Supporting Information for:

A General Method for Detecting Protease Activity via Gelation and its Application to Artificial Clotting

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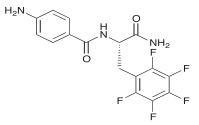
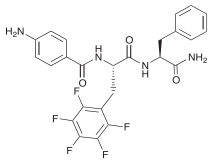
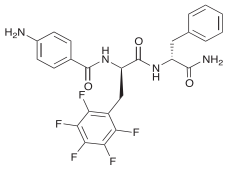
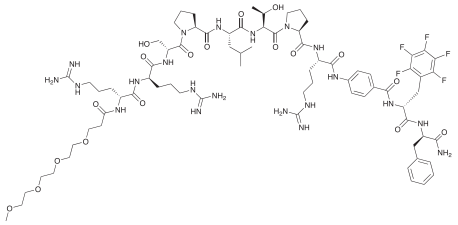
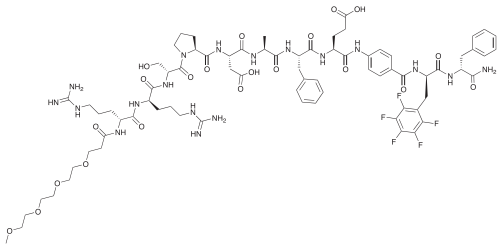
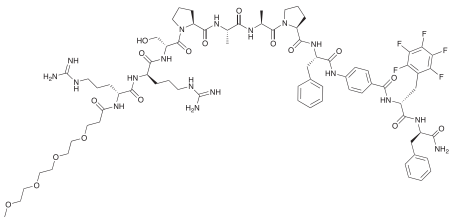
I. Structures of compounds

Table S1. Structures and functions of compounds described in this work.

Name	Structure	Use
Compound **1**		high cgc gelator
Compound **2**		low cgc gelator (L-amino acids)
Compound **3**		low cgc gelator (D-amino acids)
Compound **4**		thrombin dependent progelator
Compound **5**		Glu-C dependent progelator
Compound **6**		Chymotrypsin dependent progelator

II. Materials

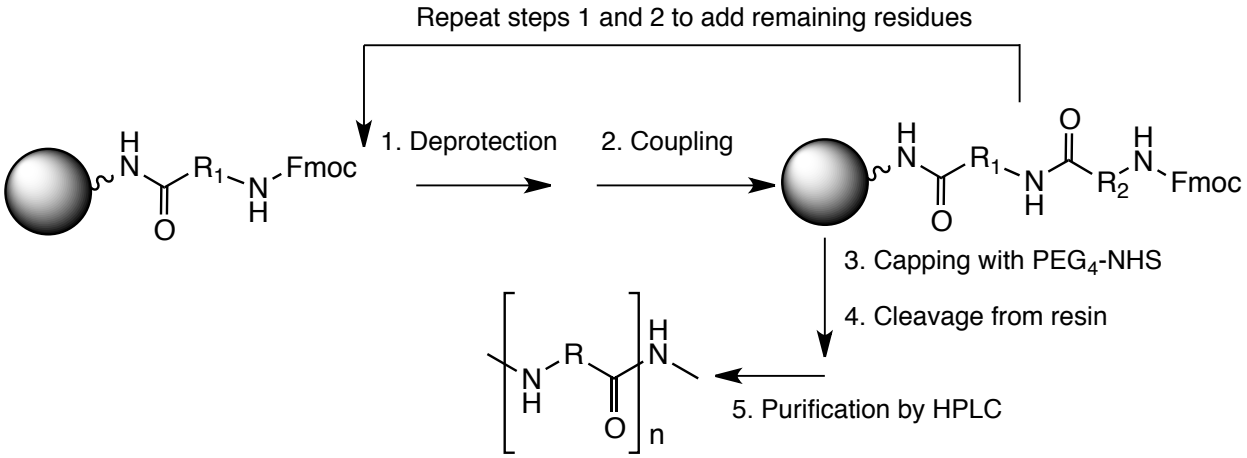
Fmoc-L-amino acids were purchased from Advanced Chem Tech. Fmoc-D-amino acids, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), *N*,*N*,*N*',*N*'-tetramethyl-O-(7-azabenzotriazol-1-yl)uronium hexafluorophosphate (HATU) and rink amide resin were purchased from ChemImpex. All solvents were used as received; N-methyl-2-pyrrolidinone (NMP) was purchased from Advanced ChemTech, diisopropylethylamine (DIEA) from Fisher, dimethylsulfoxide (DMSO) from EMD and piperidine from Sigma-Aldrich. PEG_4-NHS was purchased from Thermo Scientific. Thrombin and chymotrypsin from human pancreas were purchased from Calbiochem. Thromboplastin from rabbit brain was purchased from Sigma-Aldrich. Endoproteinase Glu-C was purchased from Worthington Biochemical Corporation. Fibrinogen-deficient plasma was purchased from Affinity Biologicals.

III. General experimental

Peptide synthesis and purification

Peptide synthesis was performed using standard solid-phase Fmoc chemistry[1,2] on rink amide resin (0.1 mmol) as illustrated in Scheme 1.

Scheme 1



1. Deprotection reaction

Fmoc deprotection was carried out using a 6 mL solution of 20 vol% piperidine in NMP incubated for at least 10 min at rt. The resin is then washed 4 times with 4 mL NMP.

2. Coupling reaction

The Fmoc amino acid (0.5 mmol) and HBTU (0.5 mmol) were dissolved in 4 mL of NMP containing DIEA (300 mM). This solution was added to rink amide resin (0.1 mmol) in a 12 mL cartridge and the mixture was shaken for at least 30 min at rt. The reaction was determined to be complete based on the Kaiser test.[3] The resin is then washed 4 times with 4 mL NMP.

Special Coupling Reaction

After coupling *p*-aminobenzoic acid (PABA) to the resin, the next amino acid coupling was performed with 1 mmol Fmoc-amino acid and 1 mmol HATU. The reaction was heated to 75 °C with a CEM Discover® microwave synthesizer for at least 50 min. The reaction was determined to be complete based on the Kaiser test.[3]

3. PEG_4 capping reaction

PEG_4-NHS (0.25 mmol) was added to 4 mL of NMP containing DIEA (300 mM) and shaken for 30 min. The resin is then washed 4 times with 4 mL NMP.

4. Cleavage reaction

The peptides were cleaved from the resin using a 5 mL solution of 95 vol% trifluoroacetic acid, 2.5 vol% H_2O, 2.5 vol% triisopropylsilane incubated for at least 2 h at rt.

5. Purification

Compounds **1-3** were subjected to rotary evaporation with 5 mL toluene to remove cleavage solution. Compounds **4-6** were precipitated from the cleavage solution by addition of 40 mL of cold Et_2O and collected by centrifugation. All precipitates were then re-suspended in 2 mL DMSO for HPLC purification. Peptides were purified by HPLC on a Waters© Xbridge Prep C18 column (19 x 250 mm) using a linear gradient of CH_3CN (5-60%) in H_2O with 0.1 vol% trifluoroacetic acid at 20 mL/min. Fractions containing the product were pooled and lyophilized.

Preparation of 200 mM PBS buffer

Na_2HPO_4 (0.1 mol), KH_2PO_4 (0.1 mol), KCl (5.4 mmol) and NaCl (0.274 mol) were added to 900 mL of H_2O. The solution was adjusted (using either HCl or NaOH) to pH 7.2 and diluted to 1000 mL with H_2O.

Heat/cool procedure for gel formation

For gel screening, a 4 mL vial was charged with the compound (3-5 mg) and DMSO (0.10 mL) to generate a homogeneous solution. To this solution, 10 mM PBS (0.9 mL) buffer was added, resulting in a white suspension. The suspension was then heated until a homogeneous solution was obtained and allowed to cool to rt. Gels were identified by vial inversion. The sample was diluted by adding 10/90 DMSO/buffer solution (0.10 mL) and retested for gelation. Dilutions were continued until the gel was no longer stable to inversion. The last concentration to give a stable gel was recorded as the critical gelation concentration (cgc).

Enzymatic gelation assay

Compounds **4-6** were dissolved in DMSO to ~ 60 mM. An aliquot (30 μL) of this solution was added to a 2 mL glass vial. To the vial was also added DMSO (10 μL) and H_2O (150 μL). To initiate gelation, a solution of 200 mM phosphate buffered saline (PBS) pH 7.2 (200 μL) was premixed with enzyme (10μL of 2 μM) and then added to the vial. The vial was carefully tilted at various time intervals to check for gelation as determined by being stable to inversion.

Rheology

Rheological measurements were taken on an AR2000ex rheometer (TA Instruments) with a 25 mm parallel plate. A pre-formed gel was loaded onto the Peltier plate at rt. The gap was then fixed at 500 μm. The sample was pre-sheared under a stress of 0.1 Pa for 30 s before conducting the frequency sweep and oscillating stress sweep experiments. All measurements were repeated 2-3 times to ensure reproducibility. The frequency sweep experiment was performed under 0.1 Pa stress with a frequency range from 0.628 rad/s to 628 rad/s (i.e., 0.1 Hz-100 Hz). The oscillating stress sweep experiment was performed at 1 Hz, with a stress range from 0.03 Pa to 150 Pa.

Atomic force microscopy

Samples were imaged in air using a Dimension Icon AFM (Veeco). Samples were drop-cast and air-dried on a glass slide, then imaged in tapping mode using silicon cantilevers (VistaProbes T300R, tip radius <10 nm, force constant 40 N/m, resonance frequency 300 kHz; nanoScience Instruments). Images were acquired from 2-3 locations in each sample.

Analytical HPLC-MS

Compound was dissolved in 50 vol% CH_3CN in H_2O to 2 mg/mL. An aliquot (10 μL) was injected into a Waters© Xbridge C18 column (2.1 x 100 mm) and eluted using a linear gradient of CH_3CN (5-95%) in H_2O with 0.1 vol% formic acid over 15 min at a flow rate of 0.3 mL/min. The nominal mass for each peak was determined using a Waters© Micromass ZQ mass spectrometer. The University of Michigan Technical Services department performed high-resolution mass analysis (HRMS-ESI) on all compounds.

NMR spectroscopy

^1H and ^{19}F NMR spectra were obtained on a Varian MR 400 operating at 400 and 376 MHz, respectively. ^{13}C NMR was obtained using a Varian vnmrs 500 operating at 125 MHz. For ^1H and ^{13}C NMR spectra, the chemical shift data are reported in units of δ (ppm) relative to tetramethylsilane and referenced with residual solvent. For ^{19}F NMR spectra, the chemical shift data are reported in units of δ

(ppm) relative to trichlorofluoromethane and are referenced according to the trifluoroacetate in the sample. Multiplicities are reported as follows: singlet (s), doublet (d), doublet of doublets (dd), triplet (t) and multiplet (m).

