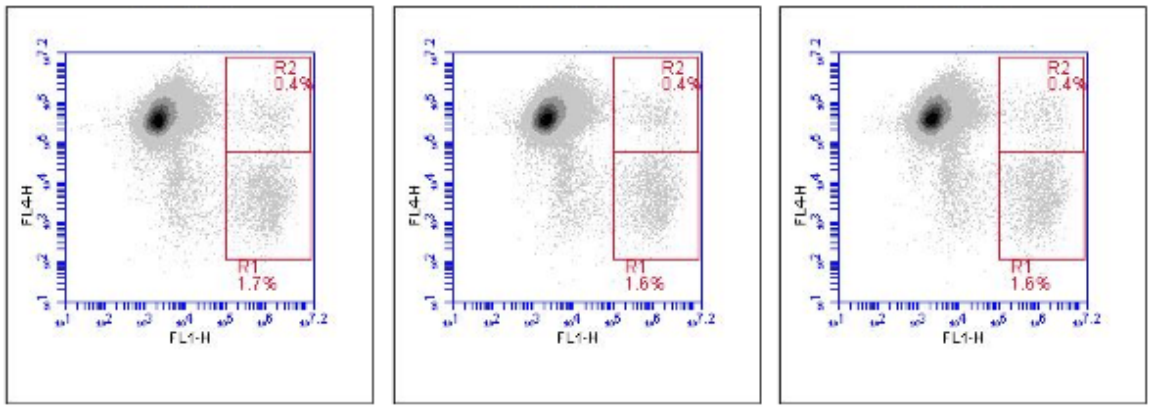
Target: HT-29, 100 nM L-uPA$_{DNP}$, Rabbit Anti-FITC antibody, triplicate:



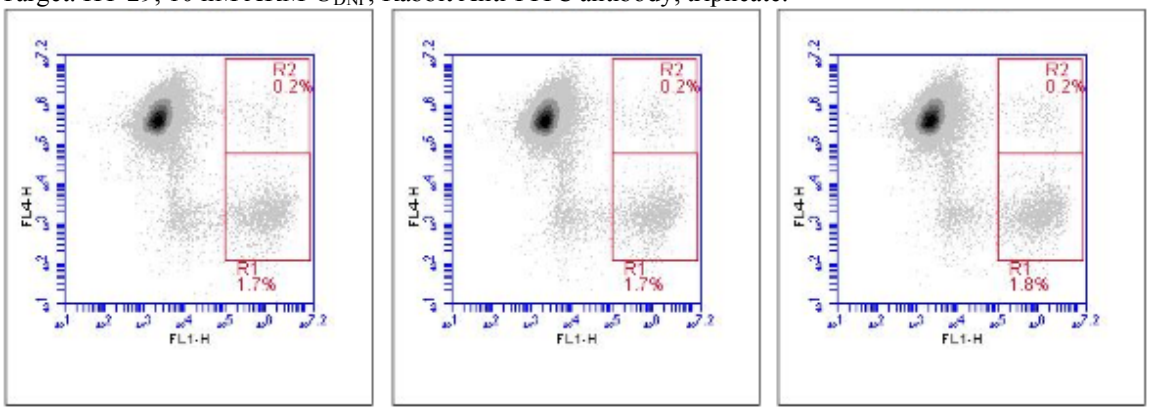
Target: HT-29, 10 nM ARM-U_{DNP}, no antibody, triplicate:



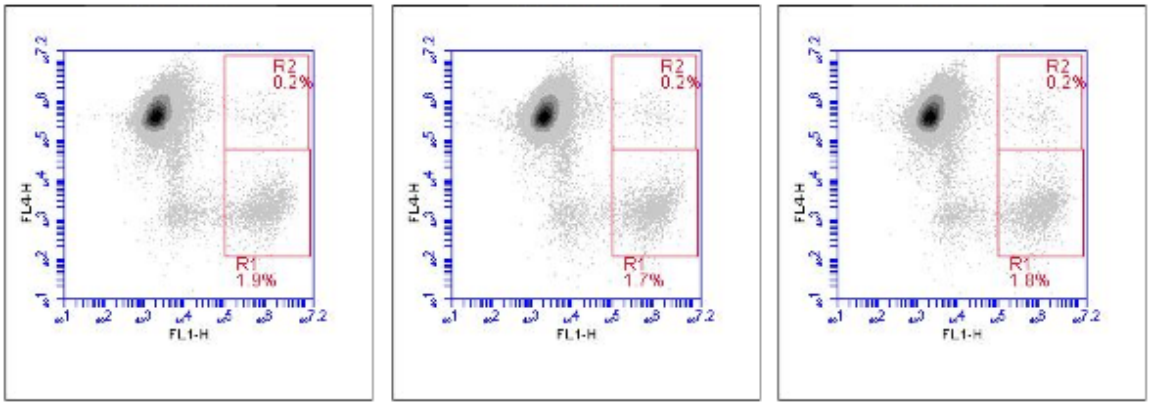
Target: HT-29, 10 nM ARM-U_{DNP}, Rabbit Anti-DNP antibody, triplicate:



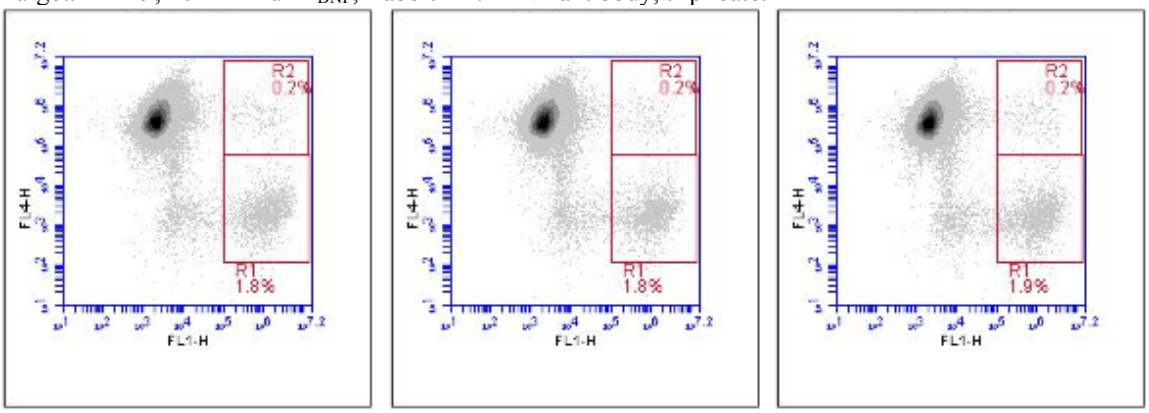
Target: HT-29, 10 nM ARM-U_{DNP}, Rabbit Anti-FITC antibody, triplicate:



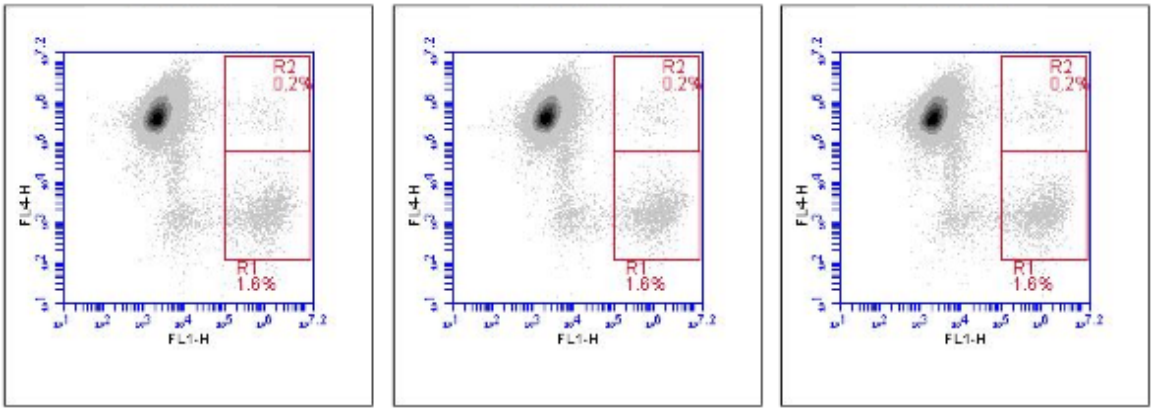
Target: HT-29, 10 nM L-uPA$_{DNP}$, no antibody, triplicate:



