

Supporting Information for:

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

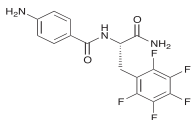
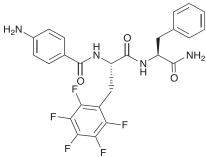
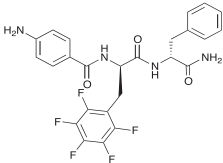
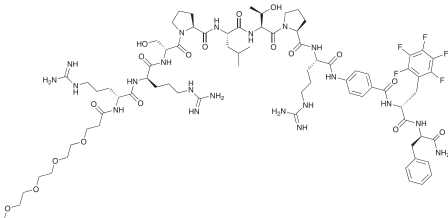
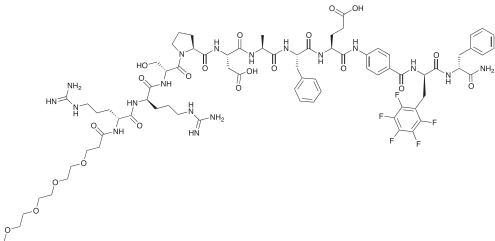
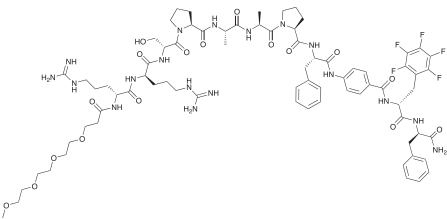
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I. Structures of compounds

**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

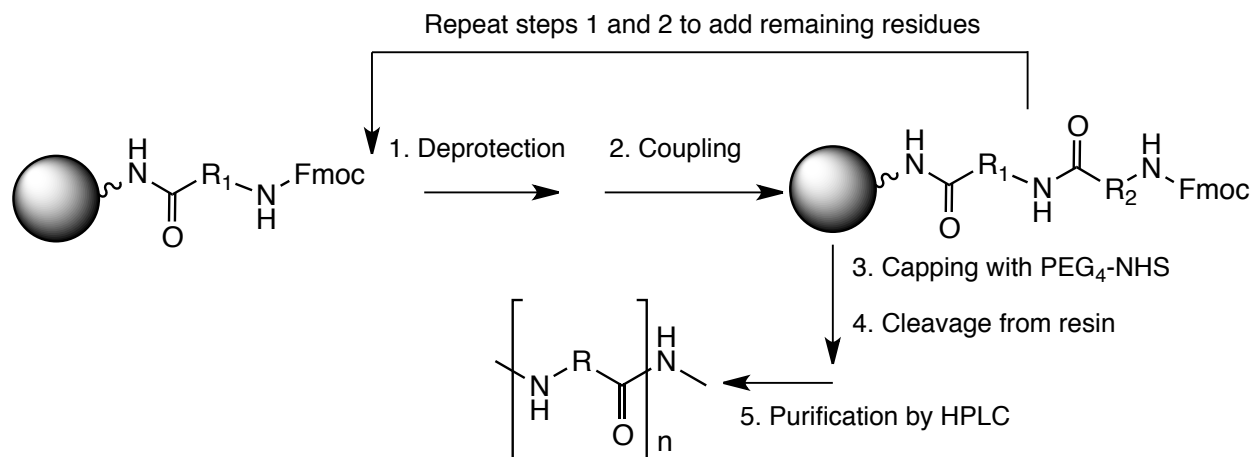
Fmoc-L-amino acids were purchased from Advanced Chem Tech. Fmoc-D-amino acids, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), *N,N,N',N'*-tetramethyl-O-(7-azabenzotriazol-1-yl)uronium hexafluorophosphate (HATU) and rink amide resin were purchased from ChemImpex. All 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<sub>4</sub>-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<sup>1,2</sup> 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.<sup>3</sup> 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<sup>®</sup> microwave synthesizer for at least 50 min. The reaction was determined to be complete based on the Kaiser test.<sup>3</sup>

### 3. PEG<sub>4</sub> capping reaction

PEG<sub>4</sub>-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<sub>2</sub>O, 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<sub>2</sub>O 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<sup>®</sup> Xbridge Prep C18 column (19 x 250 mm) using a linear gradient of CH<sub>3</sub>CN (5-60%) in H<sub>2</sub>O 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<sub>2</sub>HPO<sub>4</sub> (0.1 mol), KH<sub>2</sub>PO<sub>4</sub> (0.1 mol), KCl (5.4 mmol) and NaCl (0.274 mol) were added to 900 mL of H<sub>2</sub>O. The solution was adjusted (using either HCl or NaOH) to pH 7.2 and diluted to 1000 mL with H<sub>2</sub>O.

### 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  $\mu$ L) of this solution was added to a 2 mL glass vial. To the vial was also added DMSO (10  $\mu$ L) and H<sub>2</sub>O (150  $\mu$ L). To initiate gelation, a solution of 200 mM phosphate buffered saline (PBS) pH 7.2 (200  $\mu$ L) was premixed with enzyme (10  $\mu$ L of 2  $\mu$ M) 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  $\mu$ m. 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<sub>3</sub>CN in H<sub>2</sub>O to 2 mg/mL. An aliquot (10  $\mu$ L) was injected into a Waters<sup>®</sup> Xbridge C18 column (2.1 x 100 mm) and eluted using a linear gradient of CH<sub>3</sub>CN (5-95%) in H<sub>2</sub>O 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<sup>®</sup> Micromass ZQ mass spectrometer. The University of Michigan Technical Services department performed high-resolution mass analysis (HRMS-ESI) on all compounds.

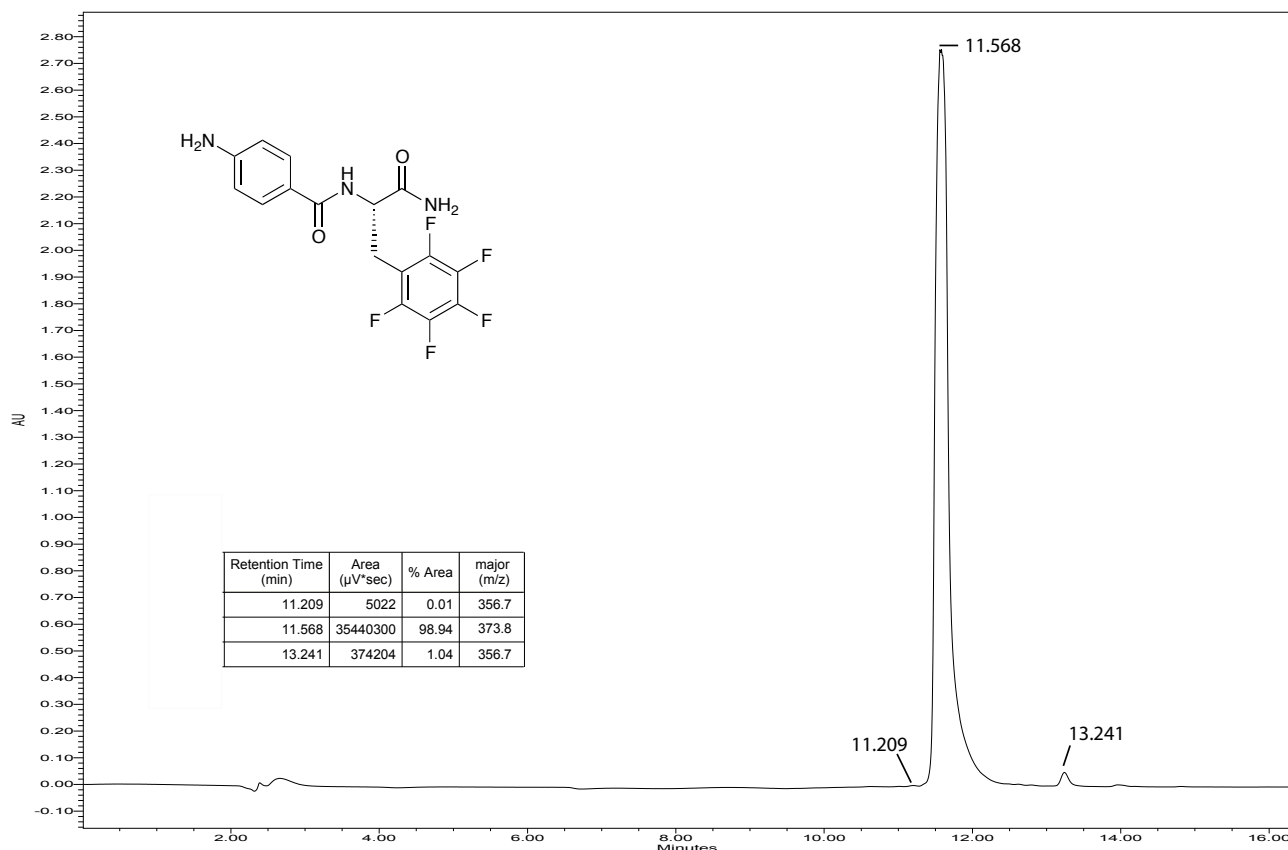
### NMR spectroscopy

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

(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

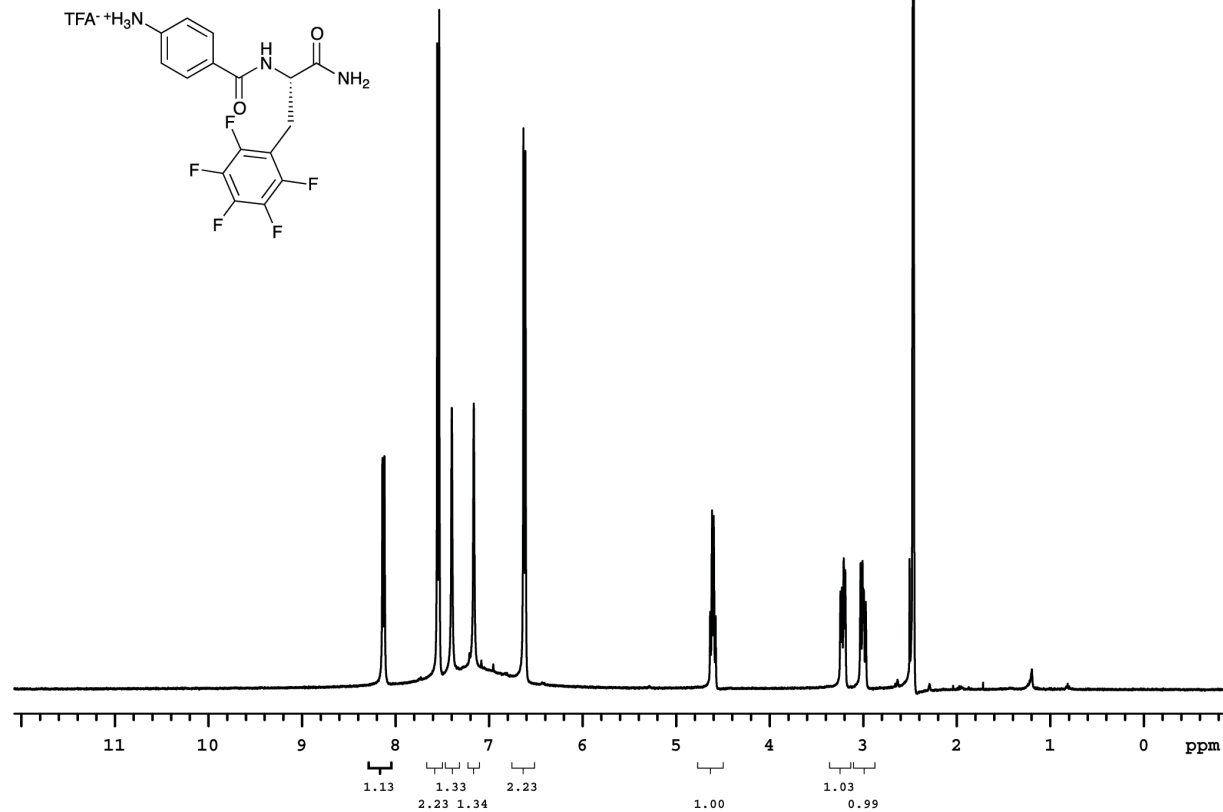
##### a. Compound 1



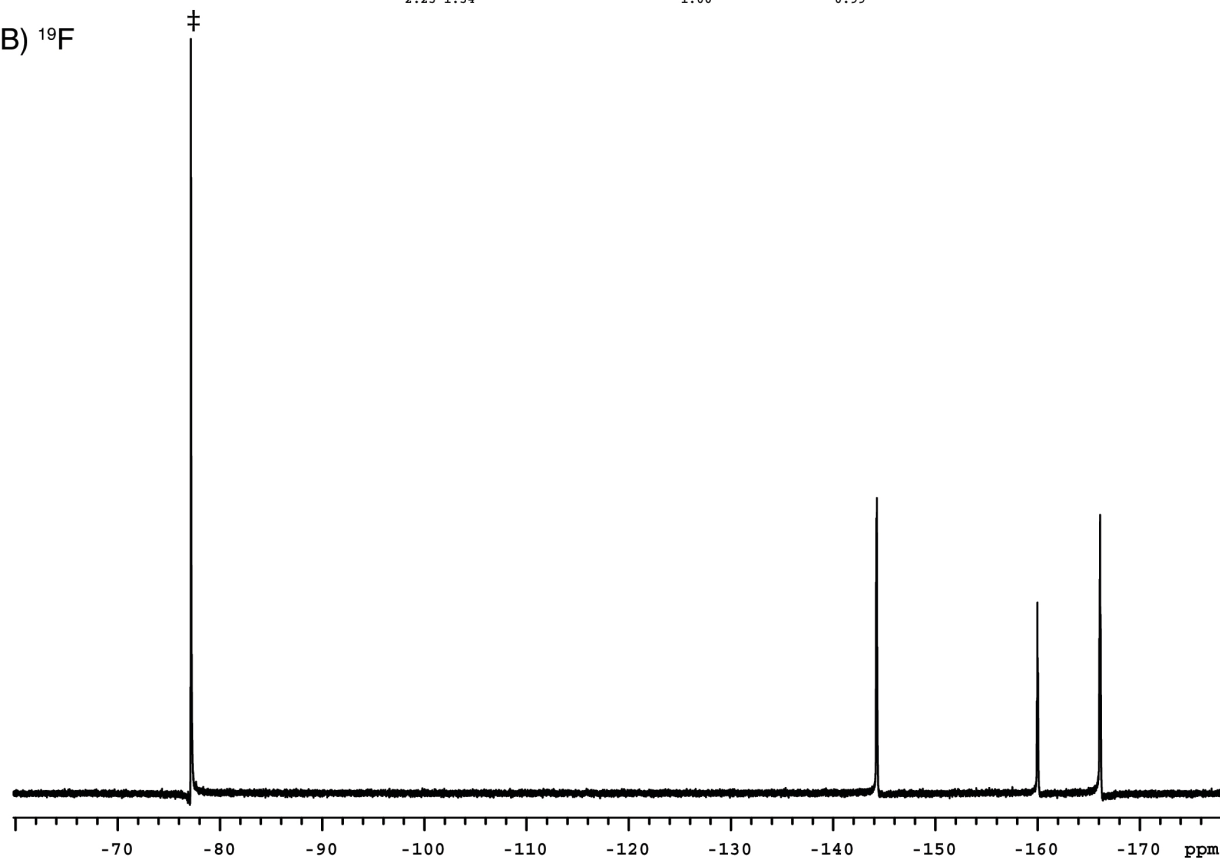
**Figure S1.** Analytical HPLC-MS trace for compound **1**. Compound **1** eluted at 11.568 min and is approximately 99% pure based on absorbance at 280 nm. The compound was subjected to ESI-MS to determine the exact mass of the molecule. HRMS-ESI (m/z): [M+H]<sup>+</sup> calcd for C<sub>16</sub>H<sub>12</sub>F<sub>5</sub>N<sub>3</sub>O<sub>2</sub>, 374.0922; found, 374.0925.

$^1\text{H}$  NMR,  $^{19}\text{F}$  NMR and  $^{13}\text{C}$  NMR spectra for compound 1

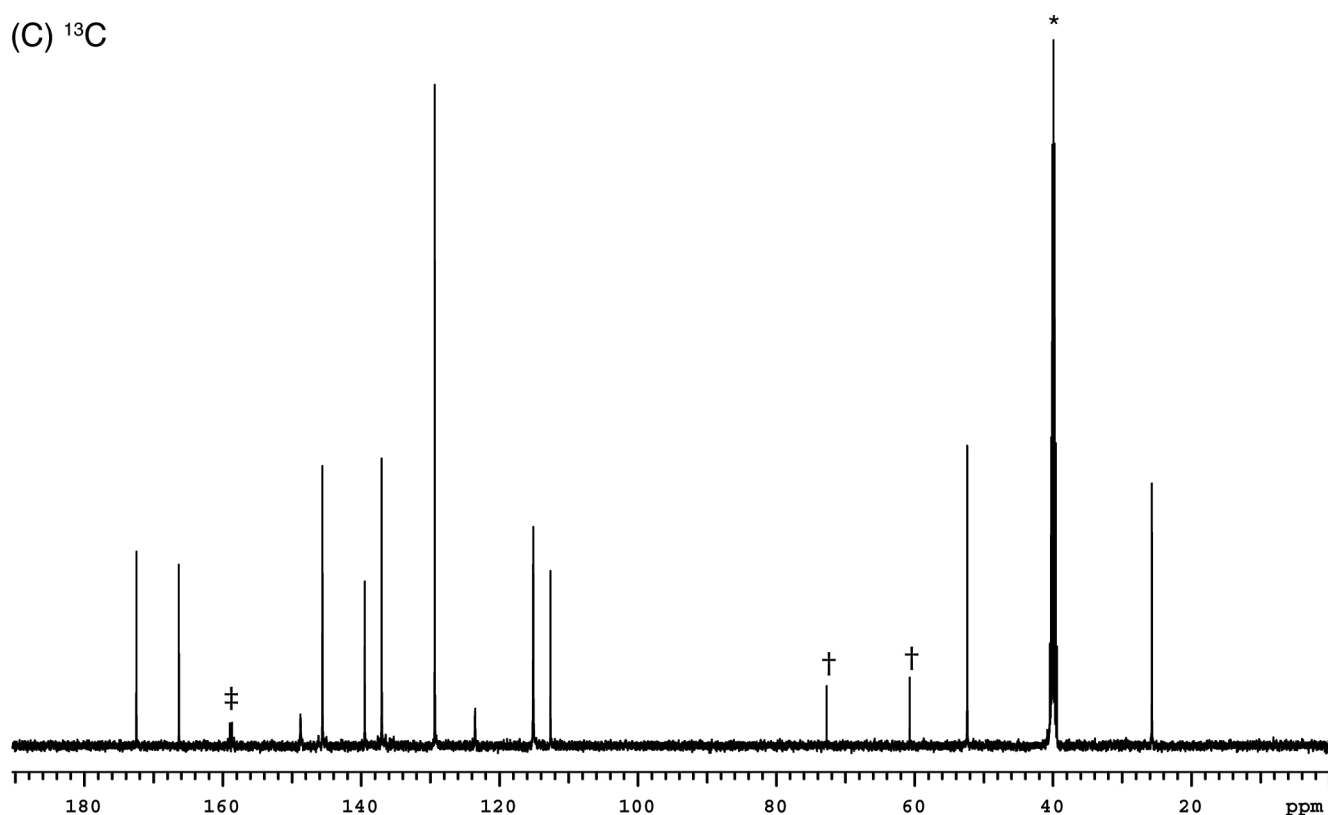
(A)  $^1\text{H}$



(B)  $^{19}\text{F}$

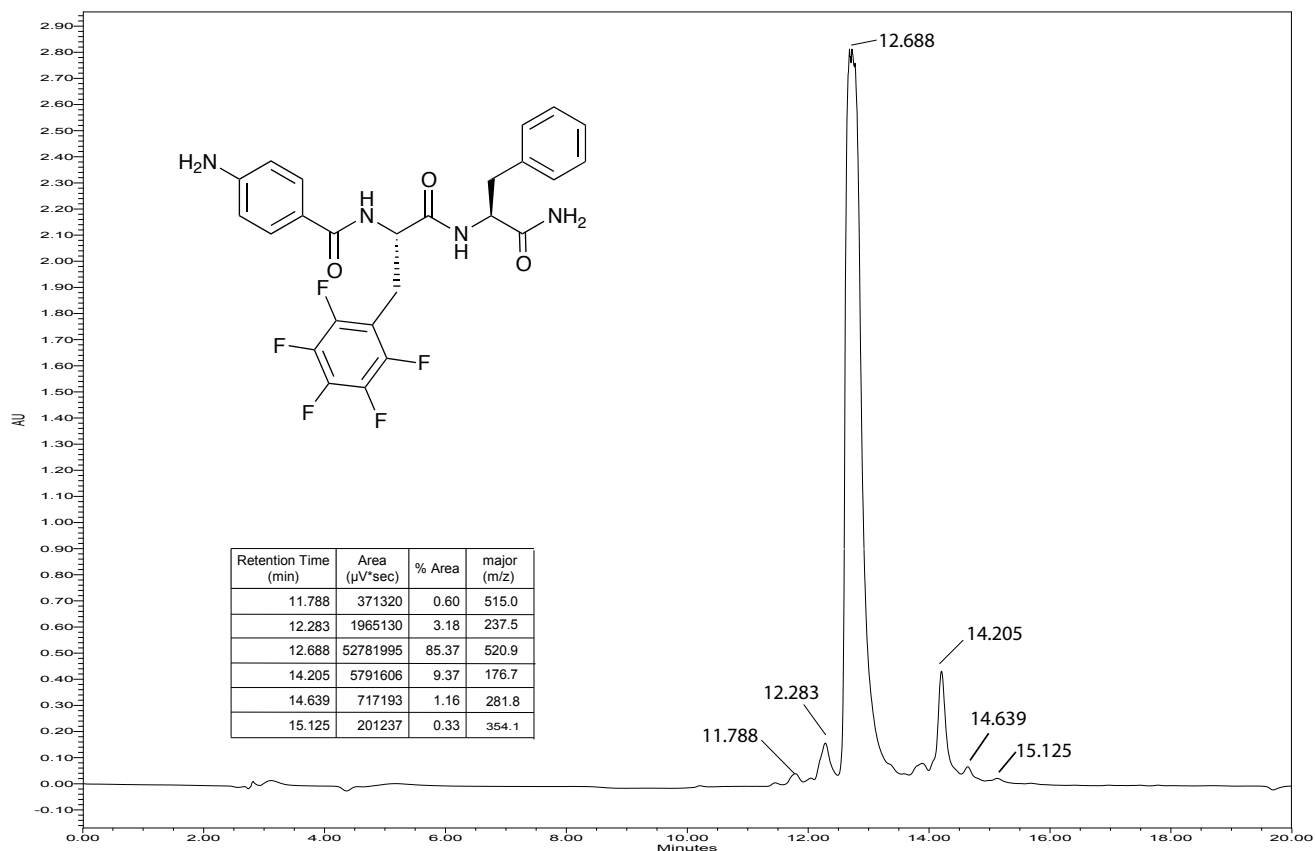


(C)  $^{13}\text{C}$



**Figure S2.** (A)  $^1\text{H}$ , (B)  $^{19}\text{F}$  and (C)  $^{13}\text{C} \{^1\text{H}, ^{19}\text{F}\}$  NMR spectra of **1**.  $^1\text{H}$  NMR ( $\text{DMSO-}d_6$ , 400 MHz):  $\delta$  (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)  $^{19}\text{F}$  NMR ( $\text{DMSO-}d_6$ , 376 MHz):  $\delta$  (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).  $^{13}\text{C} \{^1\text{H}, ^{19}\text{F}\}$  NMR ( $\text{DMSO-}d_6$ , 125 MHz):  $\delta$  (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.

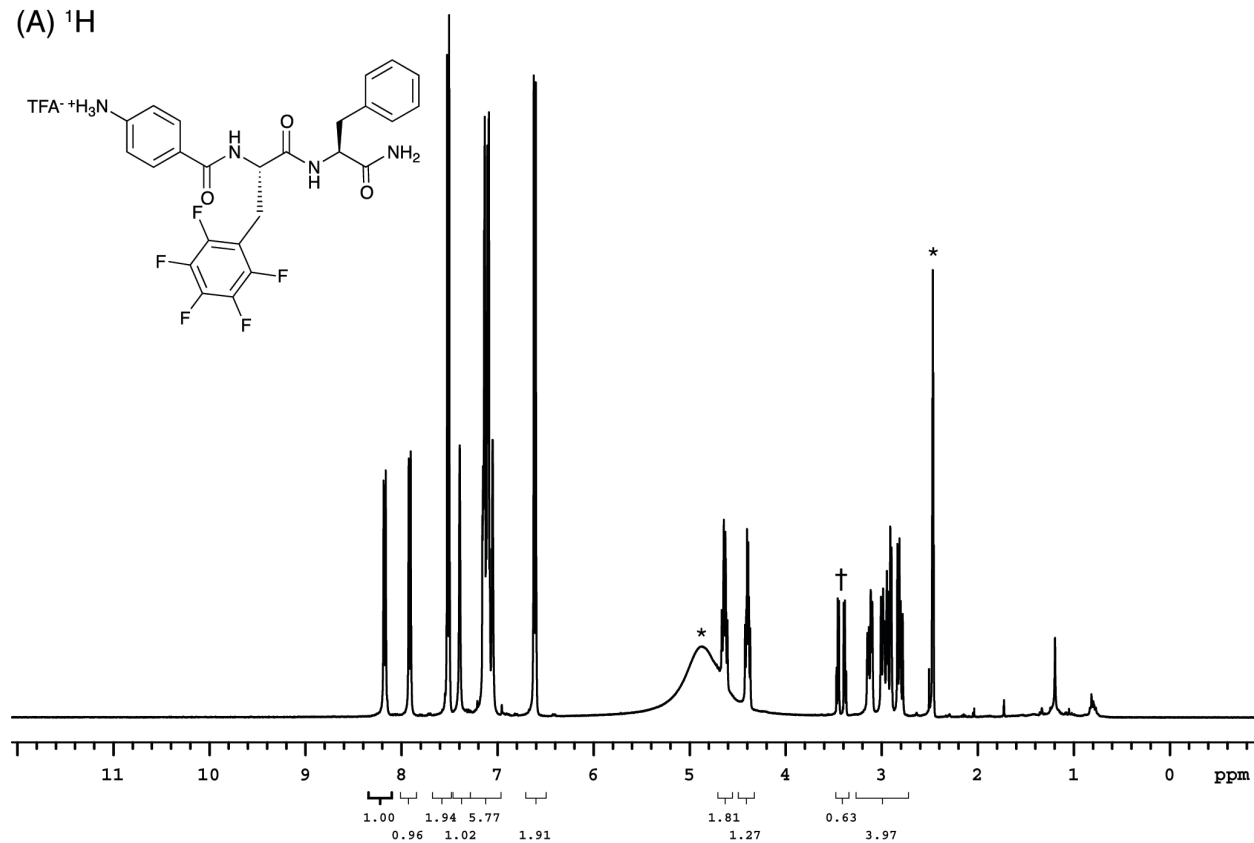
**b. Compound 2**



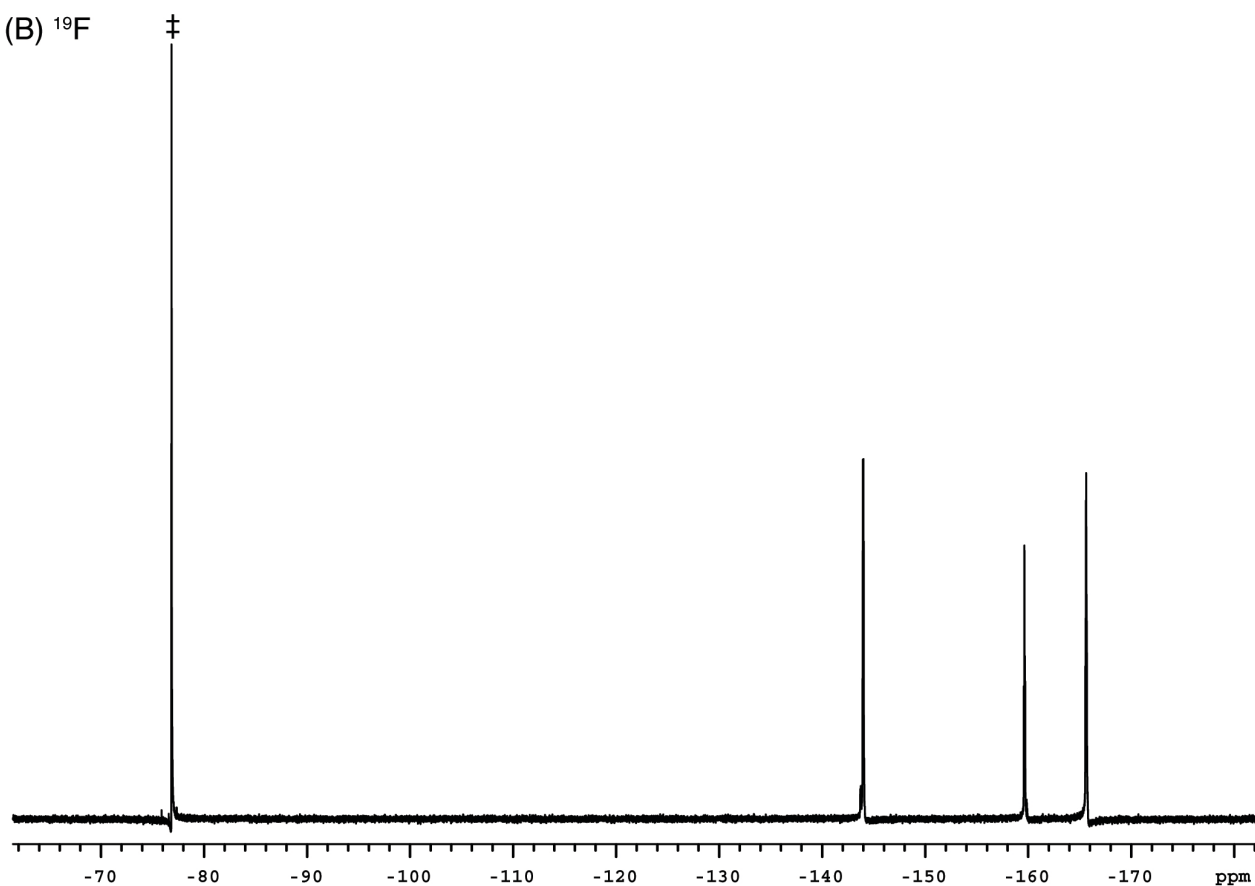
**Figure S3.** Analytical HPLC-MS trace for compound **2**. Compound **2** eluted at 12.688 min and is approximately 85% pure based on absorbance at 280 nm. The compound was subjected to ESI-MS to determine the exact mass of the molecule. HRMS-ESI (m/z): [M+H]<sup>+</sup> calcd for C<sub>25</sub>H<sub>21</sub>F<sub>5</sub>N<sub>4</sub>O<sub>3</sub>, 521.1607; found, 521.1601.

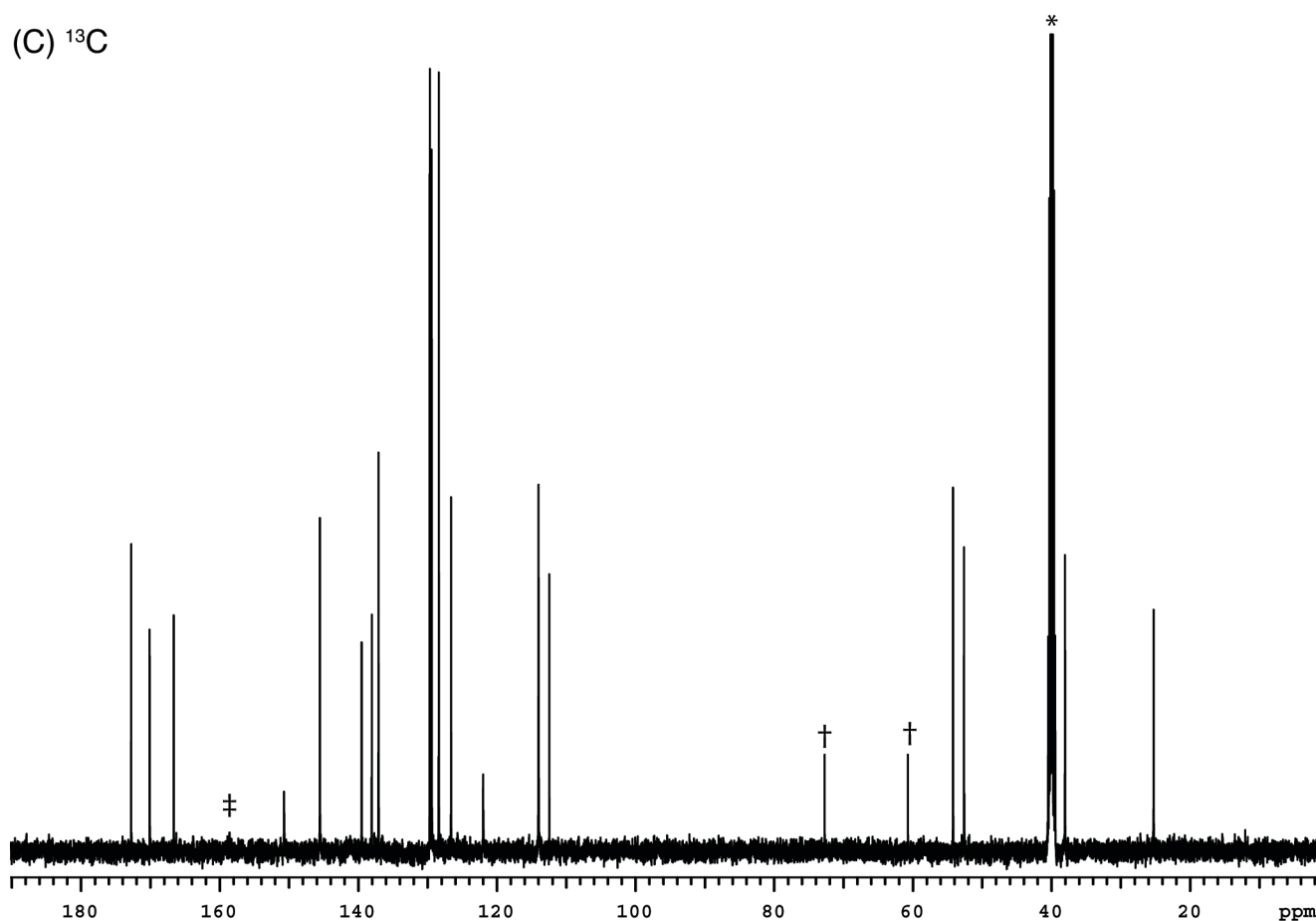
$^1\text{H}$  NMR,  $^{19}\text{F}$  NMR and  $^{13}\text{C}$  NMR spectra for compound **2**

(A)  $^1\text{H}$



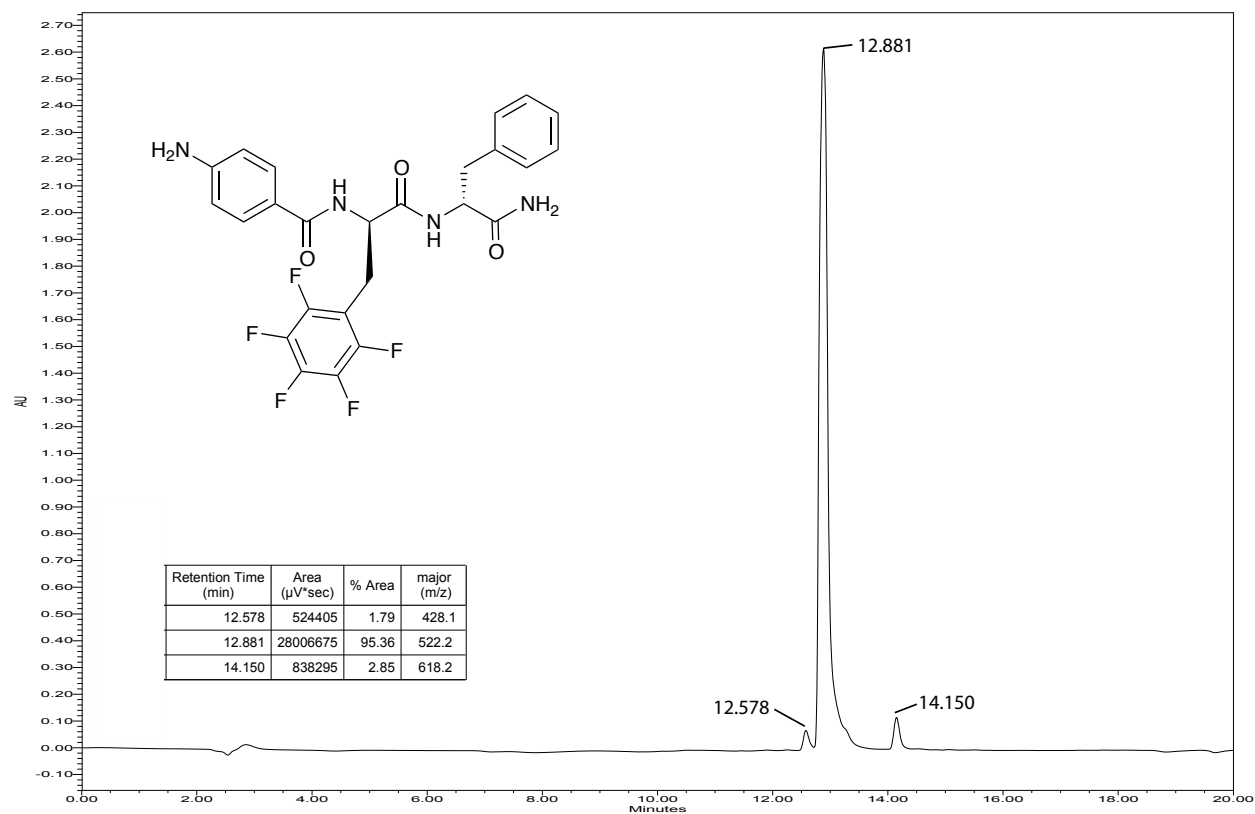
(B)  $^{19}\text{F}$





**Figure S4.** (A)  $^1\text{H}$ , (B)  $^{19}\text{F}$  and (C)  $^{13}\text{C}$   $\{^1\text{H}, ^{19}\text{F}\}$  NMR spectra of **2**. (A)  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ , 400 MHz):  $\delta$  (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)  $^{19}\text{F}$  NMR ( $\text{DMSO}-d_6$ , 376 MHz):  $\delta$  (ppm) -77.00 (s), -144.15 (d,  $J = 24.4$  Hz), -159.80 (t,  $J = 23.2$  Hz), -165.80 (m). (C)  $^{13}\text{C}$   $\{^1\text{H}, ^{19}\text{F}\}$  NMR ( $\text{DMSO}-d_6$ , 125 MHz):  $\delta$  (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

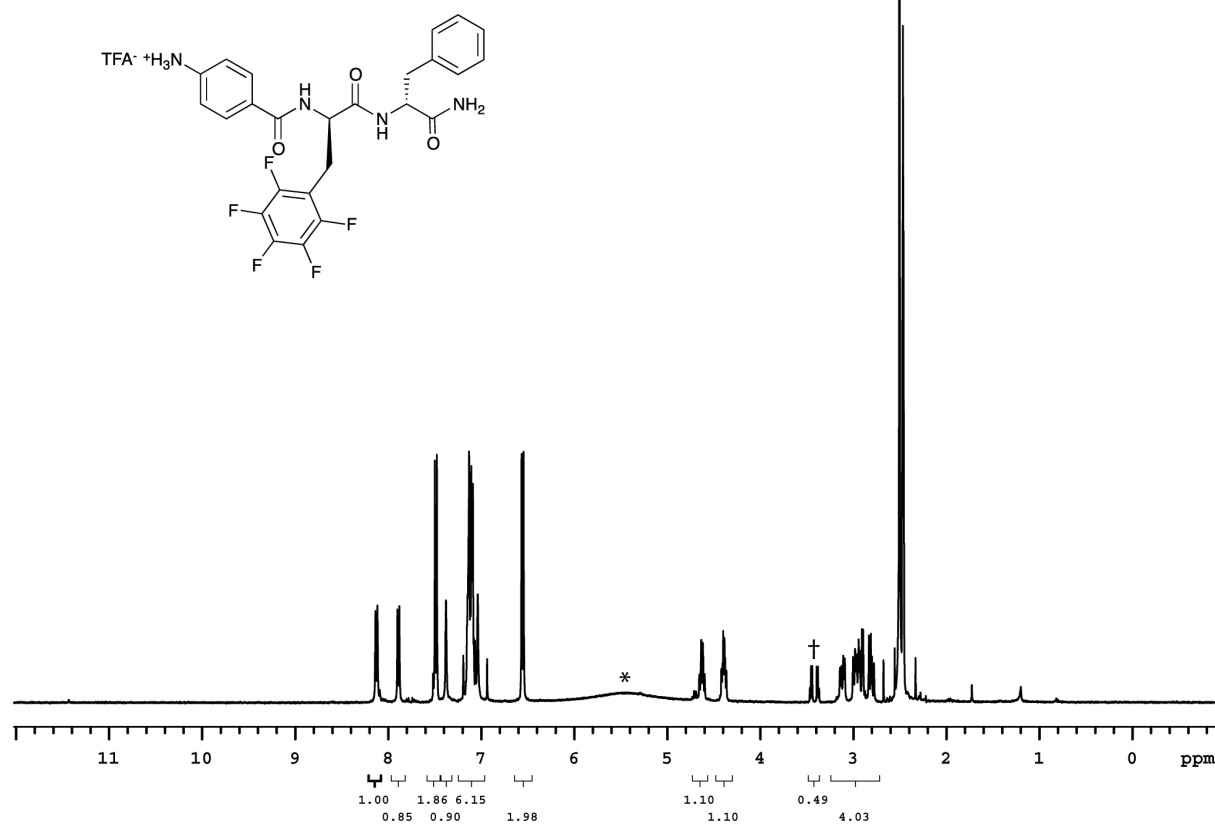


**Figure S5.** Analytical HPLC-MS trace for compound **3**. Compound **3** eluted at 12.881 min and is approximately 95% pure based on absorbance at 280 nm. The compound was subjected to ESI-MS to determine the exact mass of the molecule. HRMS-ESI (m/z): [M+H]<sup>+</sup> calcd for C<sub>25</sub>H<sub>21</sub>F<sub>5</sub>N<sub>4</sub>O<sub>3</sub>, 521.1607; found, 521.1606.

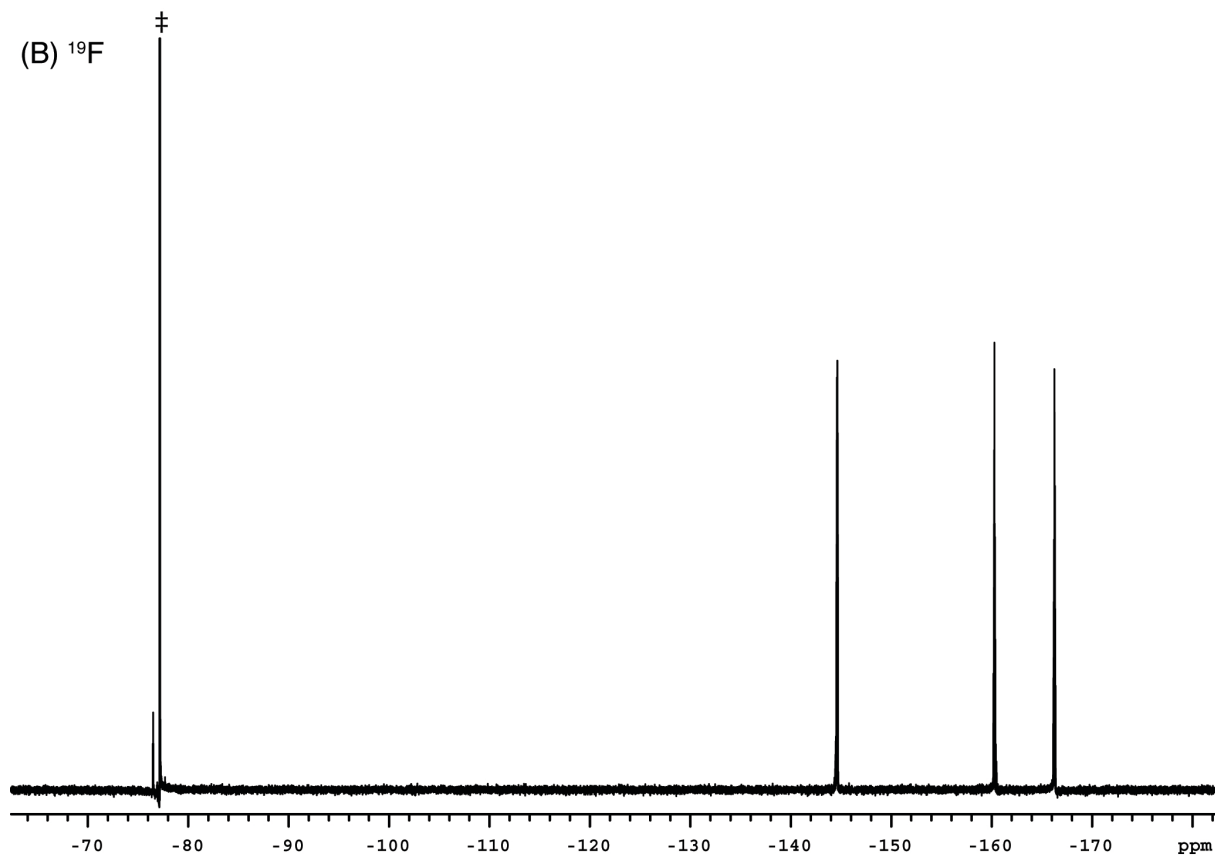


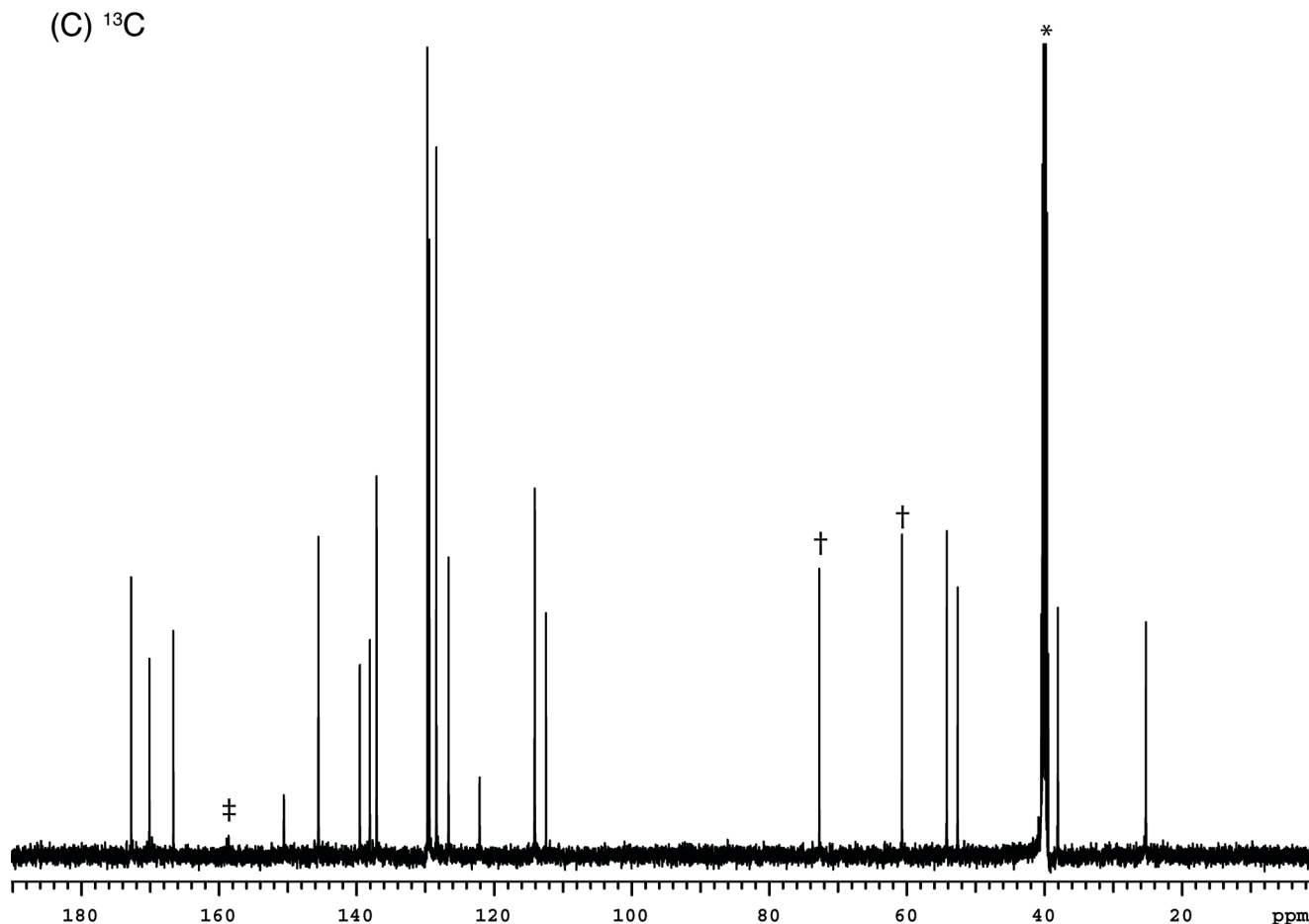
$^1\text{H}$  NMR,  $^{19}\text{F}$  NMR and  $^{13}\text{C}$  NMR spectra for compound **3**

(A)  $^1\text{H}$



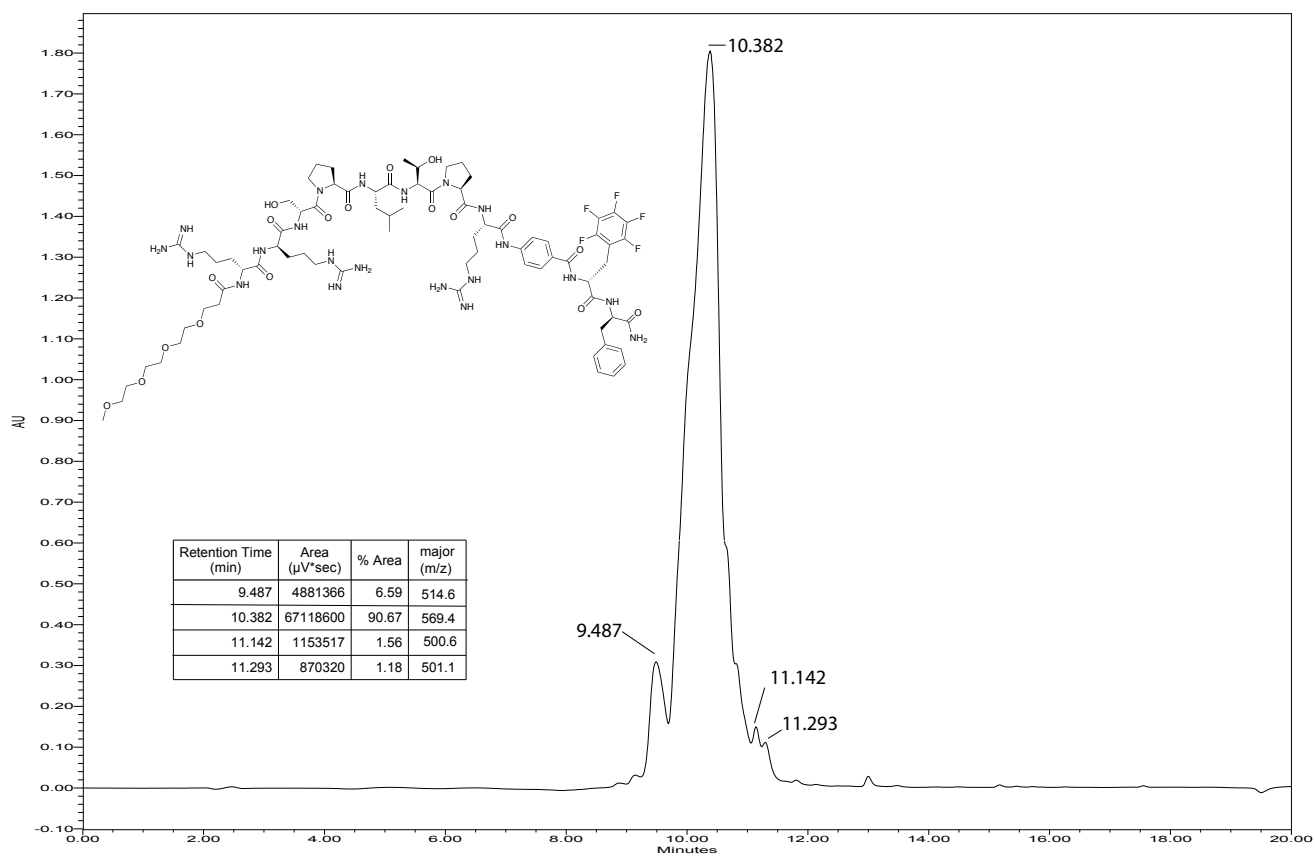
(B)  $^{19}\text{F}$





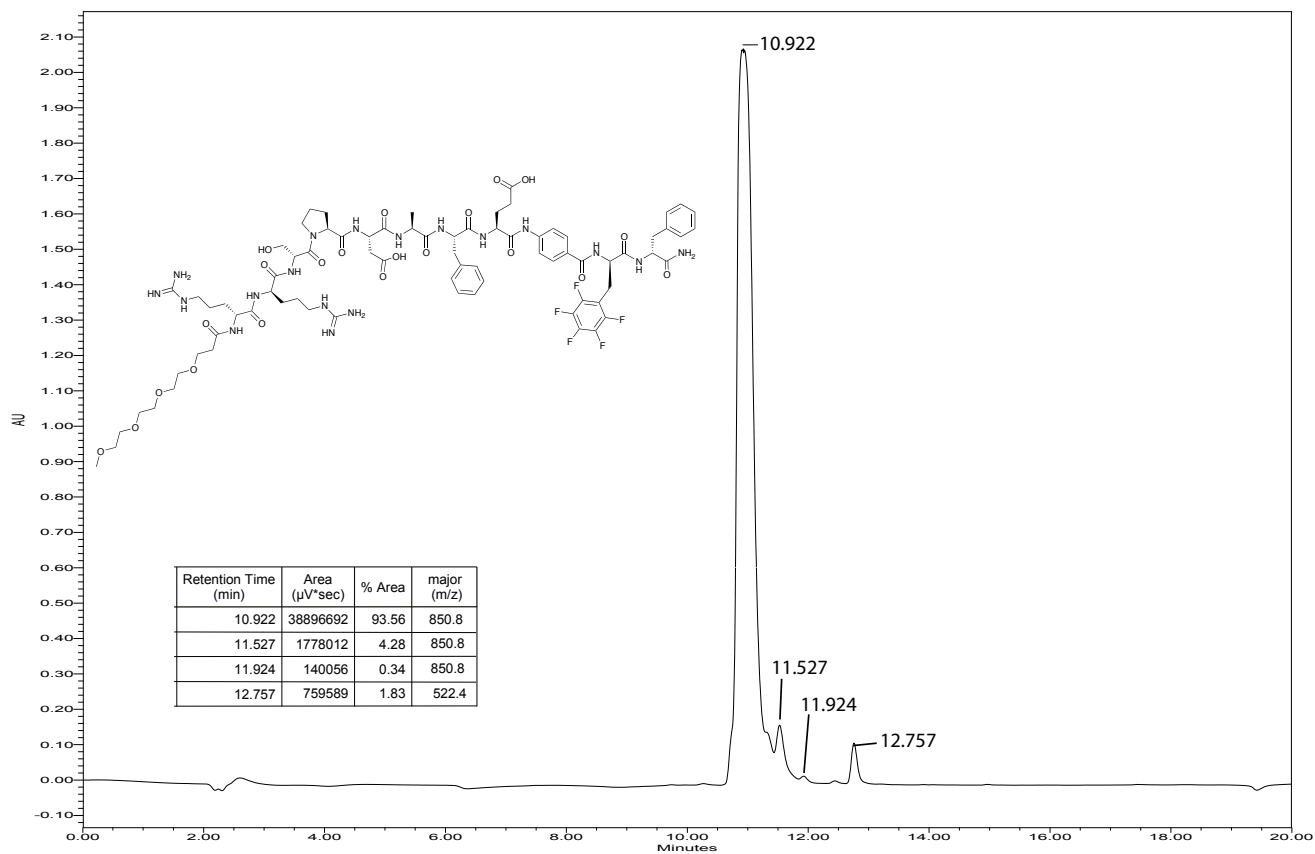
**Figure S6.** (A)  $^1\text{H}$ , (B)  $^{19}\text{F}$  and (C)  $^{13}\text{C}$   $\{^1\text{H}, ^{19}\text{F}\}$  NMR spectra of **3**.  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ , 400 MHz):  $\delta$  (ppm) 8.13 (d,  $J = 8.4$  Hz, 1H), 7.89 (d,  $J = 8.4$  Hz, 1H), 7.49 (d,  $J = 8.4$  Hz, 2H), 7.38 (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)  $^{19}\text{F}$  NMR ( $\text{DMSO}-d_6$ , 376 MHz):  $\delta$  (ppm) -77.17 (s), -144.65 (m), -160.28 (t,  $J = 23.2$  Hz), -166.25 (m). (C)  $^{13}\text{C}$   $\{^1\text{H}, ^{19}\text{F}\}$  NMR ( $\text{DMSO}-d_6$ , 125 MHz):  $\delta$  (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



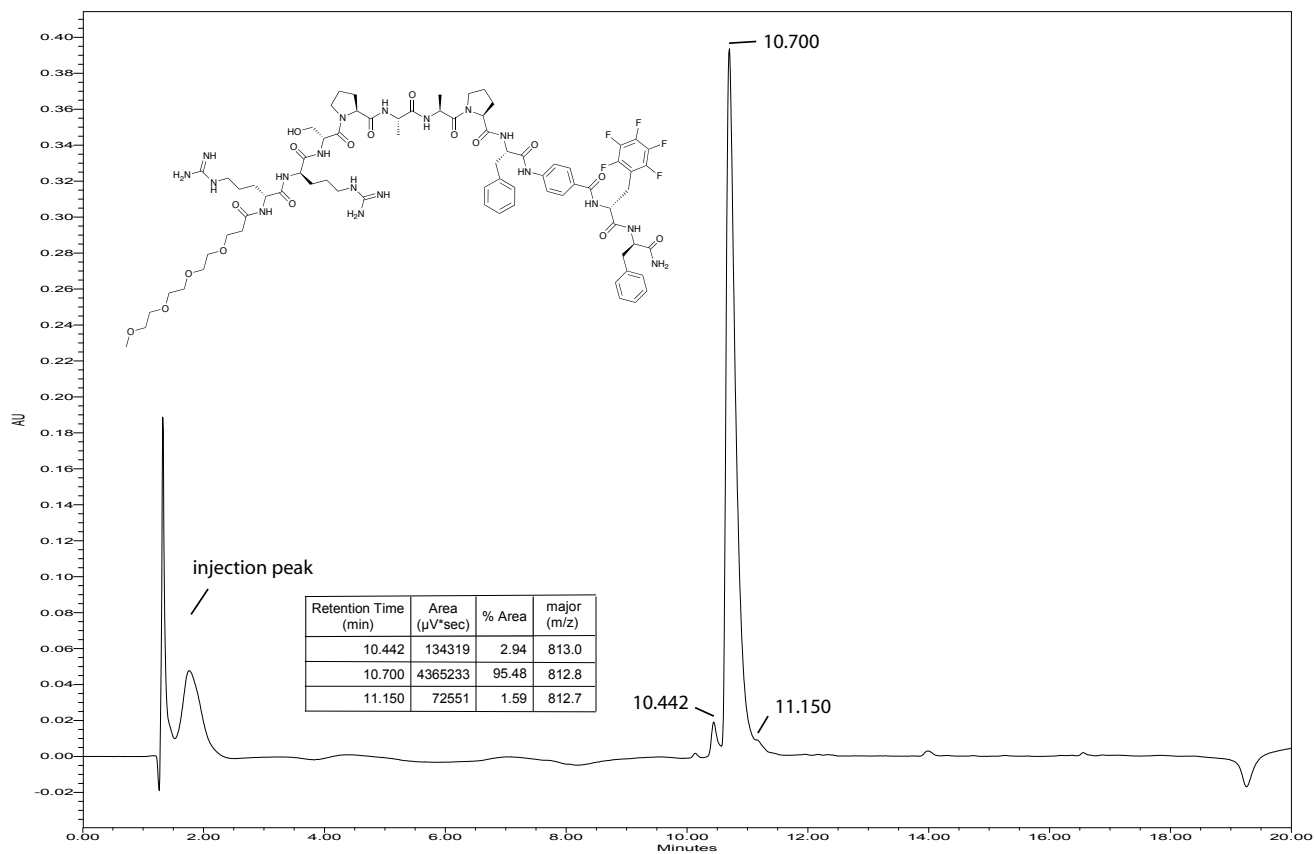
**Figure S7.** Analytical HPLC-MS trace for compound **4**. Compound **4** eluted at 10.382 min and is approximately 90% pure based on absorbance at 280 nm. The compound was subjected to ESI-MS to determine the exact mass of the molecule. HRMS-ESI (m/z):  $[M+H]^+$  calcd for  $C_{76}H_{112}F_5N_{21}O_{18}$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

e. Compound 5



**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

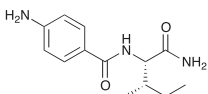
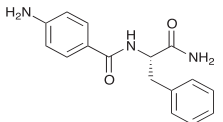
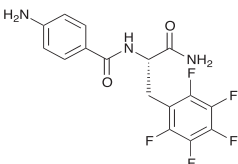
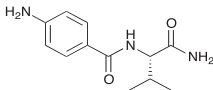
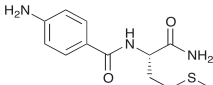
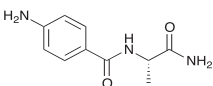
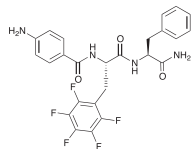
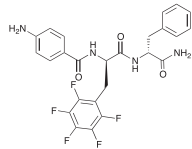


**Figure S9.** Analytical HPLC-MS trace for compound **6**. Compound **6** eluted at 10.700 min and is approximately 95% pure based on absorbance at 280 nm. The compound was subjected to ESI-MS to determine the exact mass of the molecule. HRMS-ESI (m/z):  $[M+H]^+$  calcd for  $C_{75}H_{101}F_5N_{18}O_{17}$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

## V. Gel screen for PABA-peptide

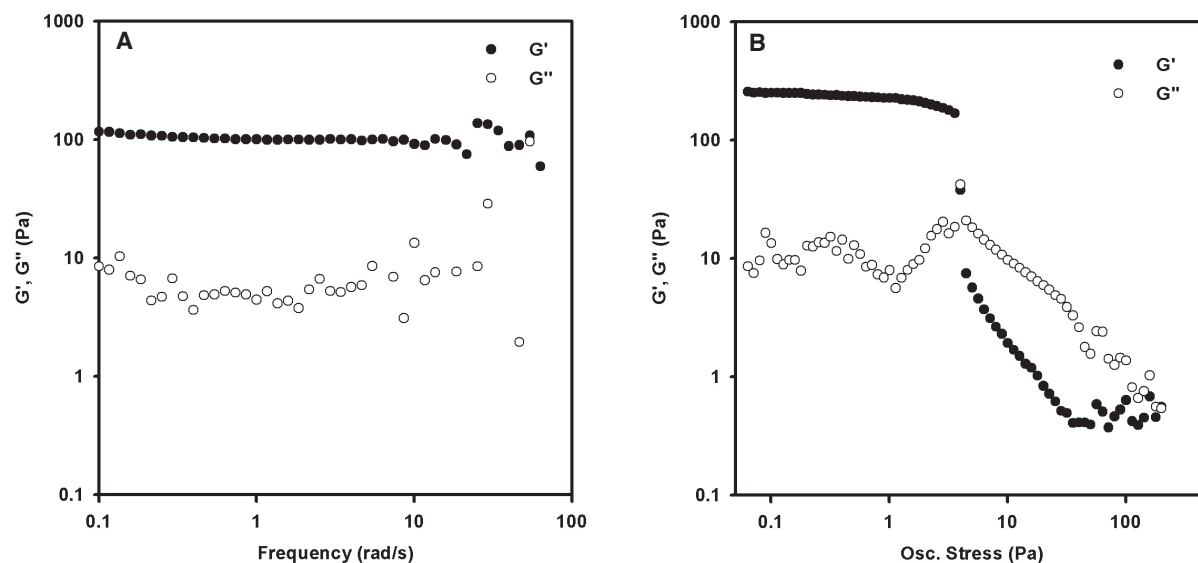
The compounds were synthesized according to the procedures described in the general experimental (pg S3–S4). In total, 6 dipeptide structures were tested and the results of the heat/cool gelation screen are listed in Table S2.

**Table S2.** Screening results for PABA-peptides

PABA-peptide	Structure	cgc
PABA-Ile-NH <sub>2</sub>		precipitated
PABA-Phe-NH <sub>2</sub>		precipitated
PABA-(F <sub>5</sub> )Phe-NH <sub>2</sub>		54 mM
PABA-Val-NH <sub>2</sub>		precipitated
PABA-Met-NH <sub>2</sub>		precipitated
PABA-Ala-NH <sub>2</sub>		precipitated
Compound 2		1.7 mM
Compound 3		1.7 mM

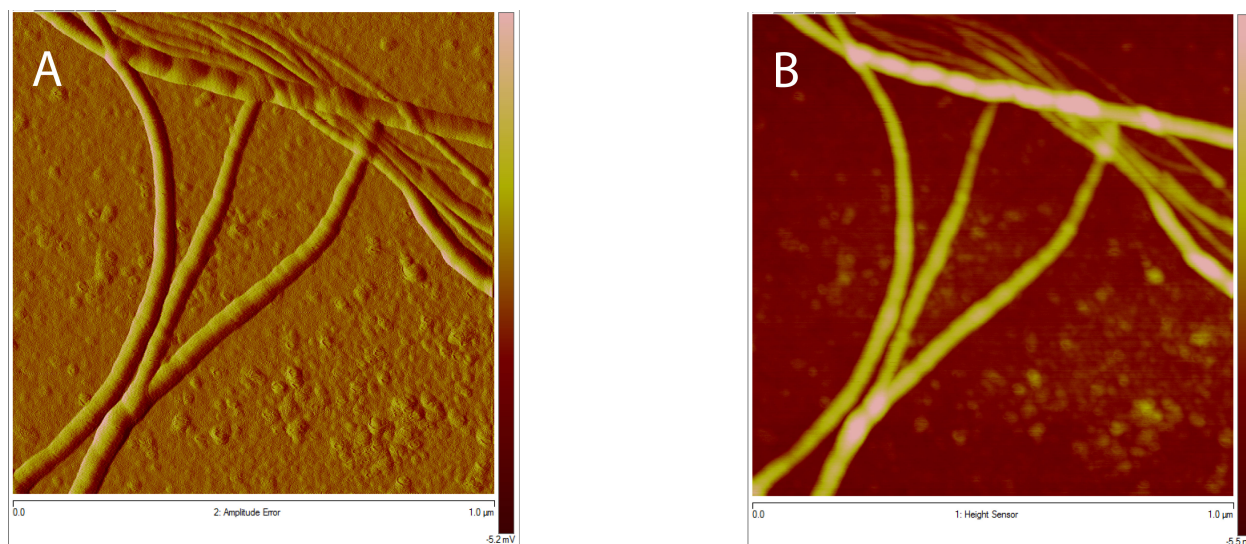
## VI. Gel characterization for compound 3

### (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



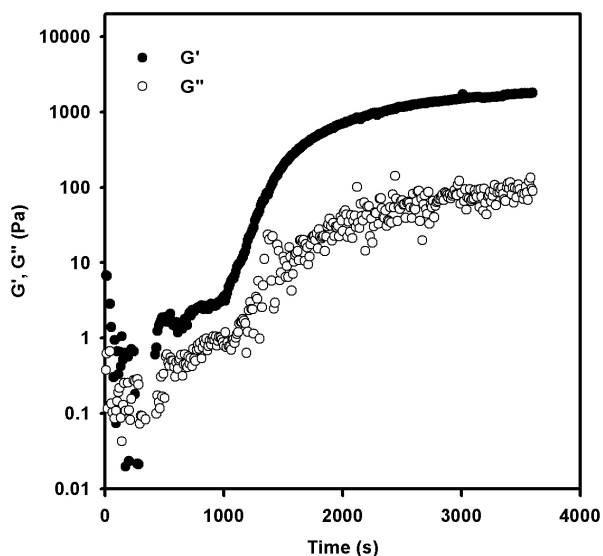
**Figure S11.** Atomic force microscopy (A) height and (B) phase images of gels formed with compound **3** (2.9 mM) in 100 mM PBS at pH 7.2 with 10 vol% DMSO.

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

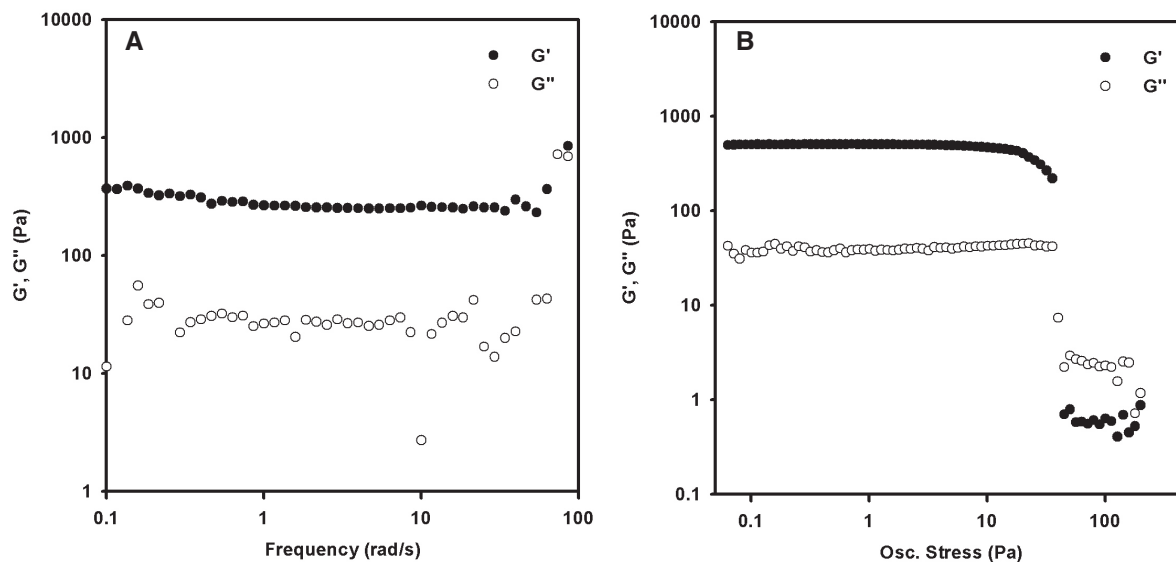
### Experimental

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



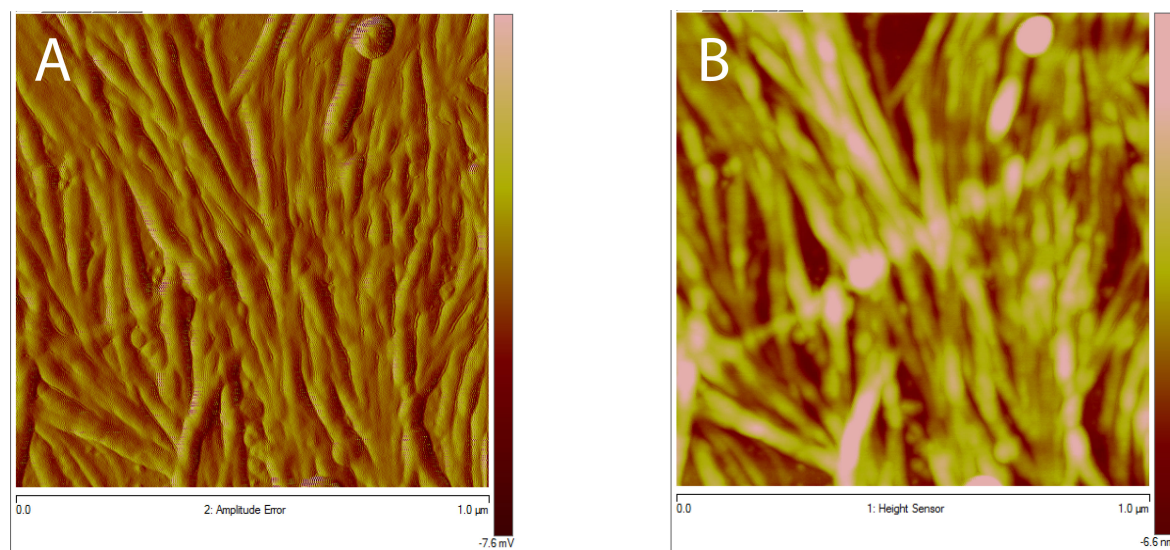
**Figure S12.** Plot of elastic modulus ( $G'$ , filled circles) and loss modulus ( $G''$ , open circles) during thrombin-triggered gelation.



**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

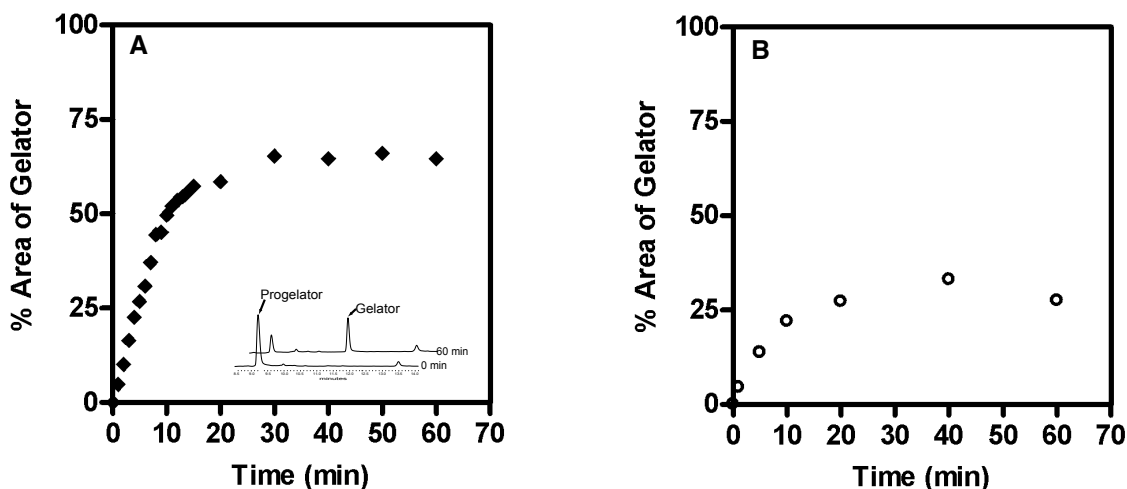


**Figure S14.** Atomic force microscopy (A) height and (B) phase images for compound **3** formed by thrombin-triggered gelation.

## 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  $\mu$ L) were removed periodically and diluted with DMSO (96  $\mu$ L) and 1M HCl (2  $\mu$ L). 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  $\mu\text{L}$ ) containing 59 nM thrombin was added to a vial containing 58.8 mM compound **4** (30  $\mu\text{L}$ ) or DMSO (30  $\mu\text{L}$ ) 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 <b>4</b>	+	+	–
Plasma	+	+	+

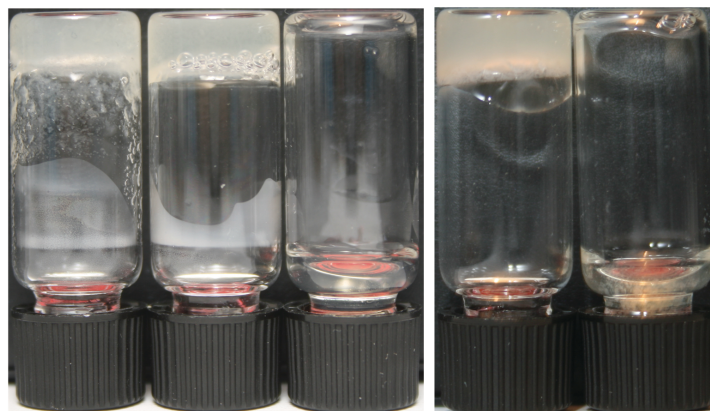


**Figure S16.** Inversion of vials containing fibrinogen-deficient plasma treated with thrombin and compound **4**. The photo was taken after 20 h incubation.

### Experimental

Fibrinogen-deficient plasma (100  $\mu\text{L}$ ) containing compound **4** (12 mM) or DMSO (30  $\mu\text{L}$ ) was treated with thromboplastin in 20 mM  $\text{CaCl}_2$  (170  $\mu\text{L}$ ) or 20 mM  $\text{CaCl}_2$  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  $\text{CaCl}_2$  after 20 h.

Compound <b>4</b>	+	+	–	+	+
Plasma	+	+	+	+	+
Thromboplastin/20mM $\text{CaCl}_2$	+	–	+	+	–
20 mM $\text{CaCl}_2$	–	+	–	–	–
$\text{H}_2\text{O}$	–	–	–	–	+

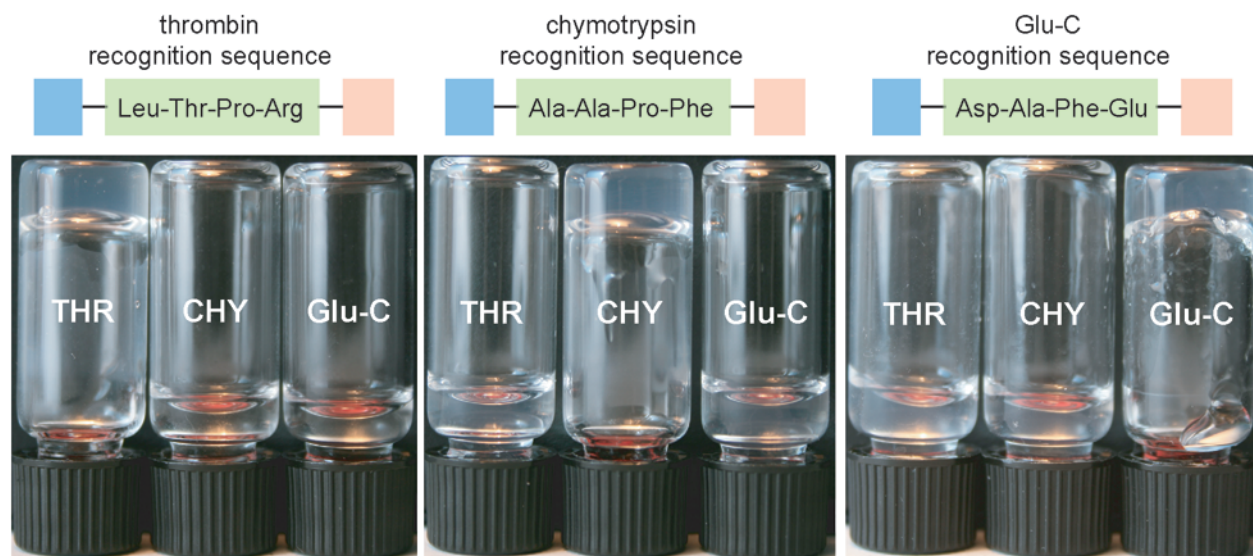


**Figure S17.** Inversion of vials containing fibrinogen-deficient plasma treated with thromboplastin and compound **4**. The photo was taken after 20 h incubation.

## 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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