IV. Characterization of peptides

a. Compound **1**

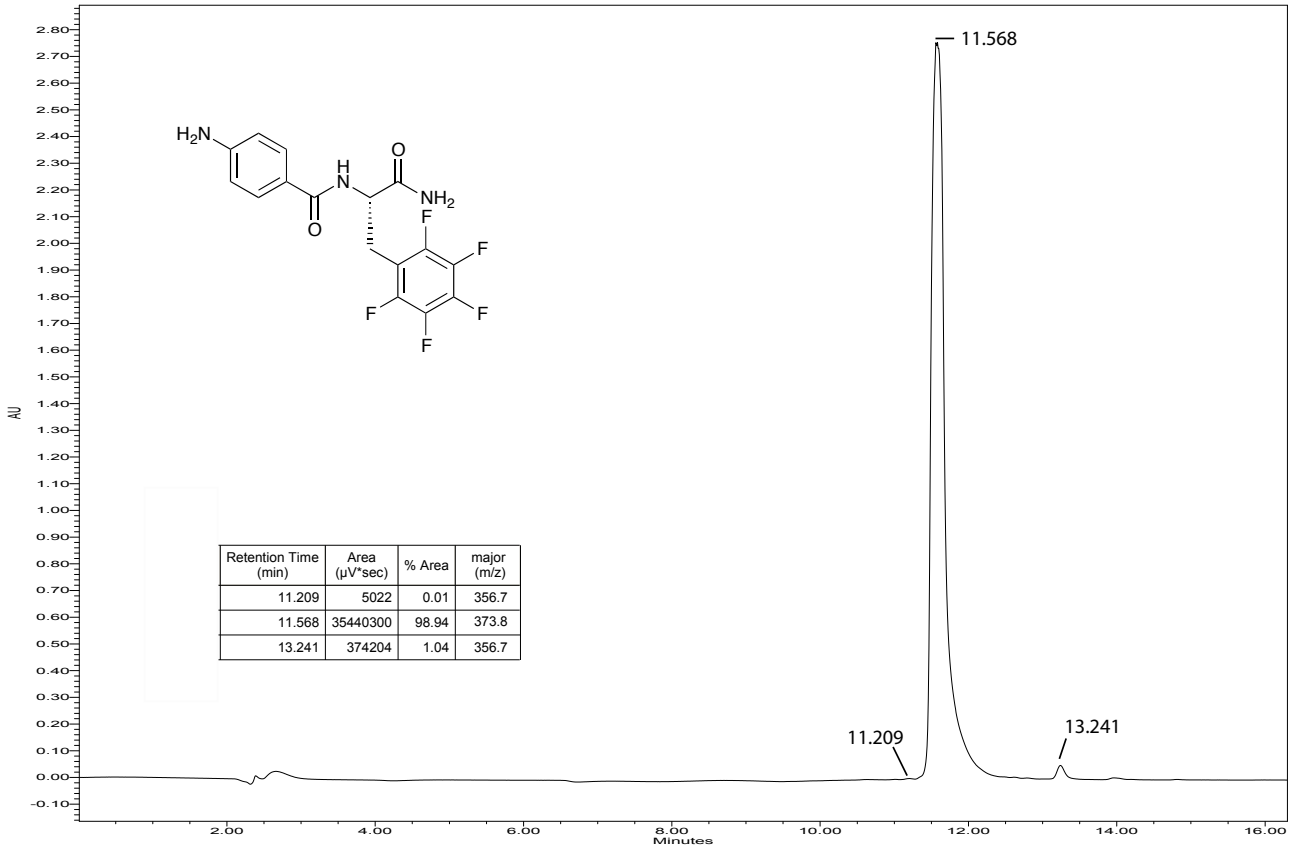
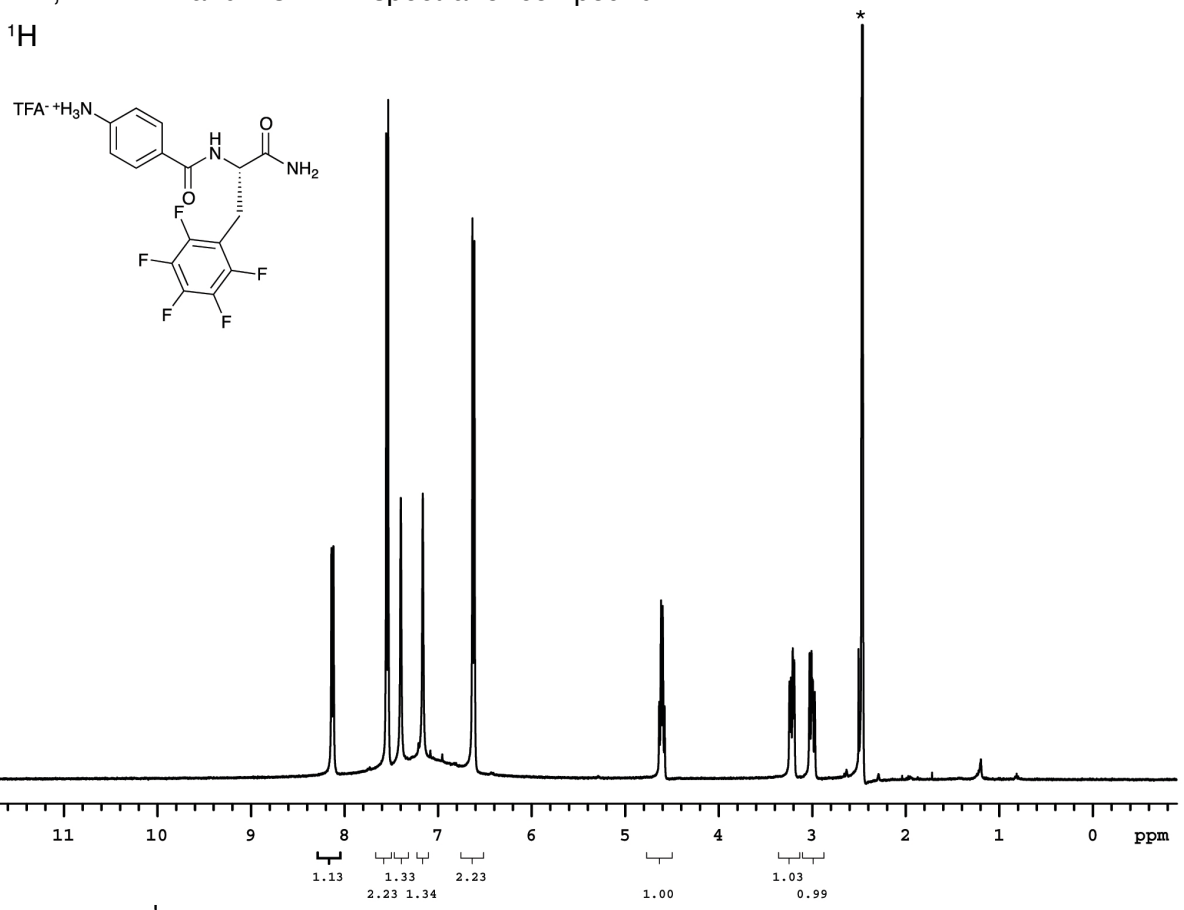


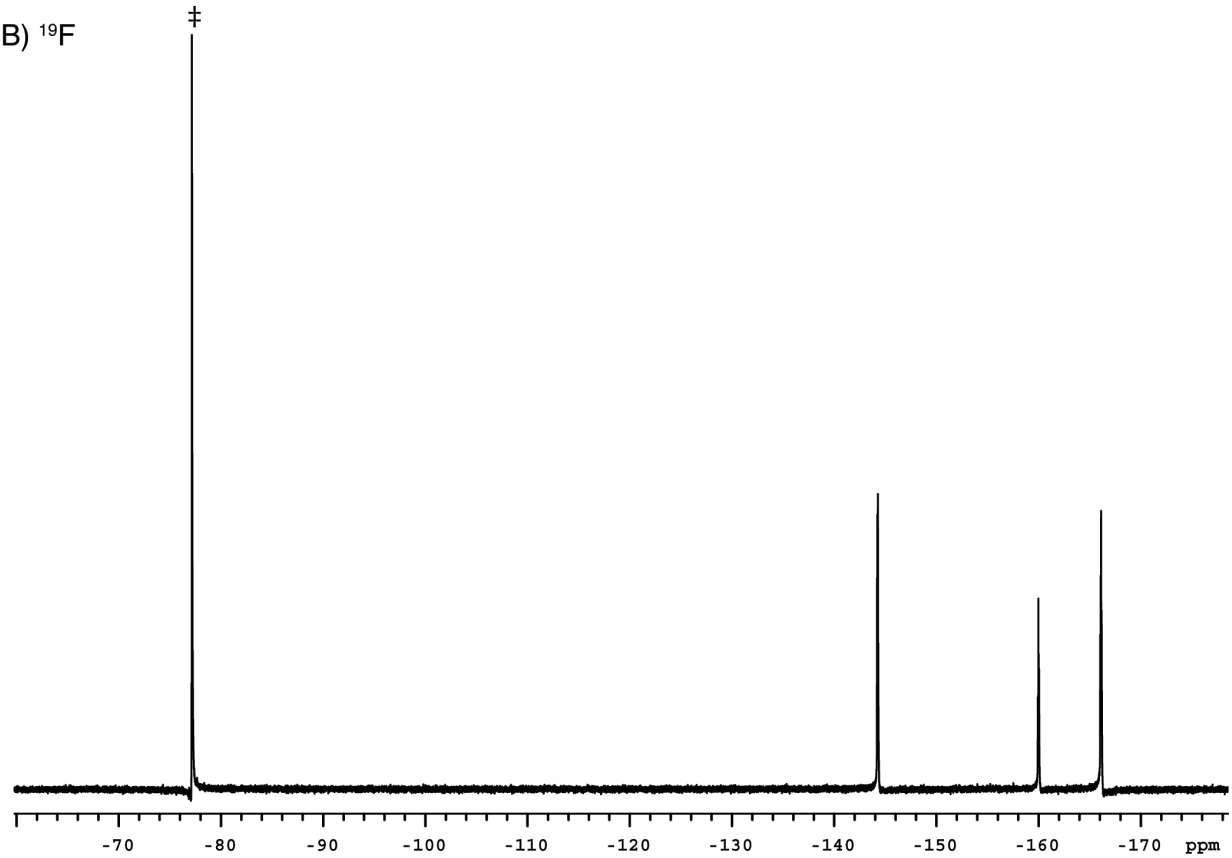
Figure S1. Analytical HPLC-MS trace for compound **1**. Compound **1** eluted at 11.568 min and is approximately 99% pure based on absorbance at 280 nm. The compound was subjected to ESI-MS to determine the exact mass of the molecule. HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{16}H_{12}F_5N_3O_2$, 374.0922; found, 374.0925.

^{1}H NMR, ^{19}F NMR and ^{13}C NMR spectra for compound **1**

(A) ^{1}H



(B) ^{19}F



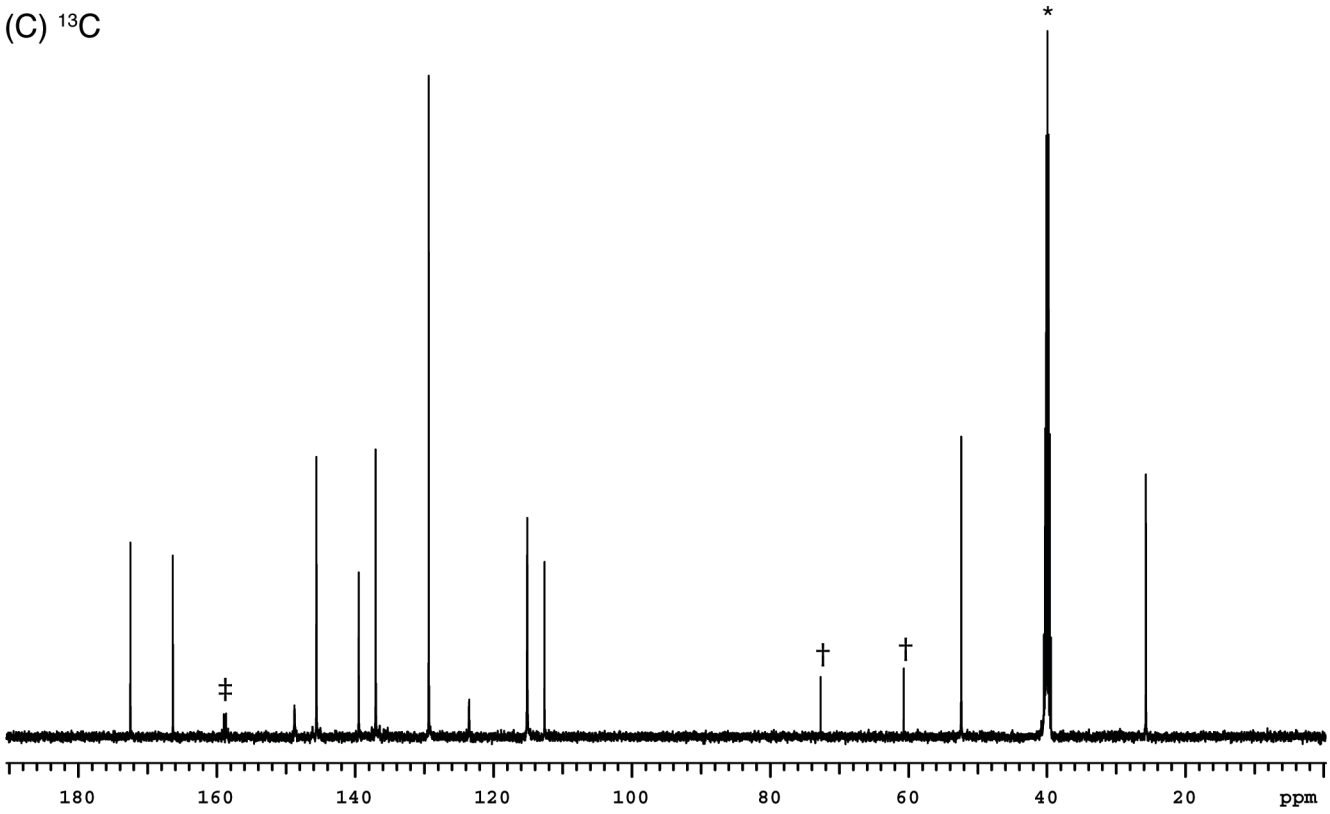
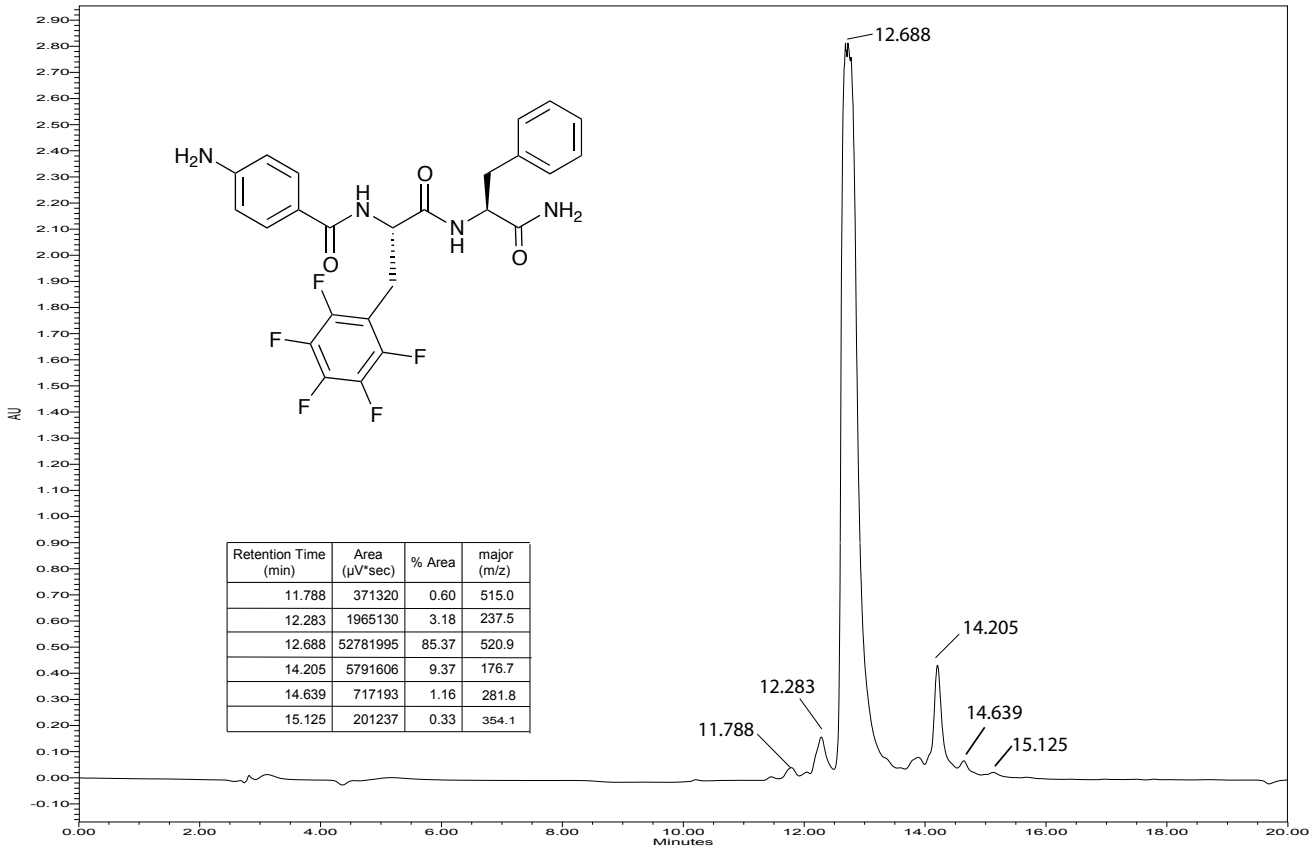


Figure S2. (A) ^{1}H, (B) ^{19}F and (C) ^{13}C {^{1}H,^{19}F} NMR spectra of **1**. ^{1}H NMR (DMSO-d_6, 400 MHz): δ (ppm) 8.13 (d, *J* = 8.4 Hz, 1H), 7.54 (d, *J* = 8.4 Hz, 2H), 7.40 (s, 1H), 7.16 (s, 1H), 6.62 (d, *J* = 8.4 Hz, 2H), 4.61 (dd, *J* = 8.0, 6.8 Hz, 1H), 3.22 (dd, *J* = 13.6, 6.4 Hz, 1H), 3.00 (dd, *J* = 13.6, 8.4 Hz, 1H). (B) ^{19}F NMR (DMSO-d_6, 376 MHz): δ (ppm) -77.00 (s), -144.09 (d, *J* = 24.8 Hz), -159.82 (t, *J* = 23.2 Hz), -165.95 (t, *J* = 23.2 Hz). ^{13}C {^{1}H, ^{19}F} NMR (DMSO-d_6, 125 MHz): δ (ppm) 172.48, 166.36, 148.76, 145.60, 139.49, 137.03, 129.36, 123.52, 115.11, 112.62, 72.72, 60.70, 52.39, 25.70. † indicates unknown impurity. ‡ indicates trifluoroacetic acid.

b. Compound **2**

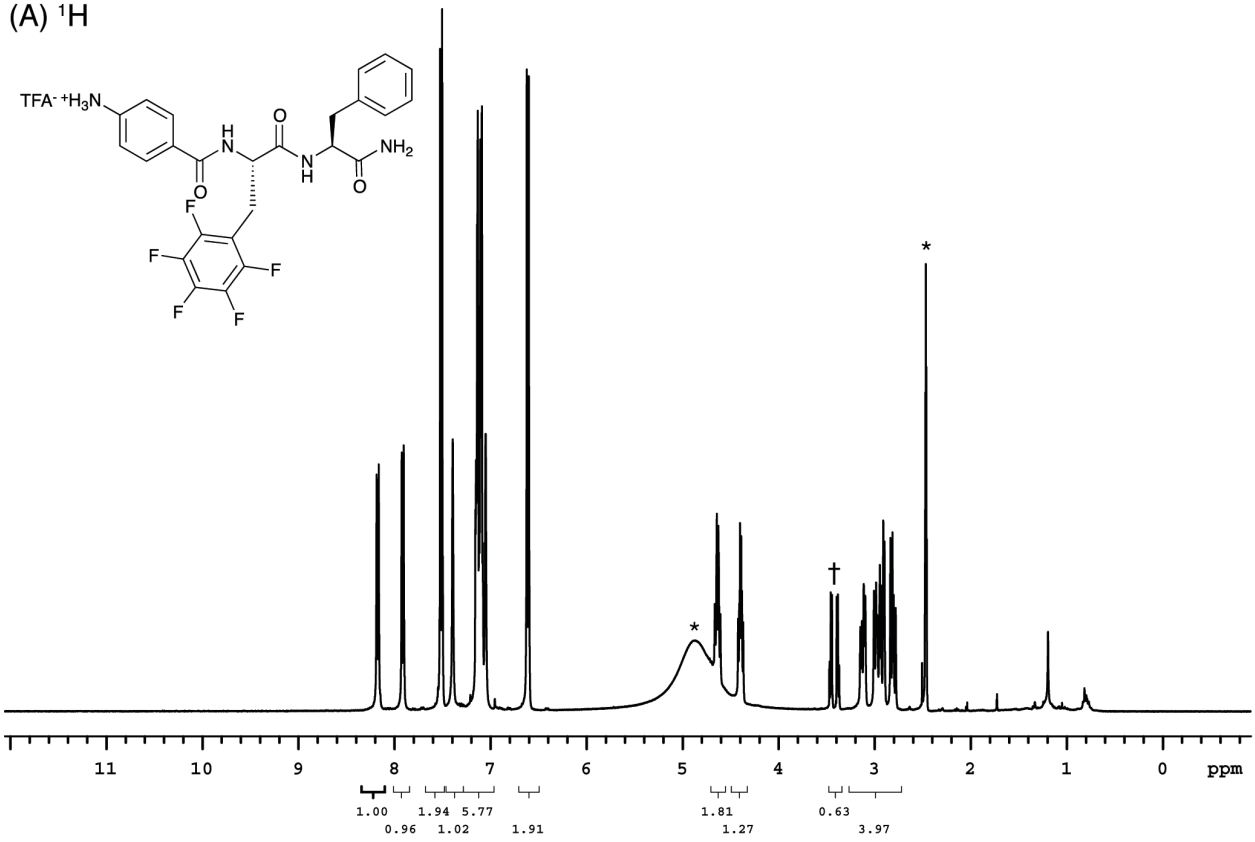


Retention Time (min)	Area (μV*sec)	% Area	major (m/z)
11.788	371320	0.60	515.0
12.283	1965130	3.18	237.5
12.688	52781995	85.37	520.9
14.205	5791606	9.37	176.7
14.639	717193	1.16	281.8
15.125	201237	0.33	354.1

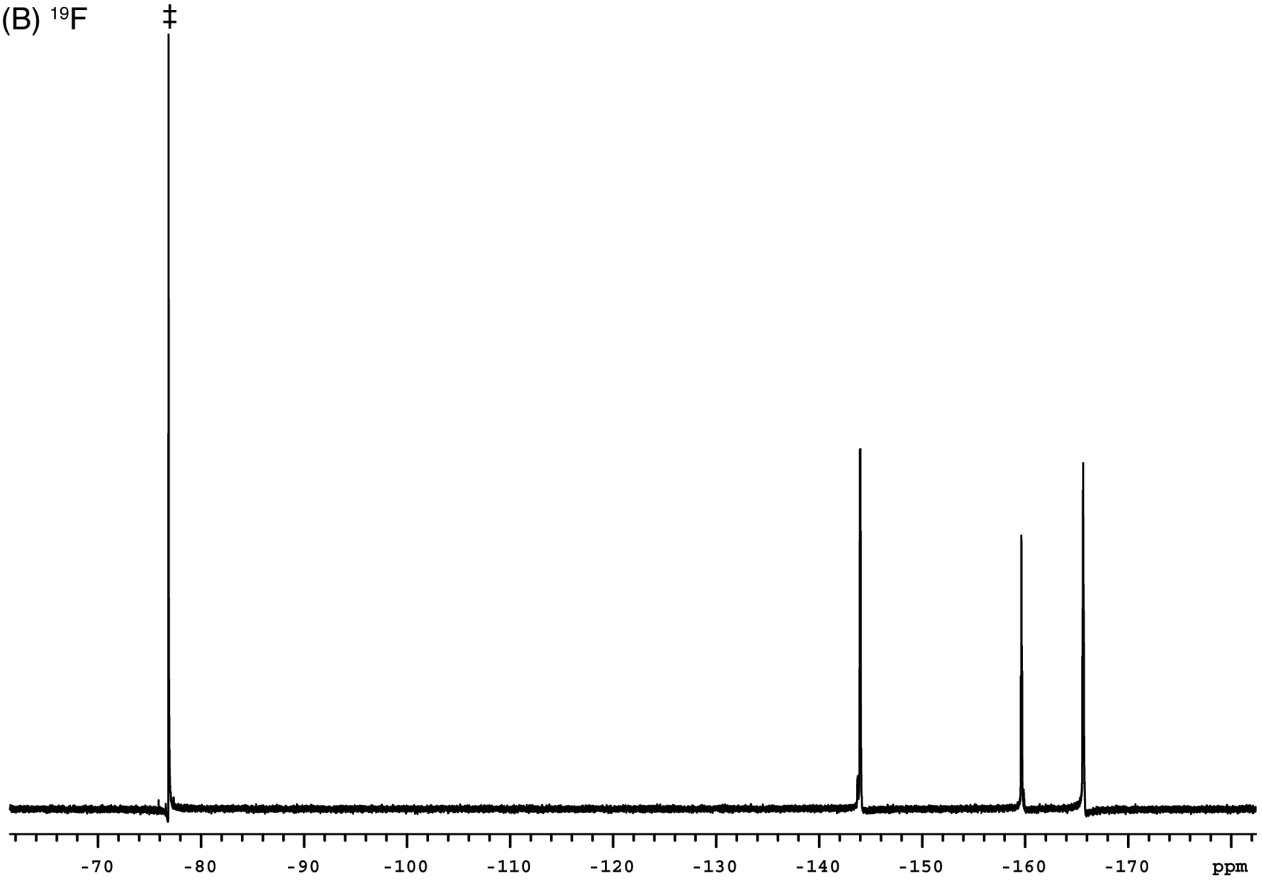
Figure S3. Analytical HPLC-MS trace for compound **2**. Compound **2** eluted at 12.688 min and is approximately 85% pure based on absorbance at 280 nm. The compound was subjected to ESI-MS to determine the exact mass of the molecule. HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{25}H_{21}F_5N_4O_3$, 521.1607; found, 521.1601.

^{1}H NMR, ^{19}F NMR and ^{13}C NMR spectra for compound **2**

(A) ^{1}H



(B) ^{19}F ‡



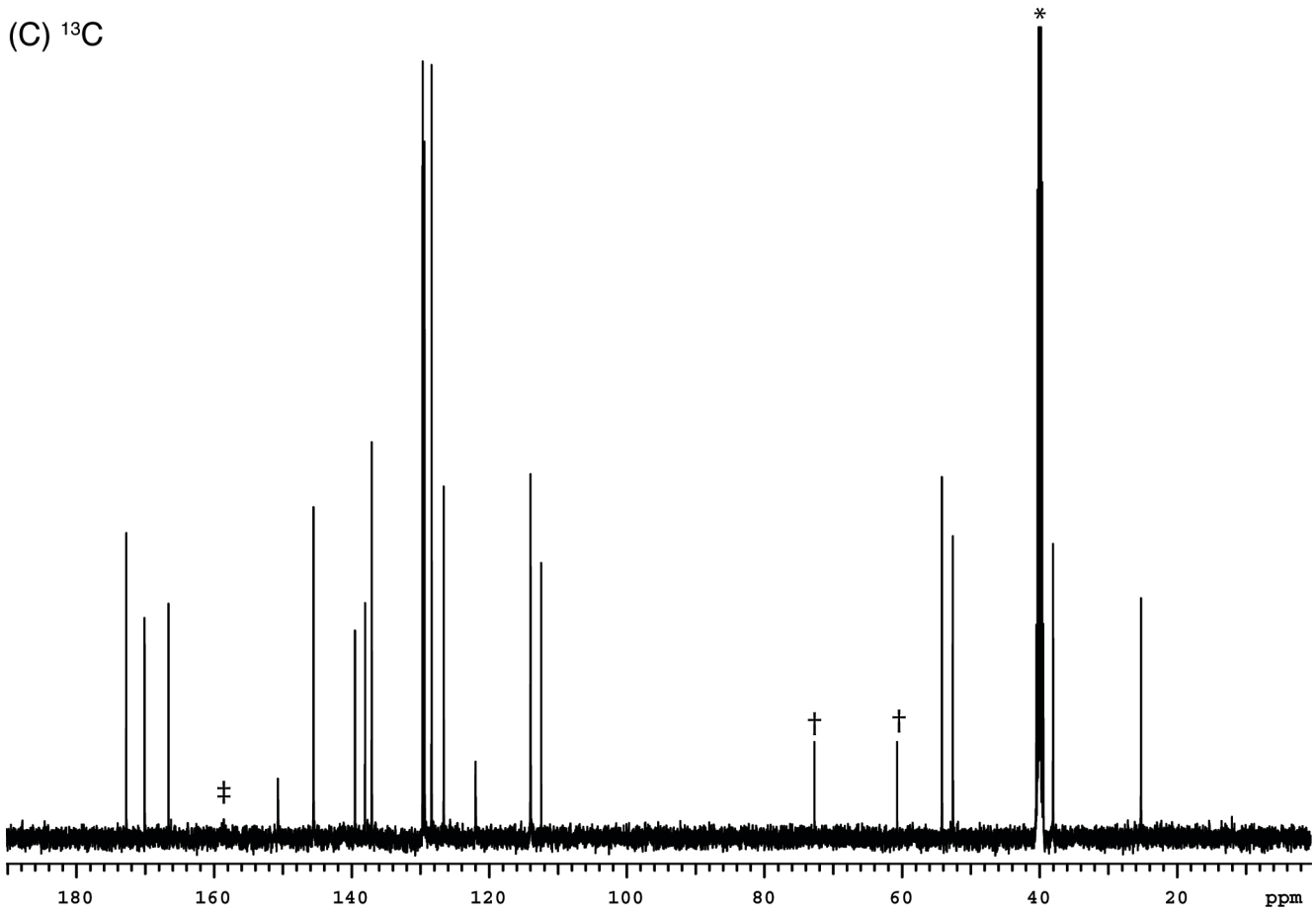


Figure S4. (A) ^{1}H, (B) ^{19}F and (C) ^{13}C {^{1}H,^{19}F} NMR spectra of **2**. (A) ^{1}H NMR (DMSO-d_6, 400 MHz): δ (ppm) 8.18 (d, J = 8.4 Hz, 1H), 7.91 (d, J = 8.4 Hz, 1H), 7.51 (d, J = 8.8 Hz, 2H), 7.39 (s, 1H), 7.10 (m, 6H), 6.61 (d, J = 8.8 Hz, 2H), 4.63 (dd, J = 8.4, 6.8 Hz, 1H), 4.40 (m, 1H), 3.42 (m, 0.63 H), 2.96 (m, 4H). (B) ^{19}F NMR (DMSO-d_6, 376 MHz): δ (ppm) -77.00 (s), -144.15 (d, J = 24.4 Hz), -159.80 (t, J = 23.2 Hz), -165.80 (m). (C) ^{13}C {^{1}H, ^{19}F} NMR (DMSO-d_6, 125 MHz): δ (ppm) 172.77, 170.11, 166.62, 150.72, 145.54, 139.52, 138.06, 137.08, 129.69, 129.42, 128.39, 126.62, 121.99, 113.99, 112.45, 72.73, 60.71, 54.19, 52.61, 38.04, 25.25. † indicates unknown impurity. ‡ indicates trifluoroacetic acid.

c. Compound **3**

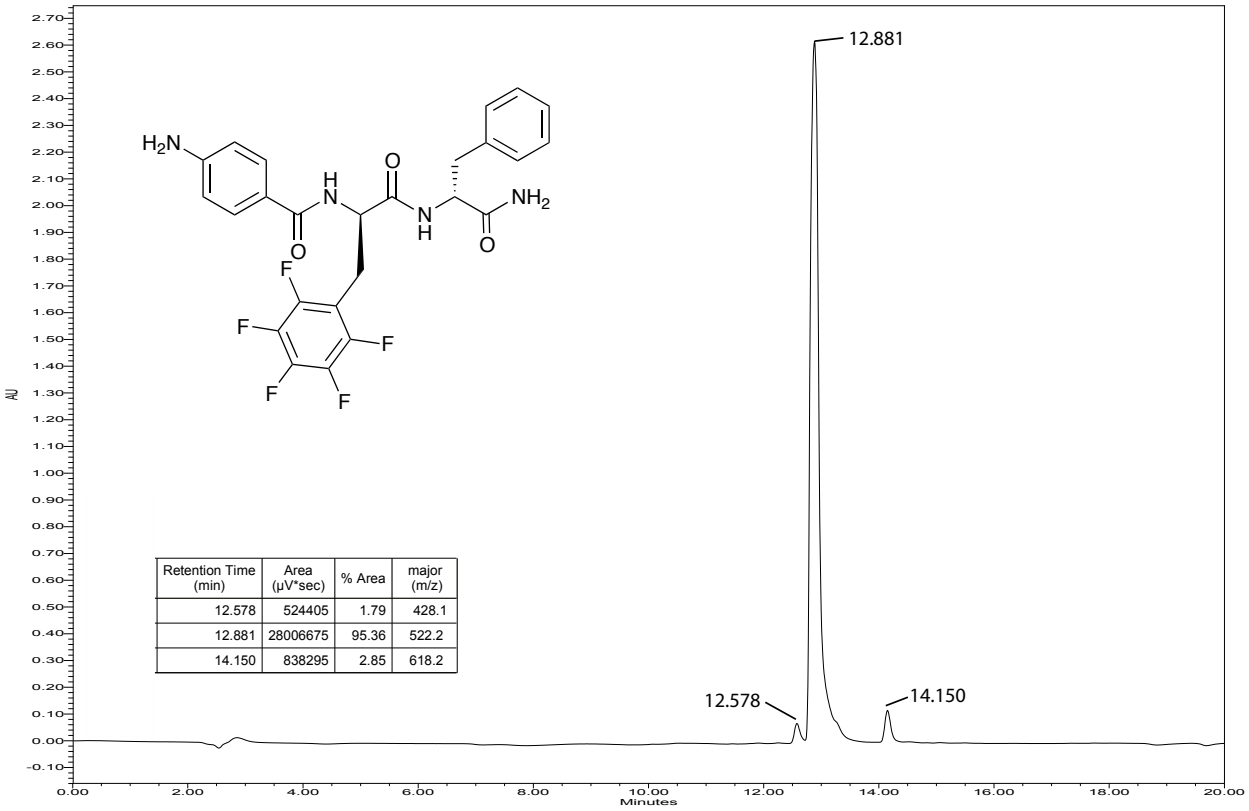
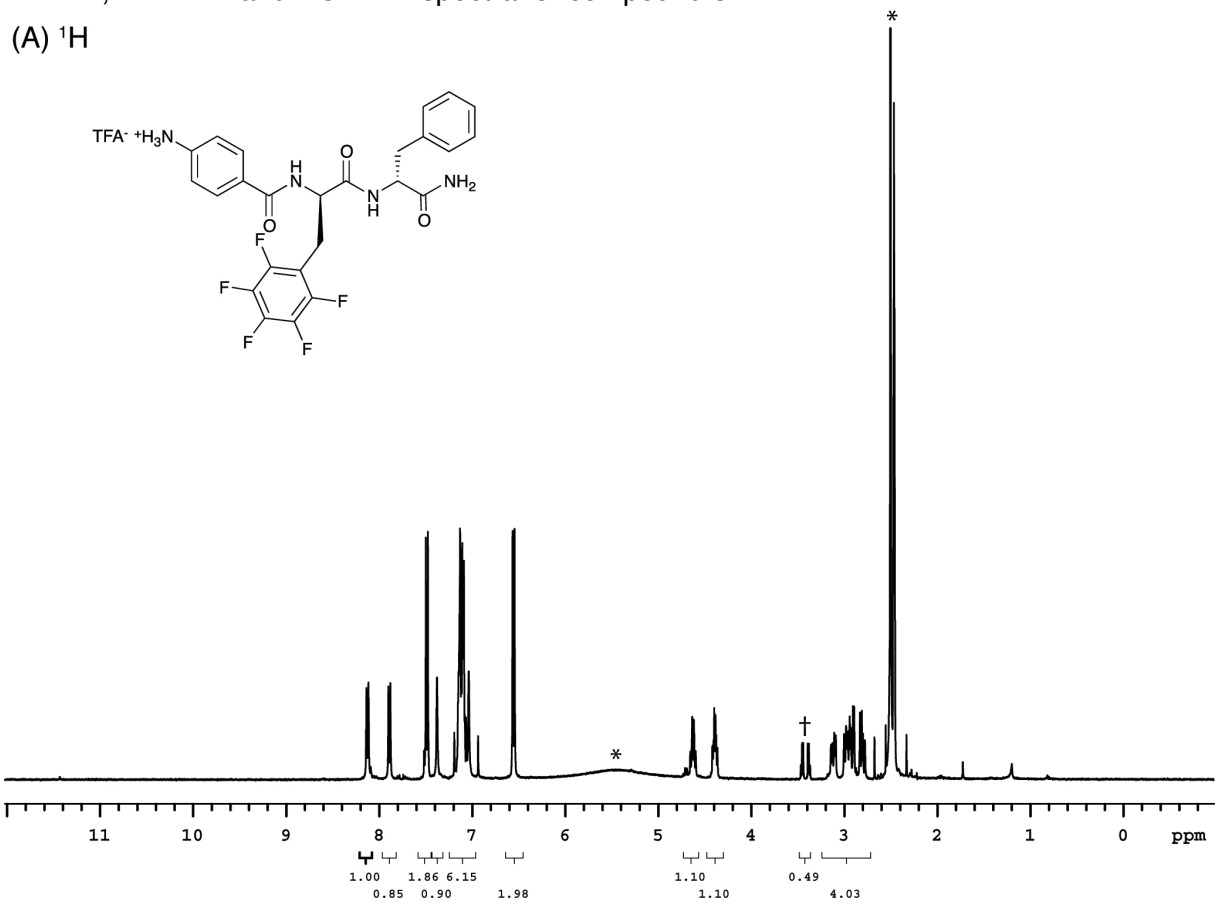


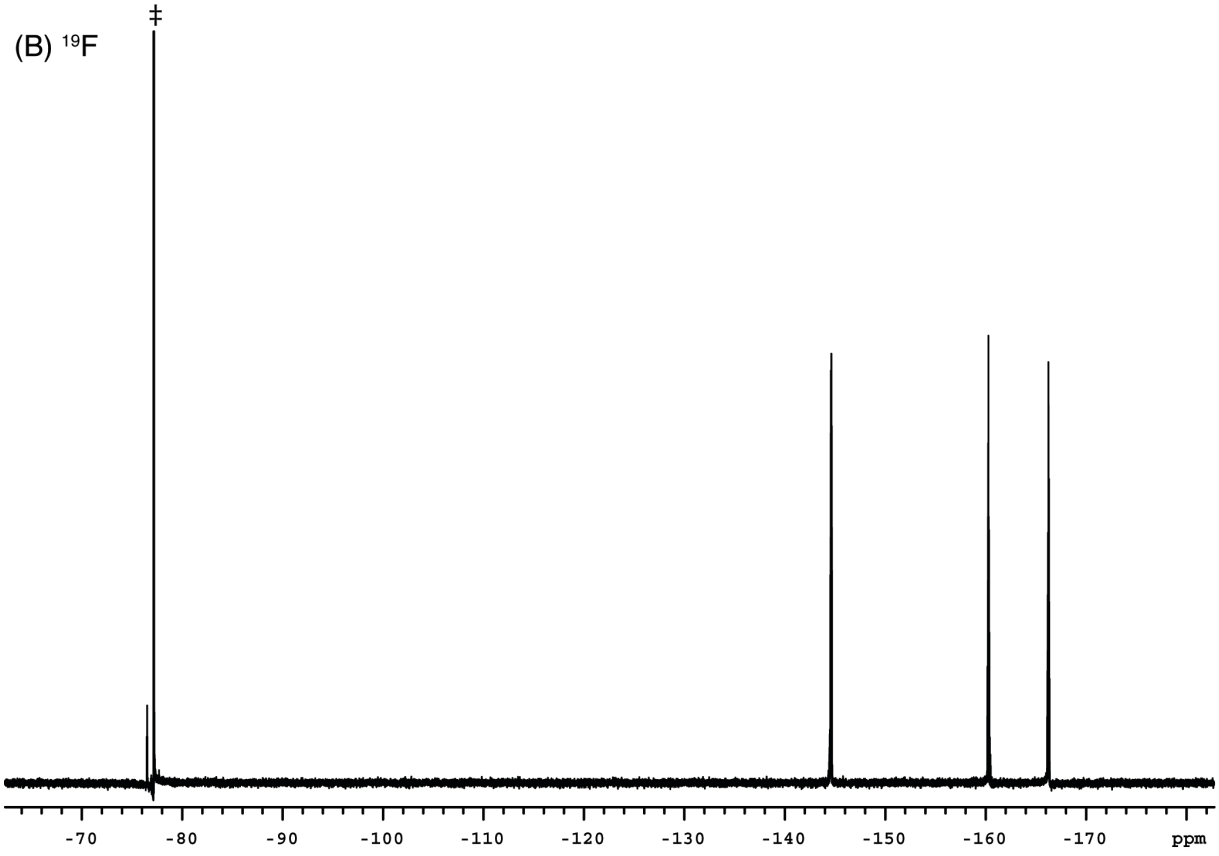
Figure S5. Analytical HPLC-MS trace for compound **3**. Compound **3** eluted at 12.881 min and is approximately 95% pure based on absorbance at 280 nm. The compound was subjected to ESI-MS to determine the exact mass of the molecule. HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{25}H_{21}F_5N_4O_3$, 521.1607; found, 521.1606.

^{1}H NMR, ^{19}F NMR and ^{13}C NMR spectra for compound **3**

(A) ^{1}H



(B) ^{19}F



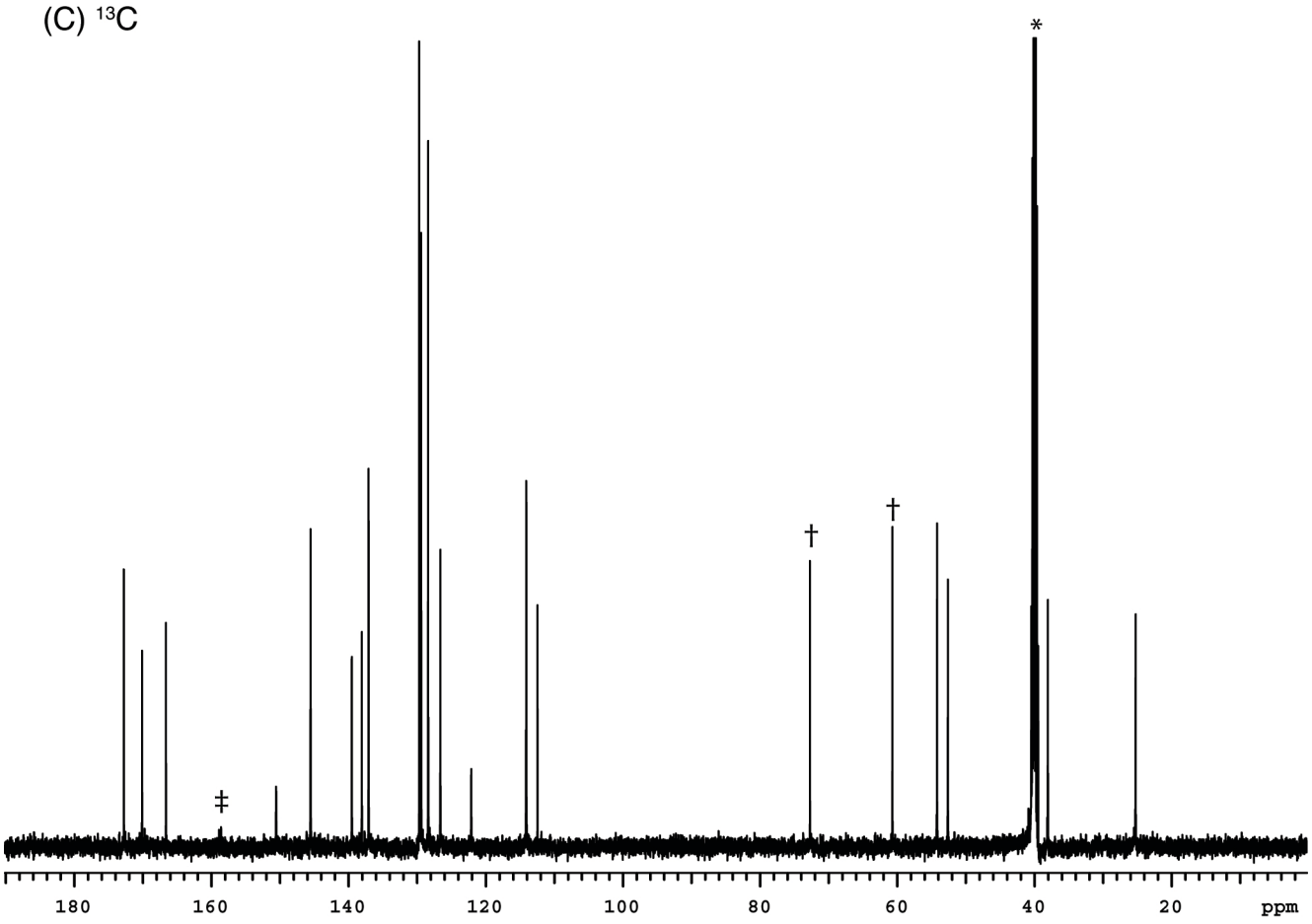


Figure S6. (A) ^{1}H, (B) ^{19}F and (C) ^{13}C {^{1}H, ^{19}F} NMR spectra of **3**. ^{1}H NMR (DMSO-d_6, 400 MHz): δ (ppm) 8.13 (d, J = 8.4 Hz, 1H), 7.89 (d, J = 8.4 Hz, 1H), 7.49 (d, J = 8.4 Hz, 2H), 7.38 (s, 1H), 7.11 (m, 6H), 6.55 (d, J = 8.4 Hz, 2H), 4.62 (dd, J = 8.4, 6.8 Hz, 1H), 4.40 (m, 1H), 3.42 (m, 0.49 H), 2.96 (m, 4H). (B) ^{19}F NMR (DMSO-d_6, 376 MHz): δ (ppm) -77.17 (s), -144.65 (m), -160.28 (t, J = 23.2 Hz), -166.25 (m). (C) ^{13}C {^{1}H, ^{19}F} NMR (DMSO-d_6, 125 MHz): δ (ppm) 172.77, 170.11, 166.62, 150.56, 145.54, 139.52, 138.06, 137.08, 129.69, 129.42, 128.38, 126.62, 122.10, 114.10, 112.45, 72.73, 60.71, 54.20, 52.61, 38.03, 25.24. † indicates unknown impurity. ‡ indicates trifluoroacetic acid.

d. Compound **4**

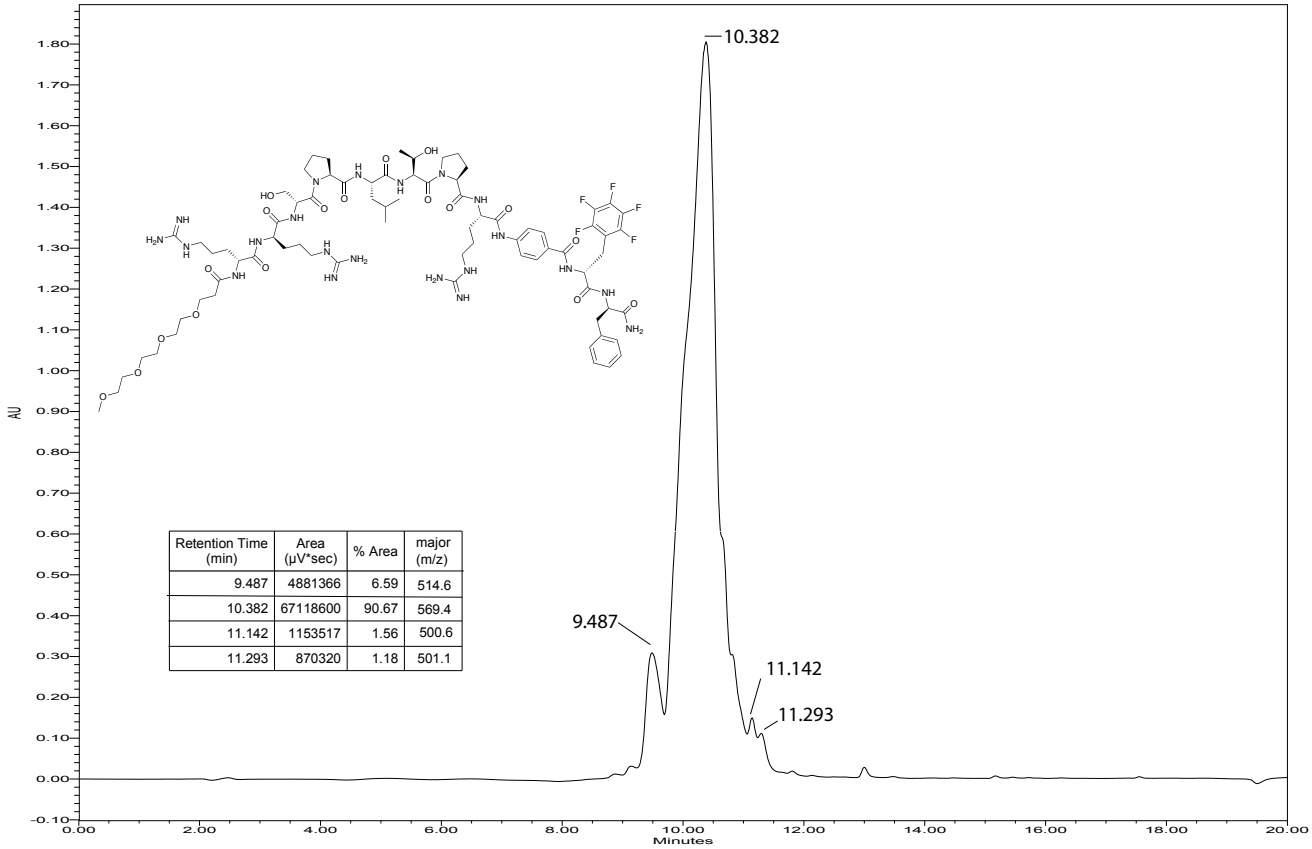
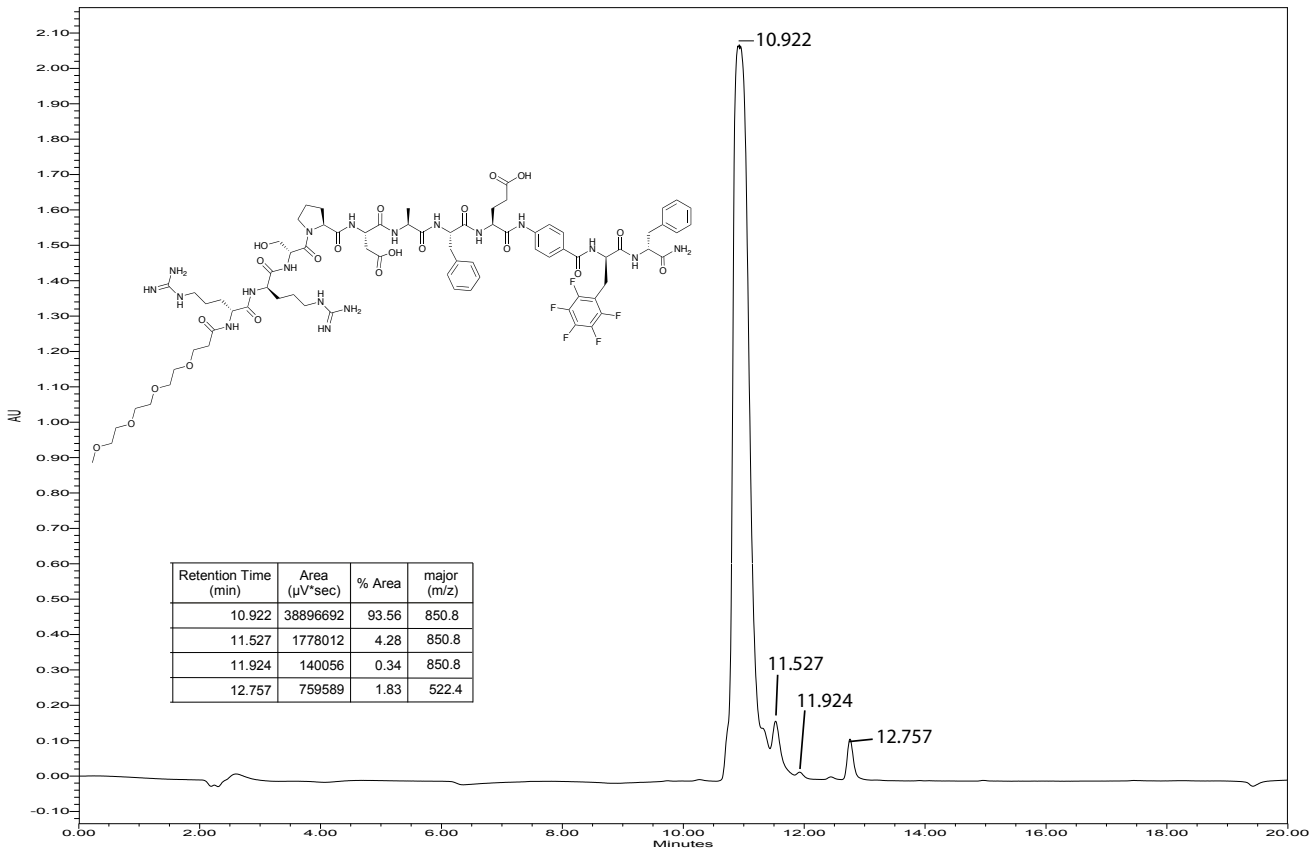


Figure S7. Analytical HPLC-MS trace for compound **4**. Compound **4** eluted at 10.382 min and is approximately 90% pure based on absorbance at 280 nm. The compound was subjected to ESI-MS to determine the exact mass of the molecule. HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{76}H_{112}F_5N_{21}O_{18}$, 1702.8487; found, 1702.8458.

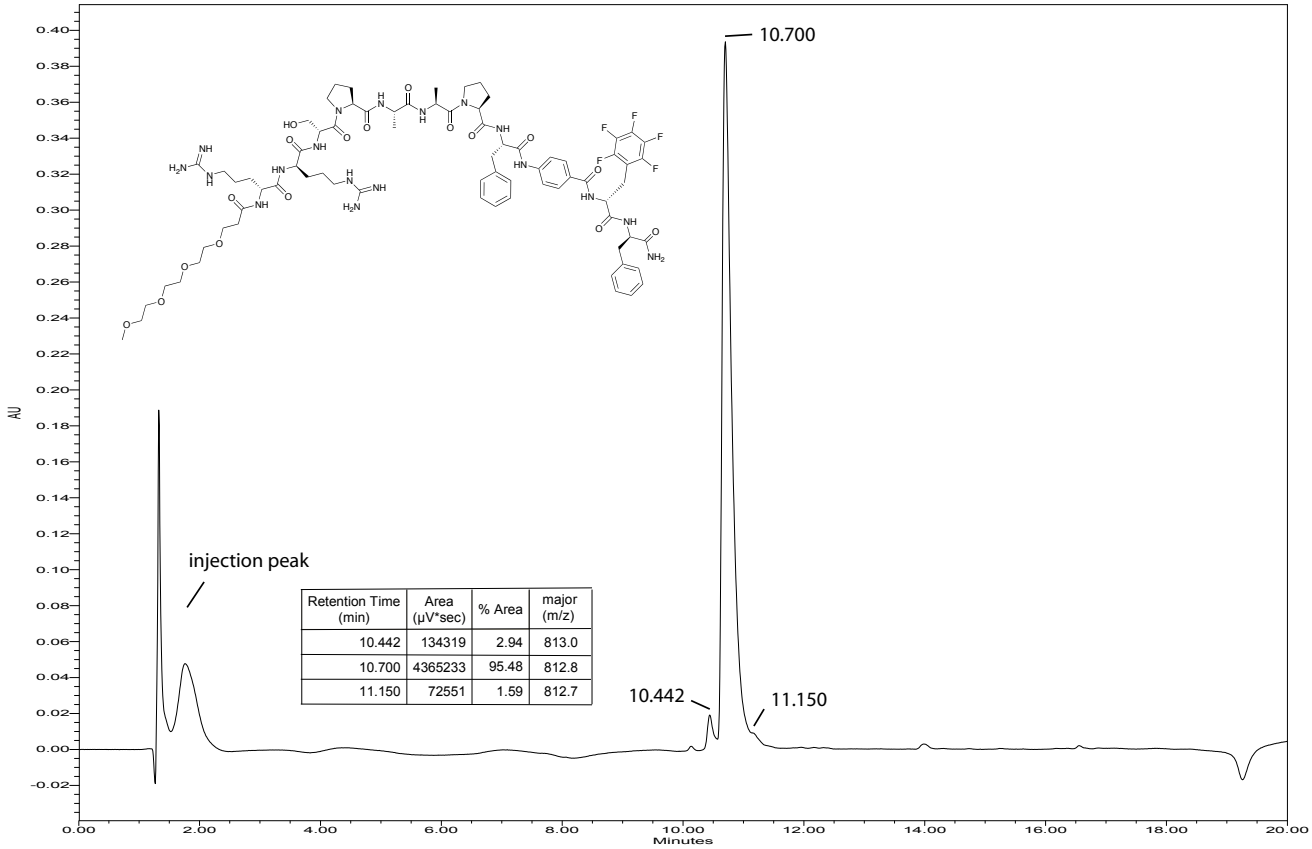
e. Compound **5**



Retention Time (min)	Area (μV*sec)	% Area	major (m/z)
10.922	38896692	93.56	850.8
11.527	1778012	4.28	850.8
11.924	140056	0.34	850.8
12.757	759589	1.83	522.4

Figure S8. Analytical HPLC-MS trace for compound **5**. Compound **5** eluted at 10.922 min and is approximately 93% pure based on absorbance at 280 nm. The compound was subjected to ESI-MS to determine the exact mass of the molecule. HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{76}H_{101}F_5N_{18}O_{21}$, 1697.7382; found, 1697.7360.

f. Compound **6**



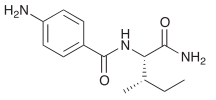
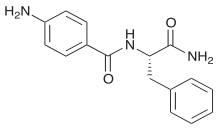
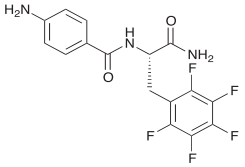
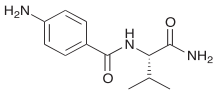
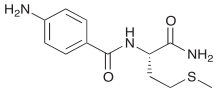
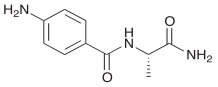
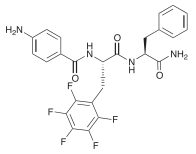
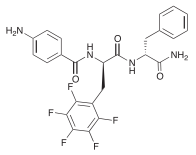
Retention Time (min)	Area (μV*sec)	% Area	major (m/z)
10.442	134319	2.94	813.0
10.700	4365233	95.48	812.8
11.150	72551	1.59	812.7

Figure S9. Analytical HPLC-MS trace for compound **6**. Compound **6** eluted at 10.700 min and is approximately 95% pure based on absorbance at 280 nm. The compound was subjected to ESI-MS to determine the exact mass of the molecule. HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{75}H_{101}F_5N_{18}O_{17}$, 1621.7585; found, 1621.7556.

V. Gel screen for PABA-peptide

The compounds were synthesized according to the procedures described in the general experimental (pg S3–S4). In total, 6 dipeptide structures were tested and the results of the heat/cool gelation screen are listed in Table S2.

Table S2. Screening results for PABA-peptides

PABA-peptide	Structure	cgc
PABA-Ile-NH_2		precipitated
PABA-Phe-NH_2		precipitated
PABA-(F_5)Phe-NH_2		54 mM
PABA-Val-NH_2		precipitated
PABA-Met-NH_2		precipitated
PABA-Ala-NH_2		precipitated
Compound **2**		1.7 mM
Compound **3**		1.7 mM

VI. Gel characterization for compound 3

(a) Rheology

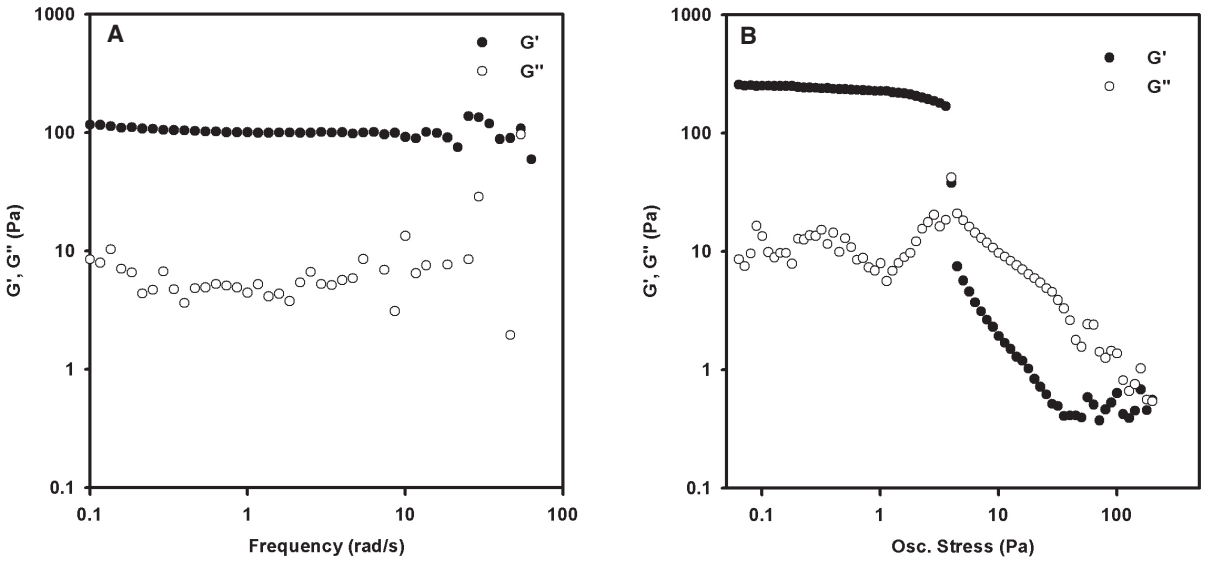


Figure S10. Rheology measurements of pre-formed gel after heat/cool procedure. Plot of elastic modulus (G′, filled circles) and loss modulus (G′′, open circles) as a function of (A) frequency and (B) oscillatory stress. ([Compound **3**] = 2.9 mM in 100 mM PBS buffer at pH 7.2 with 10 vol% DMSO).

(b) Atomic force microscopy

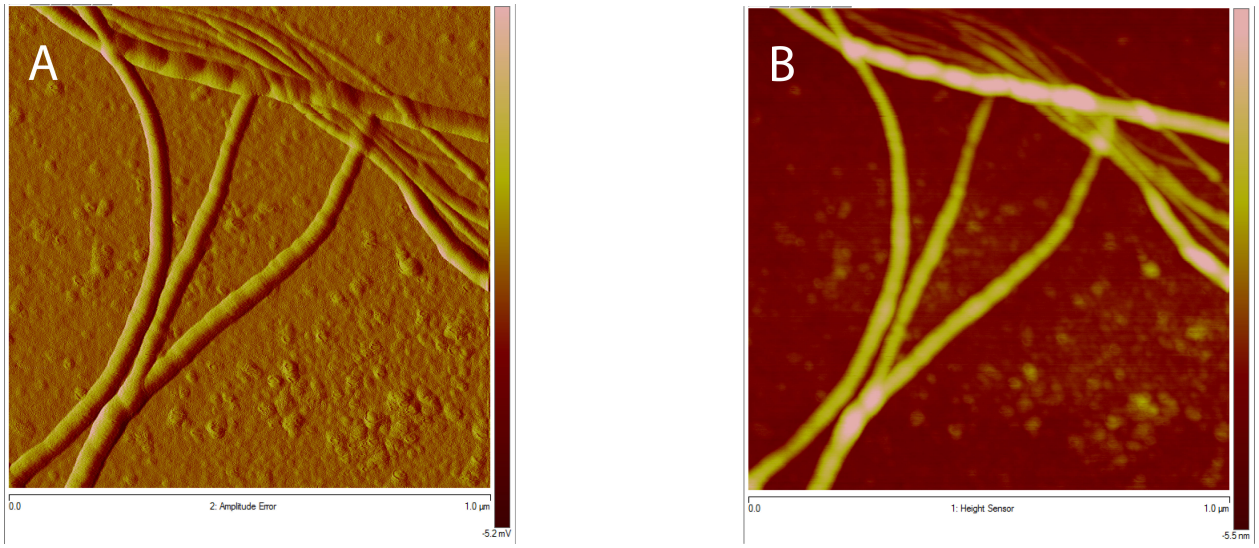


Figure S11. Atomic force microscopy (A) height and (B) phase images of gels formed with compound **3** (2.9 mM) in 100 mM PBS at pH 7.2 with 10 vol% DMSO.

VII. Gel characterization for compound 3 formed by thrombin-triggered gelation

Experimental

Reaction was carried out using 10 nM thrombin to hydrolyze compound **4** (4.4 mM) in 100 mM PBS buffer at pH 7.2 with 10 vol% DMSO. The data collected for fig. S9, S10 and S11 were from separate samples all prepared in the same manner.

(a) Rheology

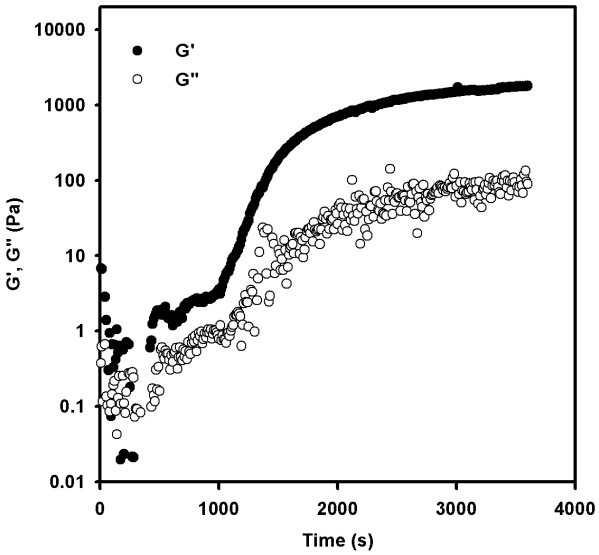


Figure S12. Plot of elastic modulus (G′, filled circles) and loss modulus (G′′, open circles) during thrombin-triggered gelation.

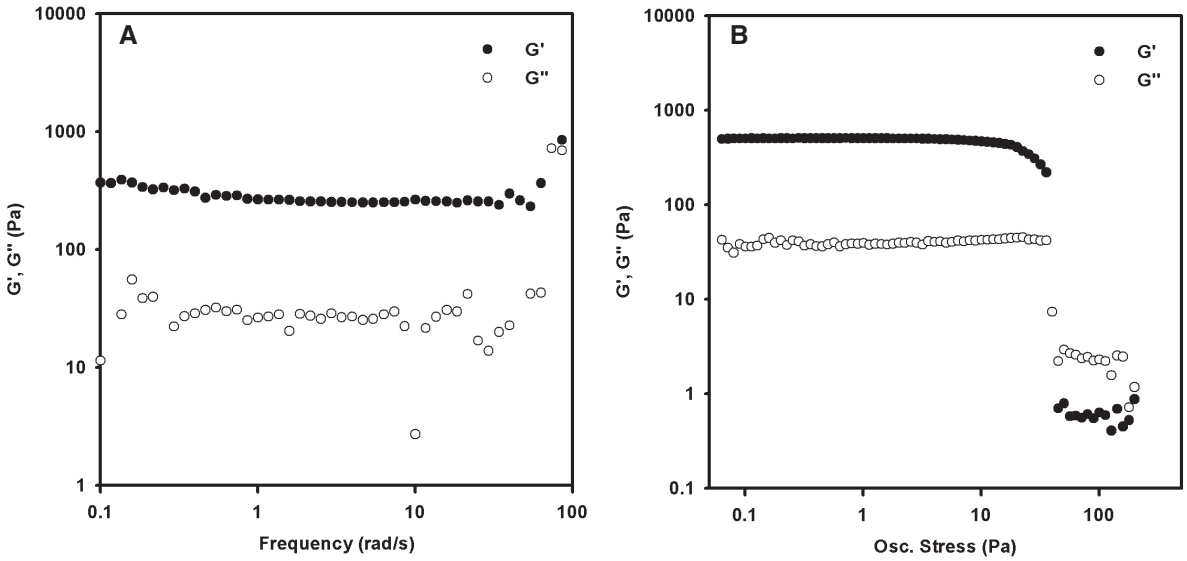


Figure S13. Plot of elastic modulus (G′, filled circles) and loss modulus (G′′, open circles) as a function of (A) frequency and (B) oscillatory stress of the gel 2 h after initiation with thrombin.

(b) Atomic force microscopy

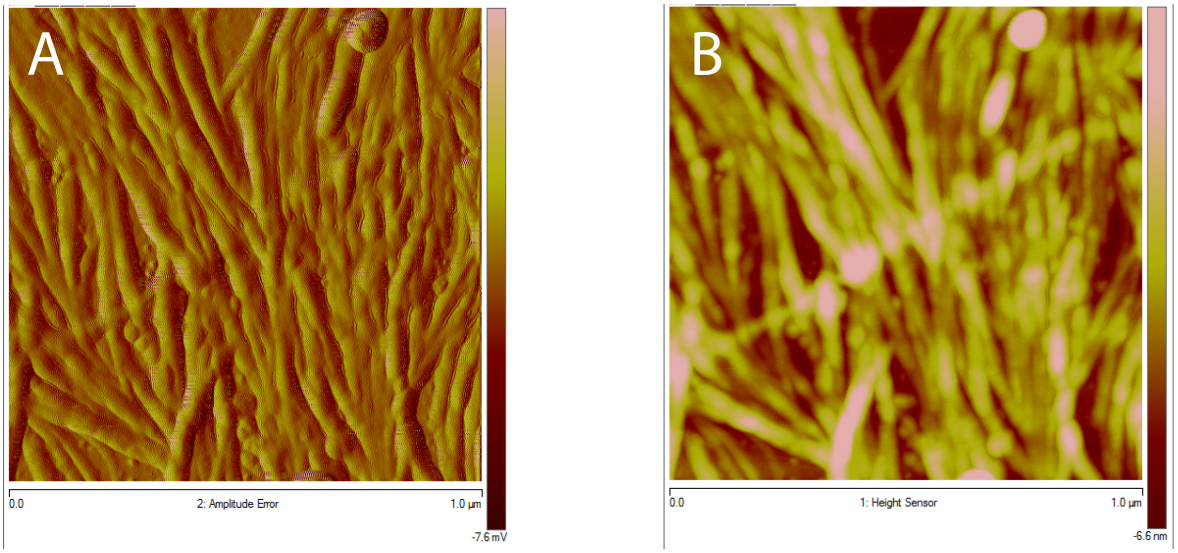


Figure S14. Atomic force microscopy (A) height and (B) phase images for compound **3** formed by thrombin-triggered gelation.

VIII. Thrombin-triggered gelation

Experimental

(A) Compound **4** (4.4 mM) was dissolved in 100 mM PBS at pH 7.2 with 10 vol% DMSO. Thrombin (50 nM) was then added. (B) Same as (A) except a thrombin inhibitor, phenylmethanesulfonyl fluoride (PMSF, 1 mM), was added. Aliquots (2 μL) were removed periodically and diluted with DMSO (96 μL) and 1M HCl (2 μL). The samples were then injected into the LC-MS to determine percent conversion.

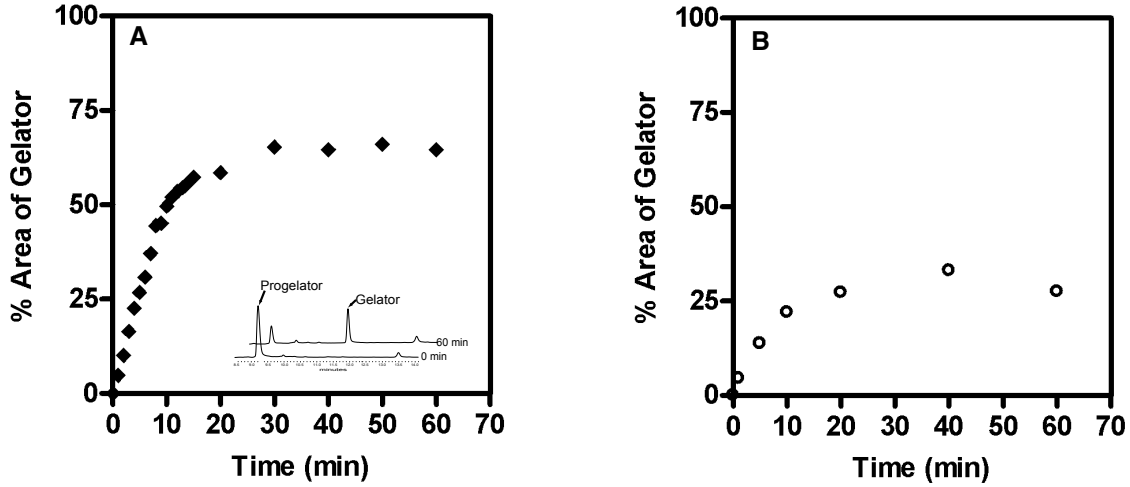


Figure S15. (A) Plot of gelator area (%) versus time for the thrombin-triggered gelation reaction. Gelation is observed within 10 min, corresponding to ~40% conversion. (B) Plot of gelator area (%) versus time for the thrombin-triggered gelation reaction with 1 mM inhibitor (PMSF). No gelation is observed.

Experimental

Compound **4** (4.4 mM) was dissolved in 100 mM PBS at pH 7.2 with 10 vol% DMSO. Different concentrations of thrombin were then added and the solutions were monitored for gelation by vial inversion. The time required for a stable gel is recorded in Table S3.

Table S3. Gelation times for reactions containing different thrombin concentrations

[Thrombin] (nM)	Gelation time (min)
0.08	>980
0.4	420
2	110
10	30
50	10

IX. Gelation of blood plasma

Experimental

Fibrinogen-deficient plasma (270 μL) containing 59 nM thrombin was added to a vial containing 58.8 mM compound **4** (30 μL) or DMSO (30 μL) and was monitored over time for gelation. The plasma containing both thrombin and compound **4** was stable to inversion after a 30-min incubation at rt. No gelation was detected in the other vials after 24 h.

Thrombin	+	–	+
Compound **4**	+	+	–
Plasma	+	+	+

Figure S16. Inversion of vials containing fibrinogen-deficient plasma treated with thrombin and compound **4**. The photo was taken after 20 h incubation.

Experimental

Fibrinogen-deficient plasma (100 μL) containing compound **4** (12 mM) or DMSO (30 μL) was treated with thromboplastin in 20 mM $CaCl_2$ (170 μL) or 20 mM $CaCl_2$ only and was monitored over time for gelation. The plasma containing both thromboplastin and peptide was stable to inversion after a 2 h incubation at rt. Gelation was also detected in the vial containing 20 mM $CaCl_2$ after 20 h.

Compound **4**	+	+	–	+	+
Plasma	+	+	+	+	+
Thromboplastin/20mM $CaCl_2$	+	–	+	+	–
20 mM $CaCl_2$	–	+	–	–	–
H_2O	–	–	–	–	+

Figure S17. Inversion of vials containing fibrinogen-deficient plasma treated with thromboplastin and compound **4**. The photo was taken after 20 h incubation.

X. Selective enzyme-triggered gelation by swapping recognition sequences

Experimental

Compounds **4-6** (4.4 mM) were dissolved in 100 mM PBS at pH 7.2 with 10 vol% DMSO and treated with thrombin (50 nM), chymotrypsin (50 nM) or Glu-C (50 nM). The reactions were incubated at rt and monitored for gelation. Thrombin treatment only gelled solutions containing compound **4**. Chymotrypsin treatment only gelled solutions containing compound **6**, while Glu-C treatment only gelled solutions containing compound **5**. Gelation of the reaction containing thrombin with **4** occurred within 10 min. Gelation of the reaction containing chymotrypsin with **6** or Glu-C with **5** occurred in 2 h.

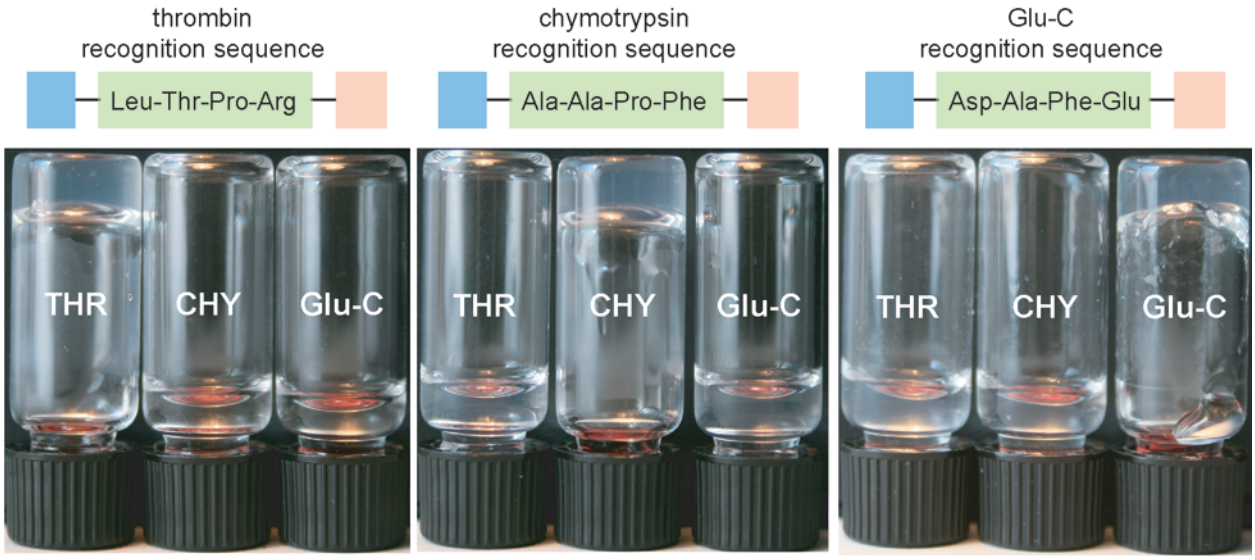


Figure S18. Inversion of vials containing thrombin (THR), chymotrypsin (CHY) or Glu-C with compound **4** (Leu-Thr-Pro-Arg), **5** (Asp-Ala-Phe-Glu) or **6** (Ala-Ala-Pro-Phe). The photos were taken after 20 h incubation.

XI. References

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