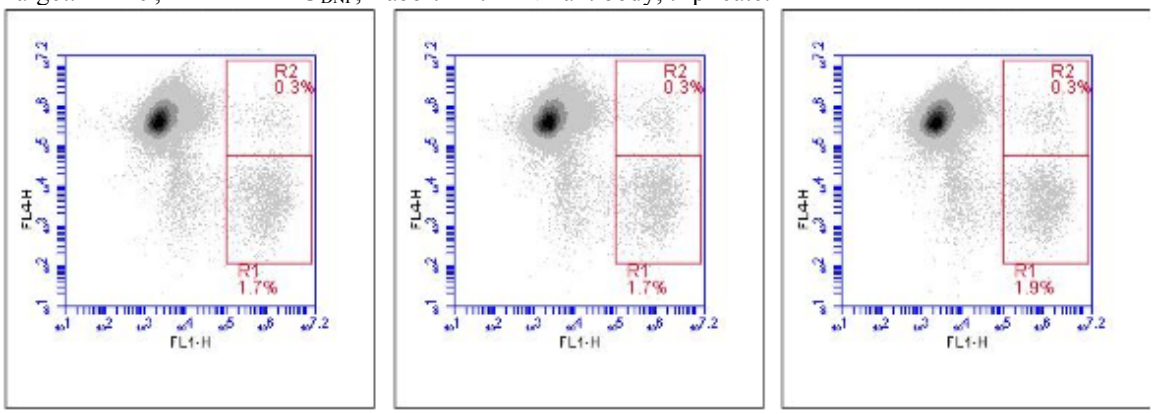
Target: HT-29, 10 nM L-uPA$_{DNP}$, Rabbit Anti-DNP antibody, triplicate:



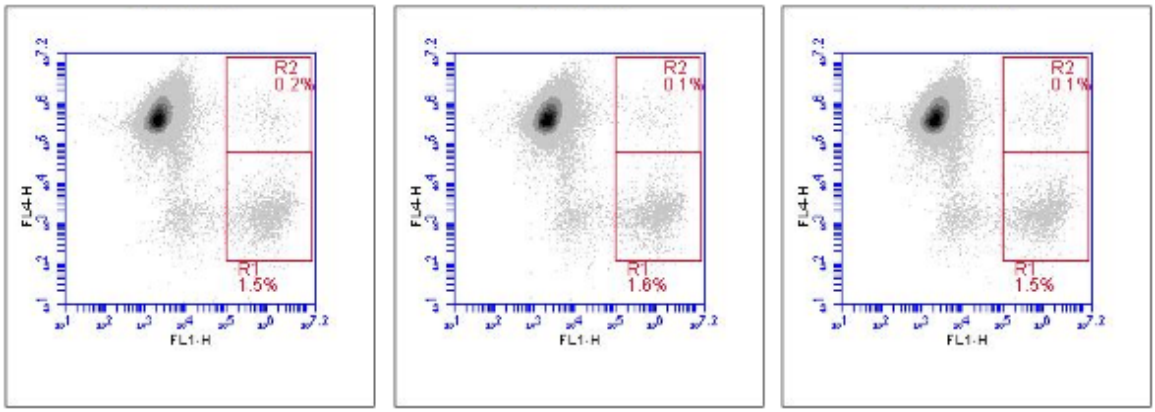
Target: HT-29, 1 nM ARM-U$_{DNP}$, no antibody, triplicate:



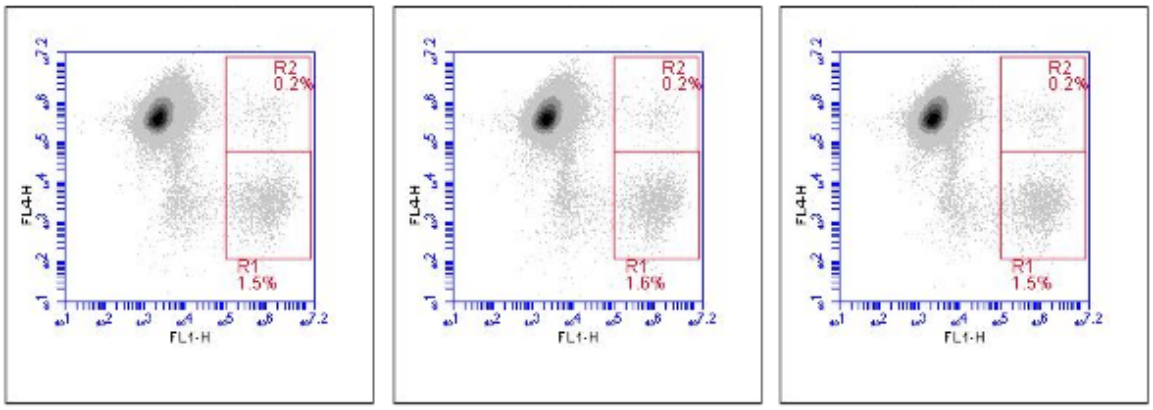
Target: HT-29, 1 nM ARM-U_{DNP}, Rabbit Anti-DNP antibody, triplicate:



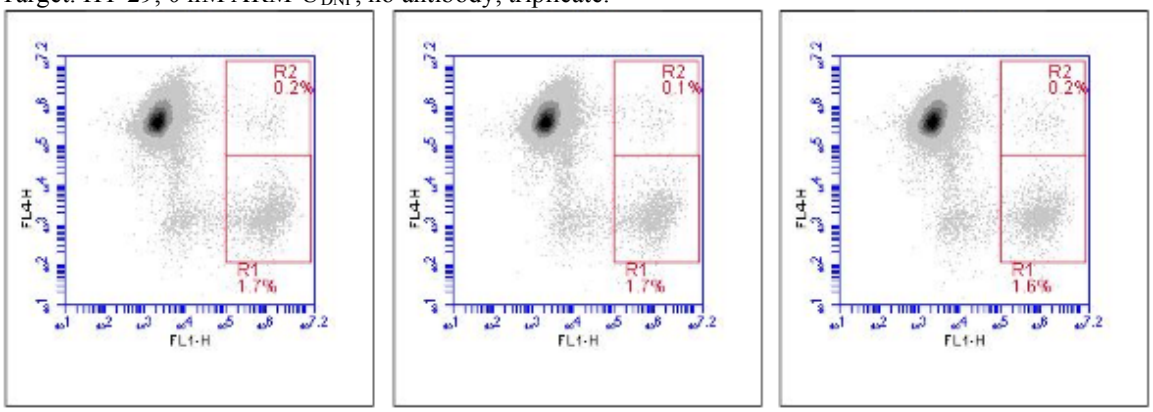
Target: HT-29, 0.1 nM ARM-U_{DNP}, no antibody, triplicate:



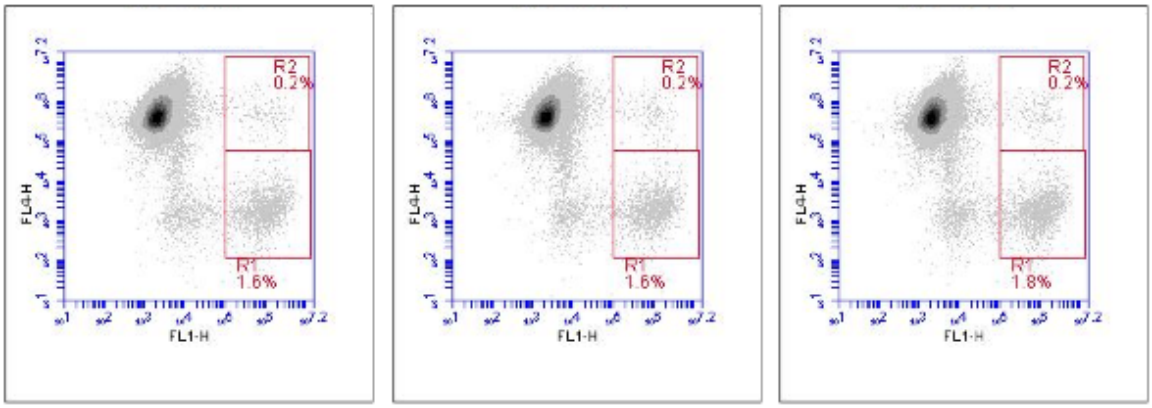
Target: HT-29, 0.1 nM ARM-U_{DNP}, Rabbit Anti-DNP antibody, triplicate:



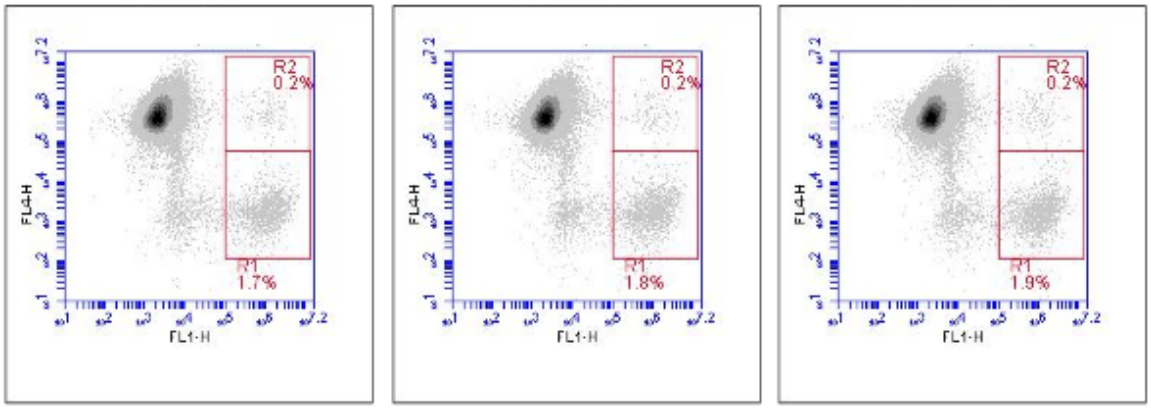
Target: HT-29, 0 nM ARM-U_{DNP}, no antibody, triplicate:



Target: HT-29, 0 nM ARM-U_{DNP}, Rabbit Anti-DNP antibody, triplicate:

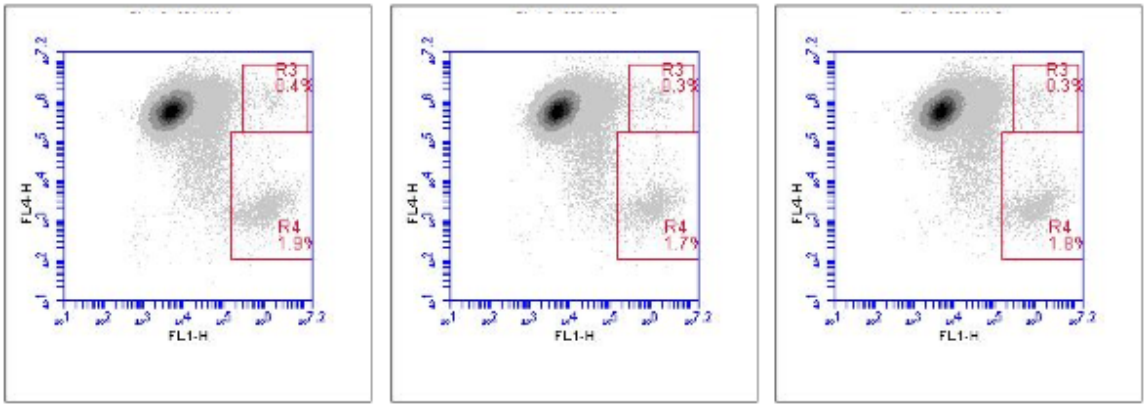


Target: HT-29, 0 nM ARM-U_{DNP}, Rabbit Anti-FITC antibody, triplicate:

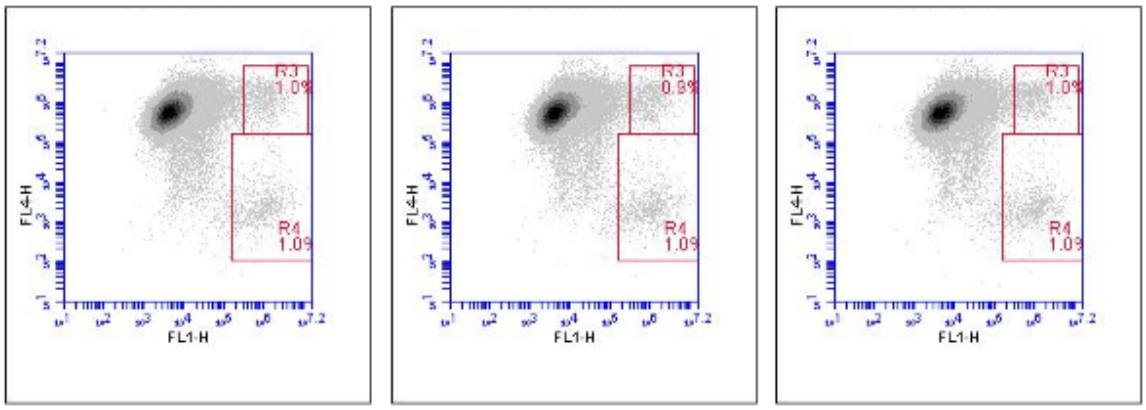


ARM-U_{Fluor} to Target HT-29 Cells

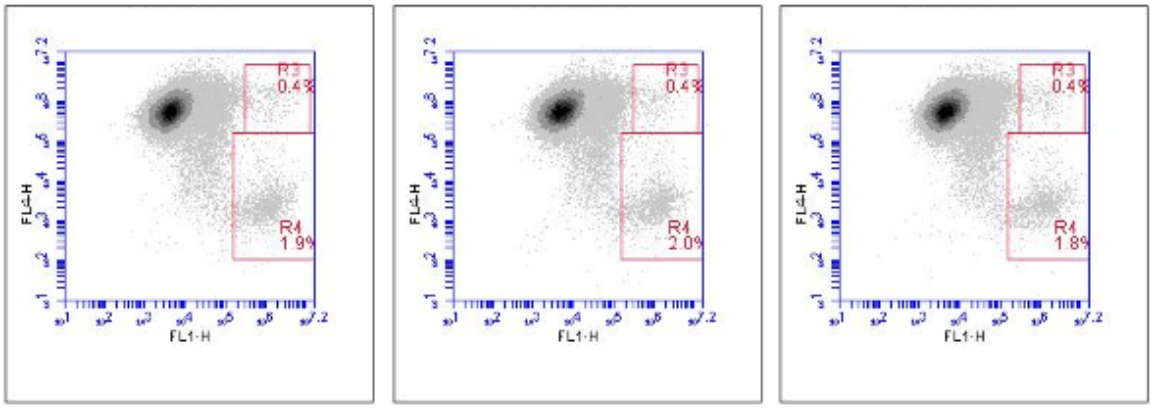
Target: HT-29, FITC, no antibody, triplicate:



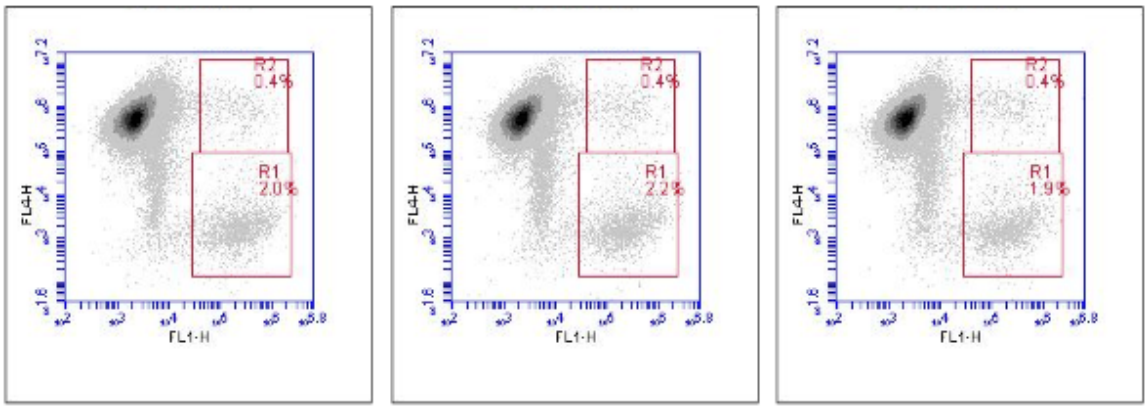
Target: HT-29, FITC, Rabbit anti-FITC, triplicate:



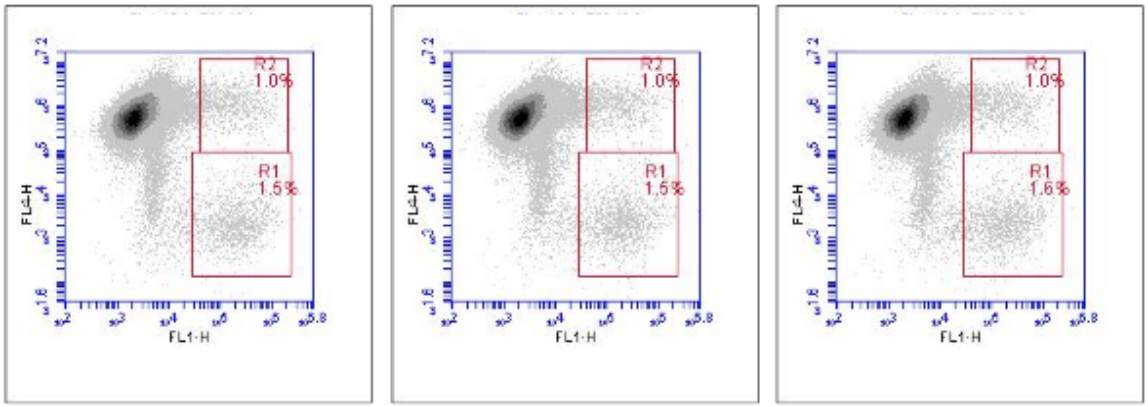
Target: HT-29, FITC, Rabbit anti-DNP, triplicate:



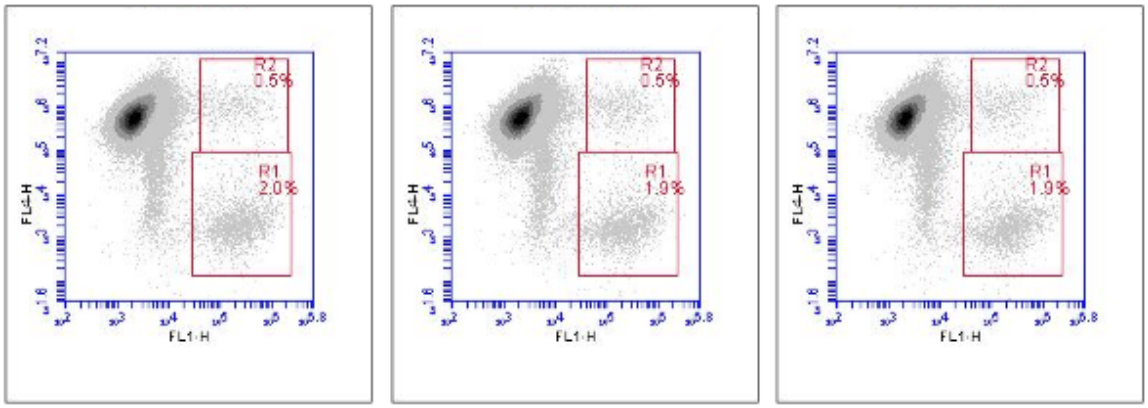
Target: HT-29, 100 nM ARM-U_{Fluor}, no antibody, triplicate:



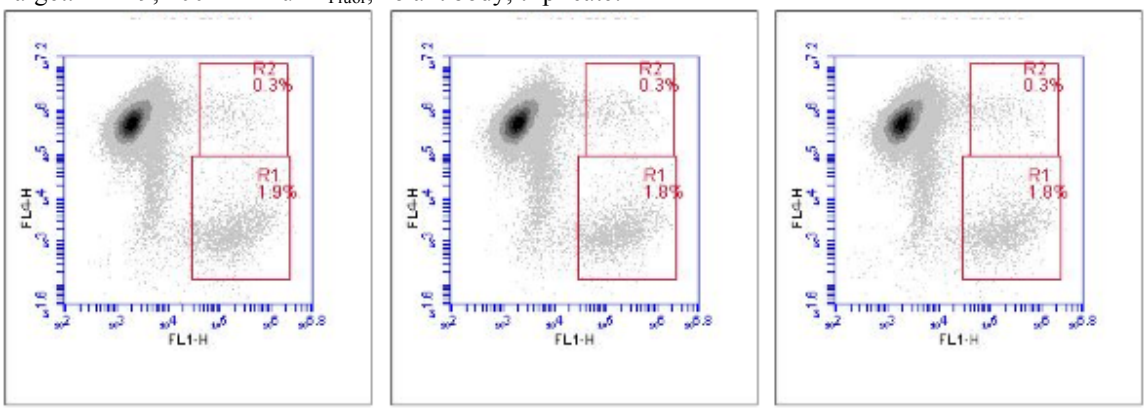
Target: HT-29, 100 nM ARM-U_{Fluor}, Rabbit anti-FITC antibody, triplicate:



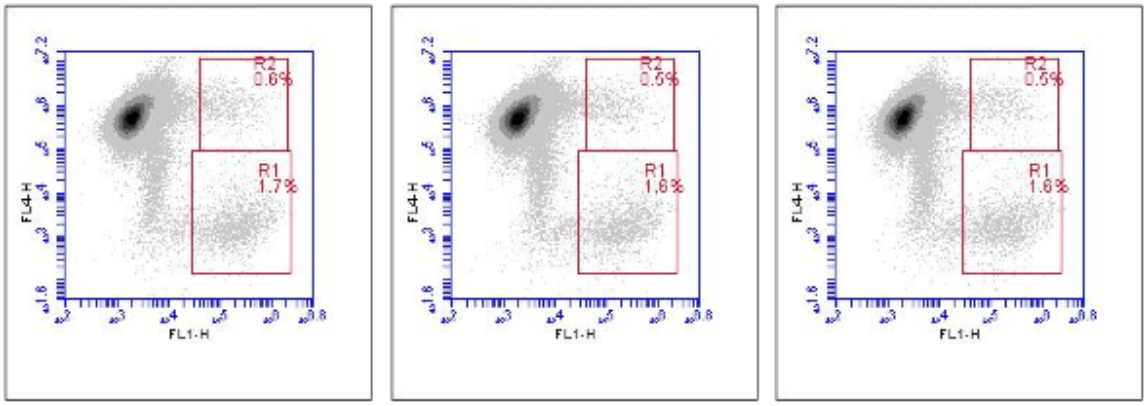
Target HT-29, 100 nM ARM-U_{Fluor}, Rabbit anti-DNP antibody, triplicate:



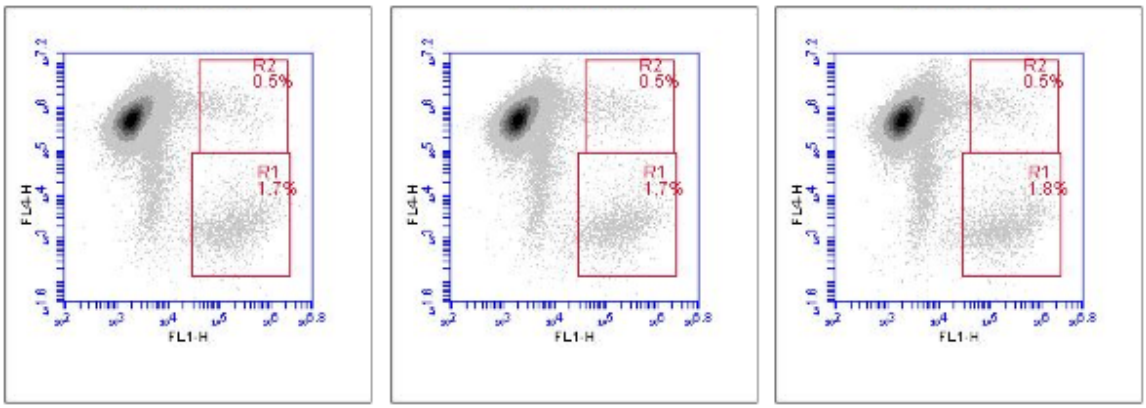
Target: HT-29, 100 nM L-uPA$_{Fluor}$, no antibody, triplicate:



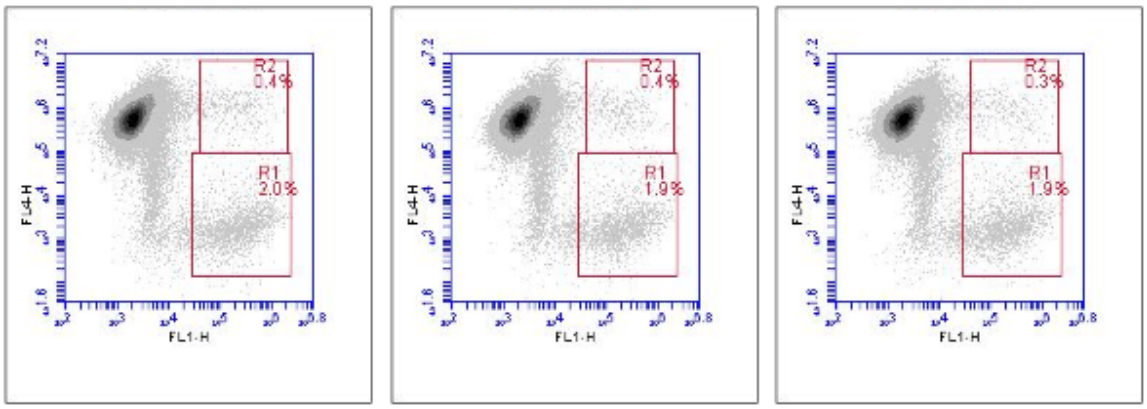
Target: HT-29, 100 nM L-uPA$_{Fluor}$, Rabbit anti-FITC, triplicate:



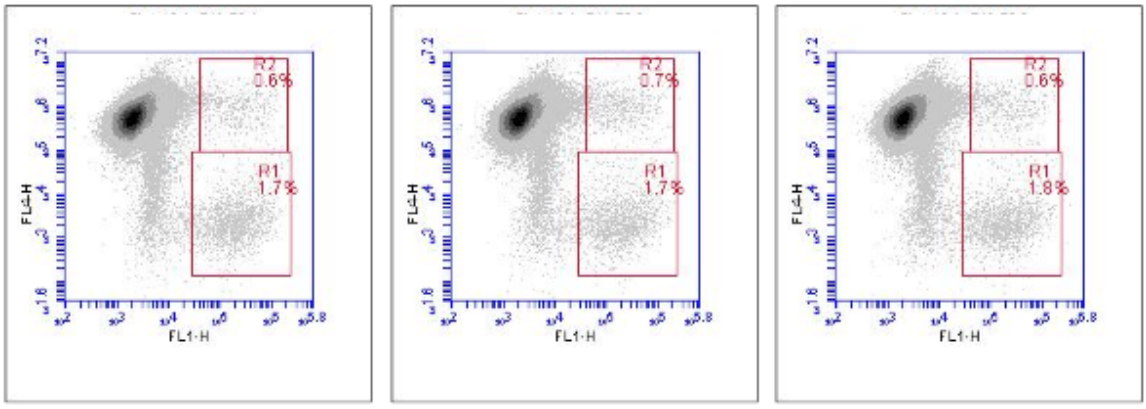
Target: HT-29, 100 nM L-uPA$_{Fluor}$, Rabbit anti-DNP, triplicate:



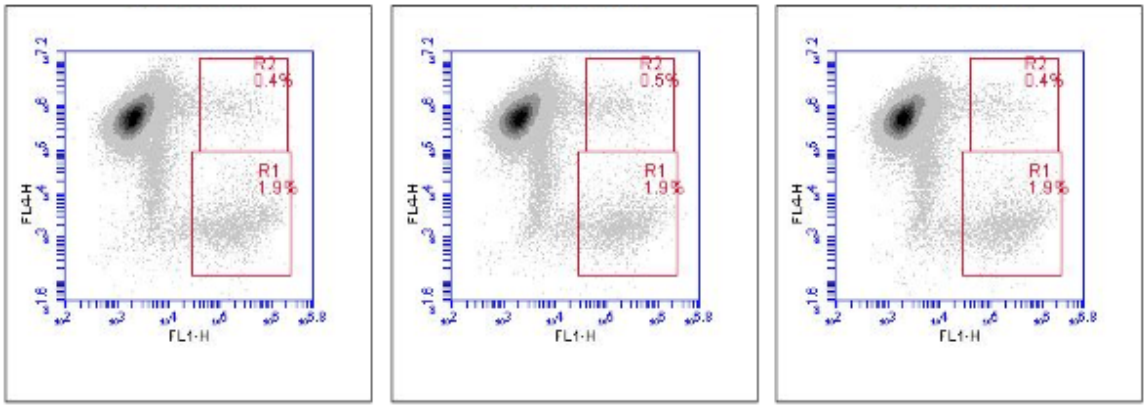
Target: HT-29, 10 nM ARM-U_{Fluor}, no antibody, triplicate:



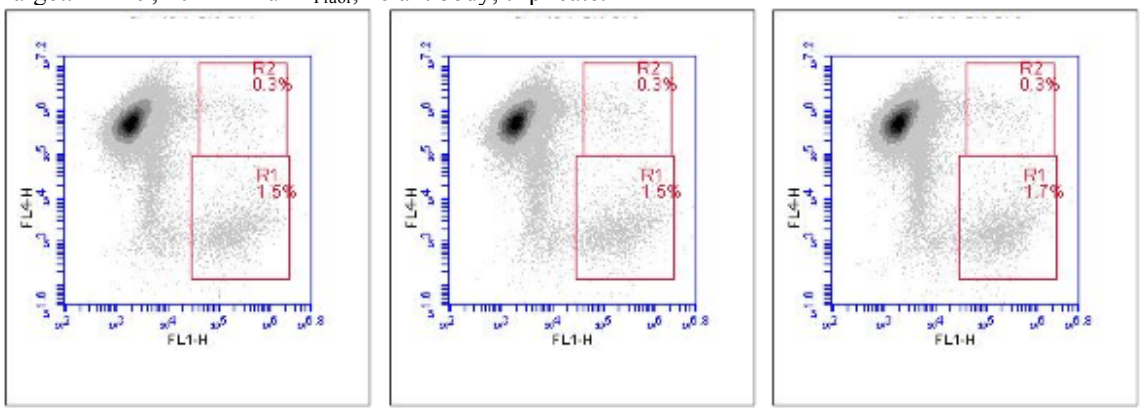
Target: HT-29, 10 nM ARM-U_{Fluor}, Rabbit anti-FITC antibody, triplicate:



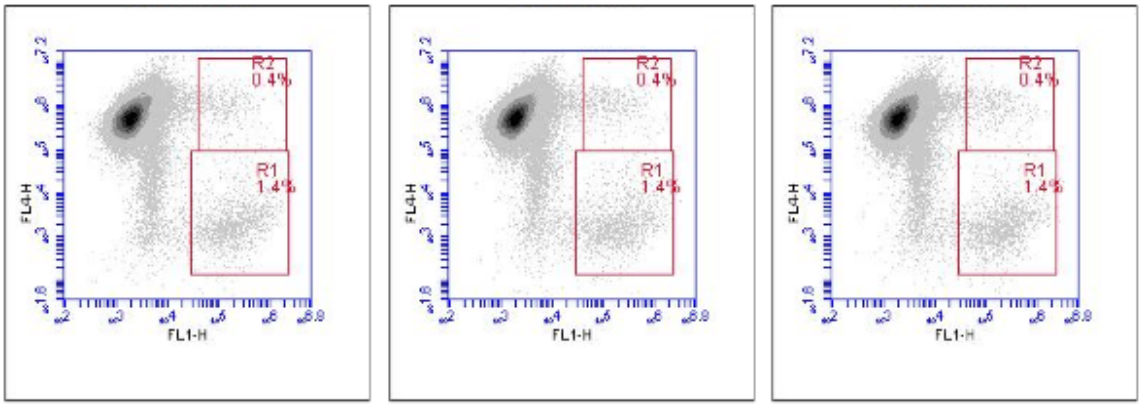
Target: HT-29, 10 nM ARM-U_{Fluor}, Rabbit anti-FITC antibody, triplicate:



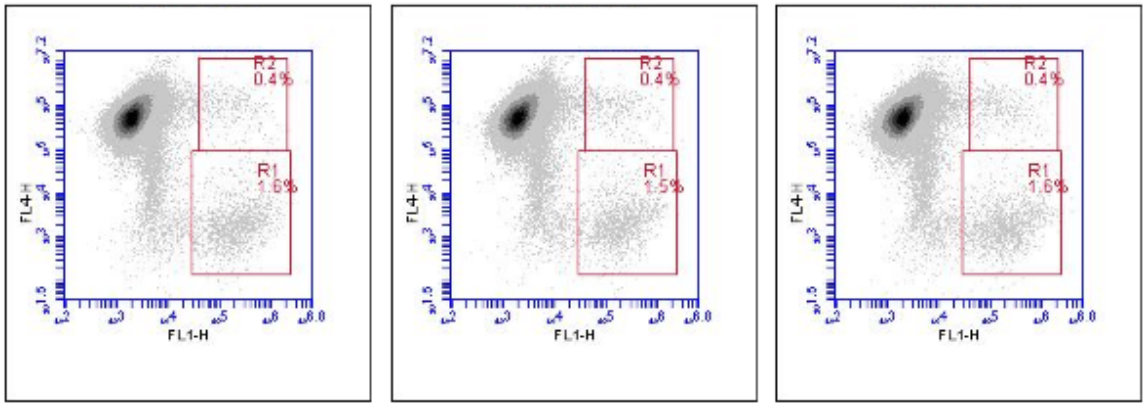
Target: HT-29, 10 nM L-uPA$_{Fluor}$, no antibody, triplicate:



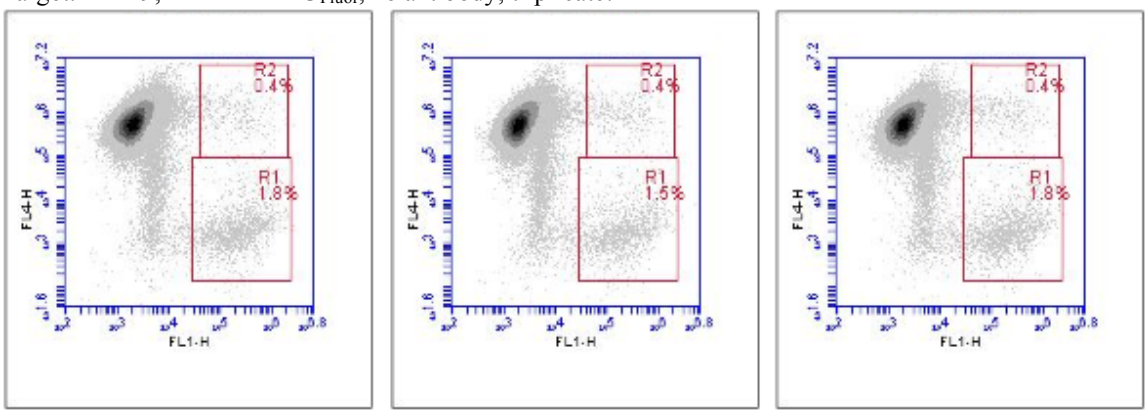
Target: HT-29, 10 nM L-uPA$_{Fluor}$, Rabbit anti-FITC, triplicate:



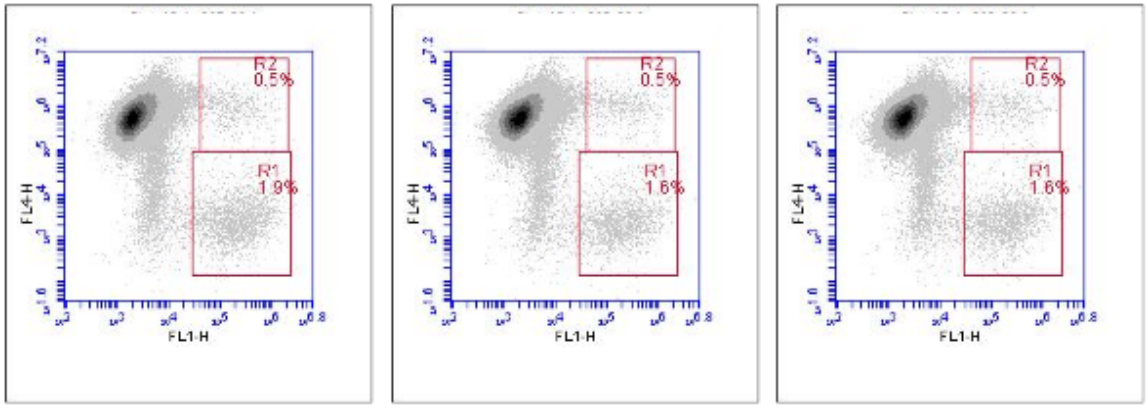
Target: HT-29, 10 nM L-uPA$_{Fluor}$, Rabbit anti-DNP, triplicate:



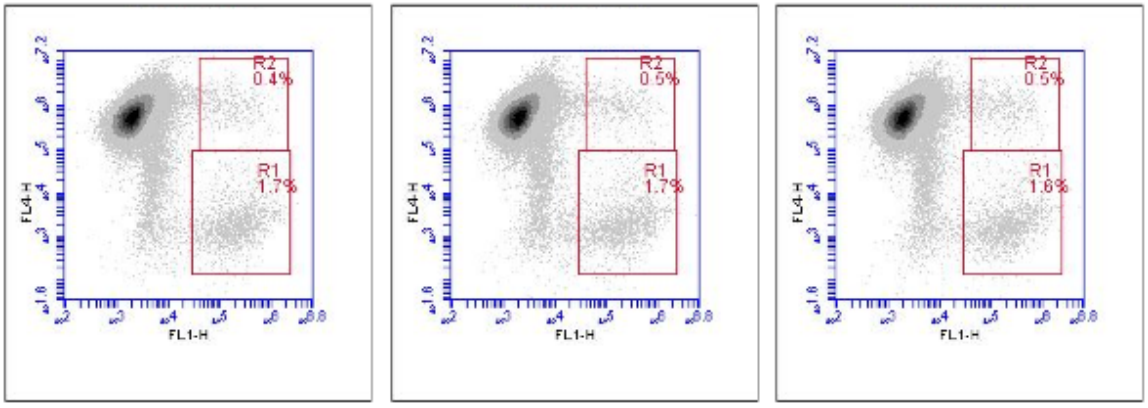
Target: HT-29, 1 nM ARM-U_{Fluor}, no antibody, triplicate:



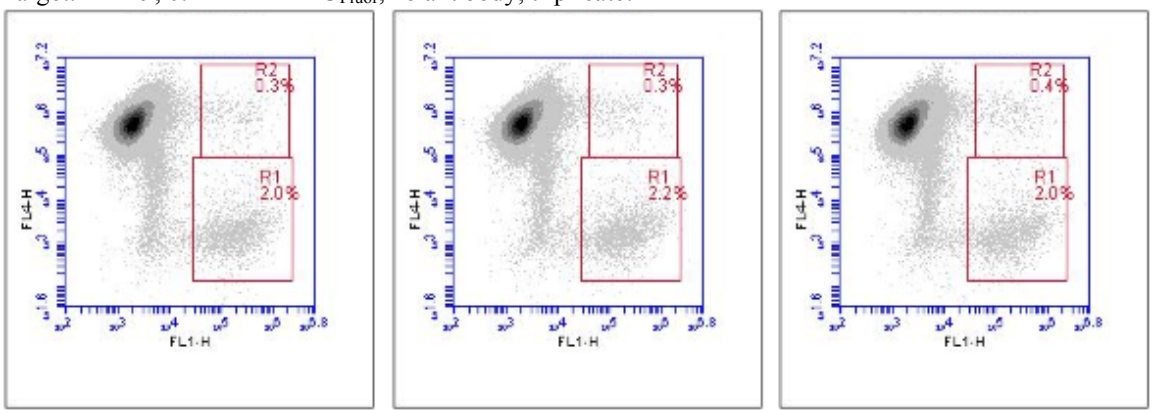
Target: HT-29, 1 nM ARM-U_{Fluor}, Rabbit anti-FITC antibody, triplicate:



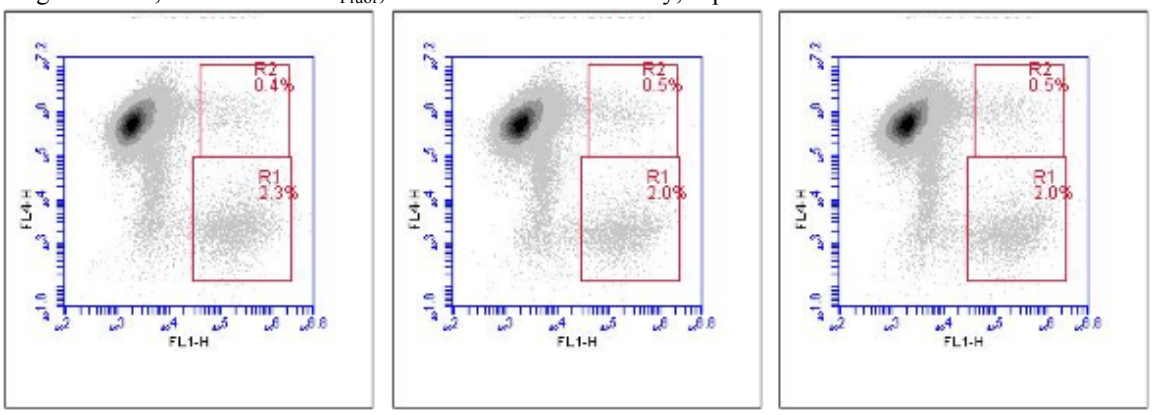
Target: HT-29, 1 nM ARM-U_{Fluor}, Rabbit anti-DNP antibody, triplicate:



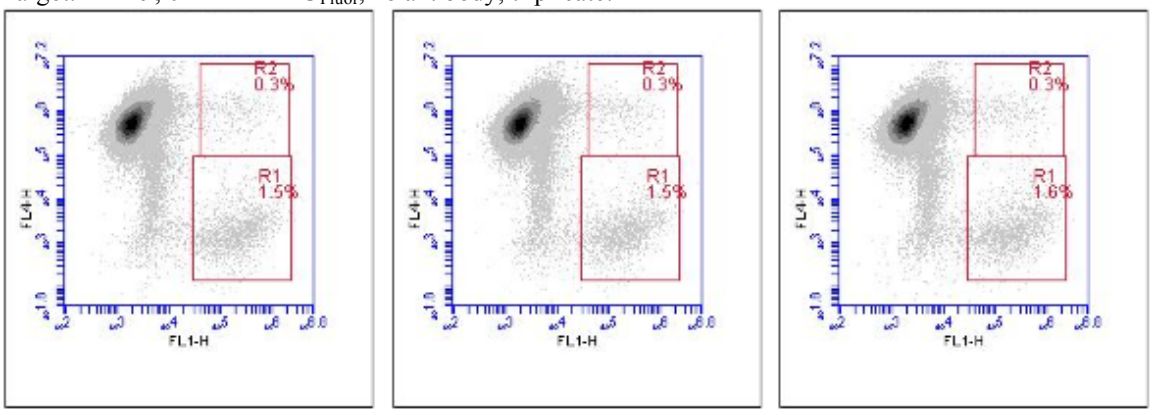
Target: HT-29, 0.1 nM ARM-U_{Fluor}, no antibody, triplicate:



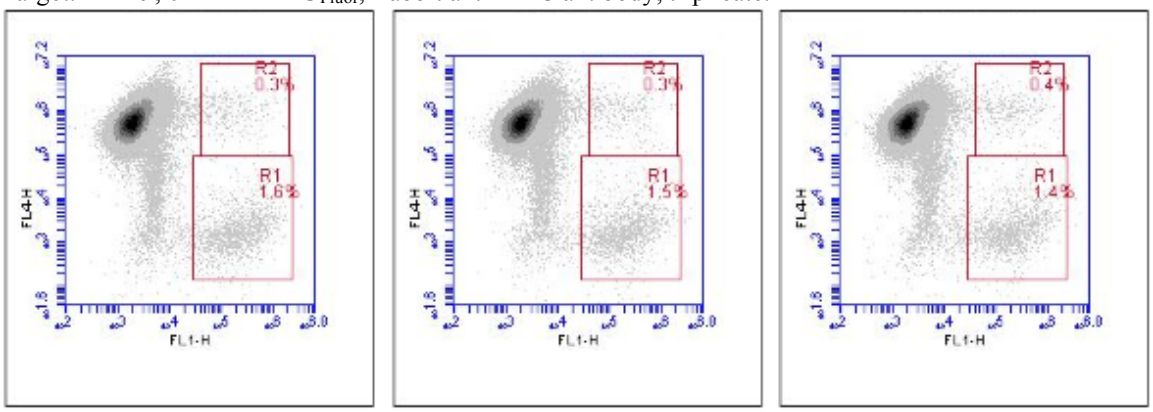
Target: HT-29, 0.1 nM ARM-U_{Fluor}, Rabbit anti-FITC antibody, triplicate:



Target: HT-29, 0 nM ARM-U_{Fluor}, no antibody, triplicate:

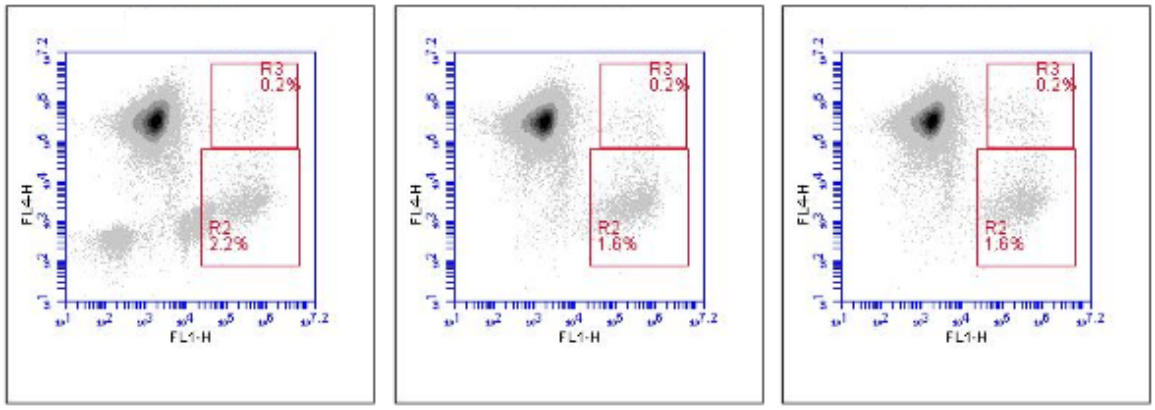


Target: HT-29, 0 nM ARM-U_{Fluor}, Rabbit anti-FITC antibody, triplicate:

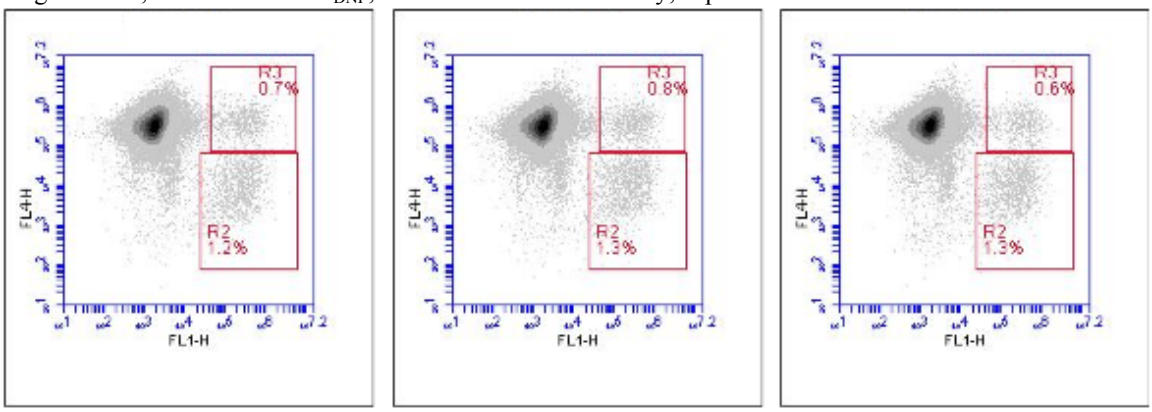


ARM-U_{DNP} to Target A172 Cells using Serum

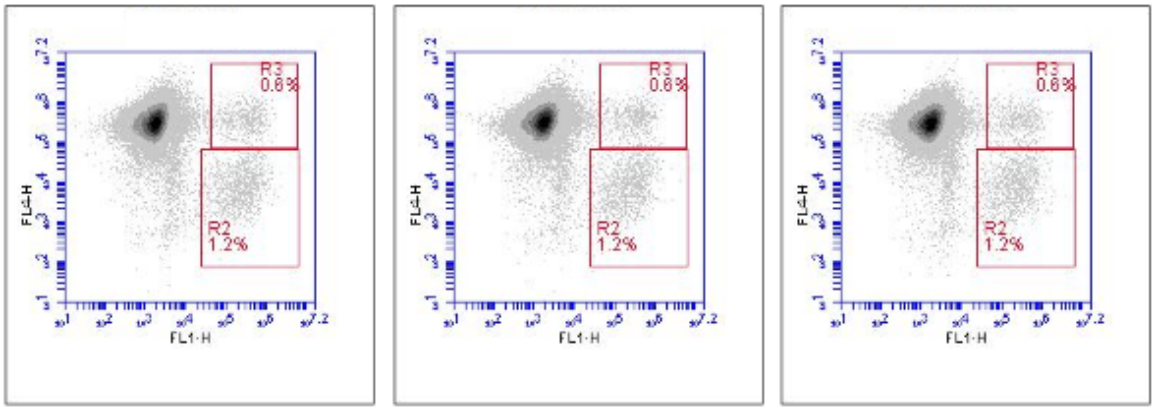
Target: A172, 100 nM ARM-U_{DNP}, no antibody, triplicate:



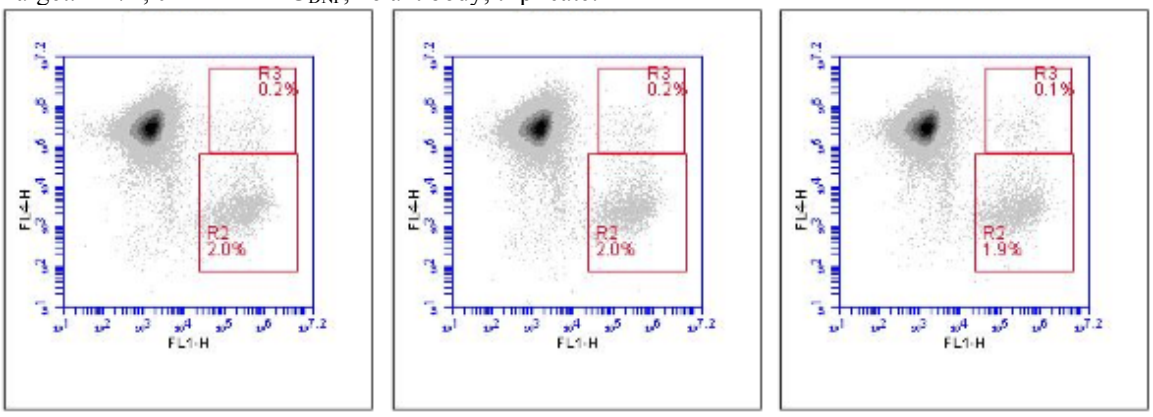
Target: A172, 100 nM ARM-U_{DNP}, Rabbit Anti-DNP antibody, triplicate:



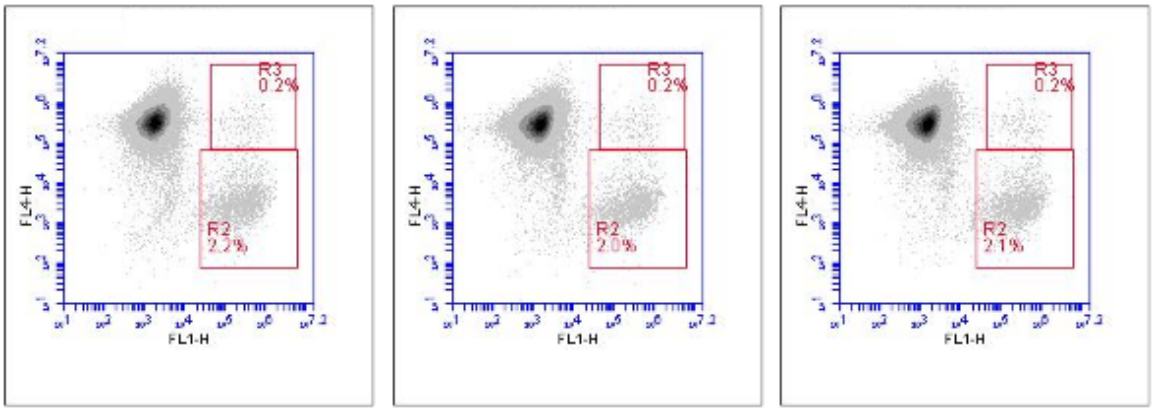
Target: A172, 100 nM ARM-U_{DNP}, 0.5% Rabbit DNP immunized serum, triplicate:



Target: A172, 0 nM ARM-U_{DNP}, no antibody, triplicate:



Target: A172, 0 nM ARM-U_{DNP}, Rabbit Anti-DNP antibody, triplicate:



Target: A172, 0 nM ARM-U_{DNP}, 0.5% Rabbit DNP immunized serum, triplicate: