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Supporting Information

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Supporting information for:

Monovalent and Bivalent Fibrin-specific MRI Contrast Agents for Detection of Thrombus

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Synthesis: Peptide synthesis was carried out on an Advanced ChemTech ACT-348 Ω Synthesizer using Rink amide resin and Fmoc amino acid chemistry amino acids (L-amino acids purchased from Advanced ChemTech or Bachem. Reversed-phase analytical HPLC analyses were obtained with an HP-1100 System and a Microsorb Dynamax C-18 column (4.6 X 250 mm, 5 µm particle size). Reversed-phase semipreparative purifications were performed on a Rainin HPLC System using a Microsorb Dynamax C-18 column (10.0 X 250 mm, 5 µm particle size). Buffers were prepared using house distilled water after purifying through a NANOpure Ultrapure Water System.

Synthesis of compound tetra-DTPA, bis-oxime 1:

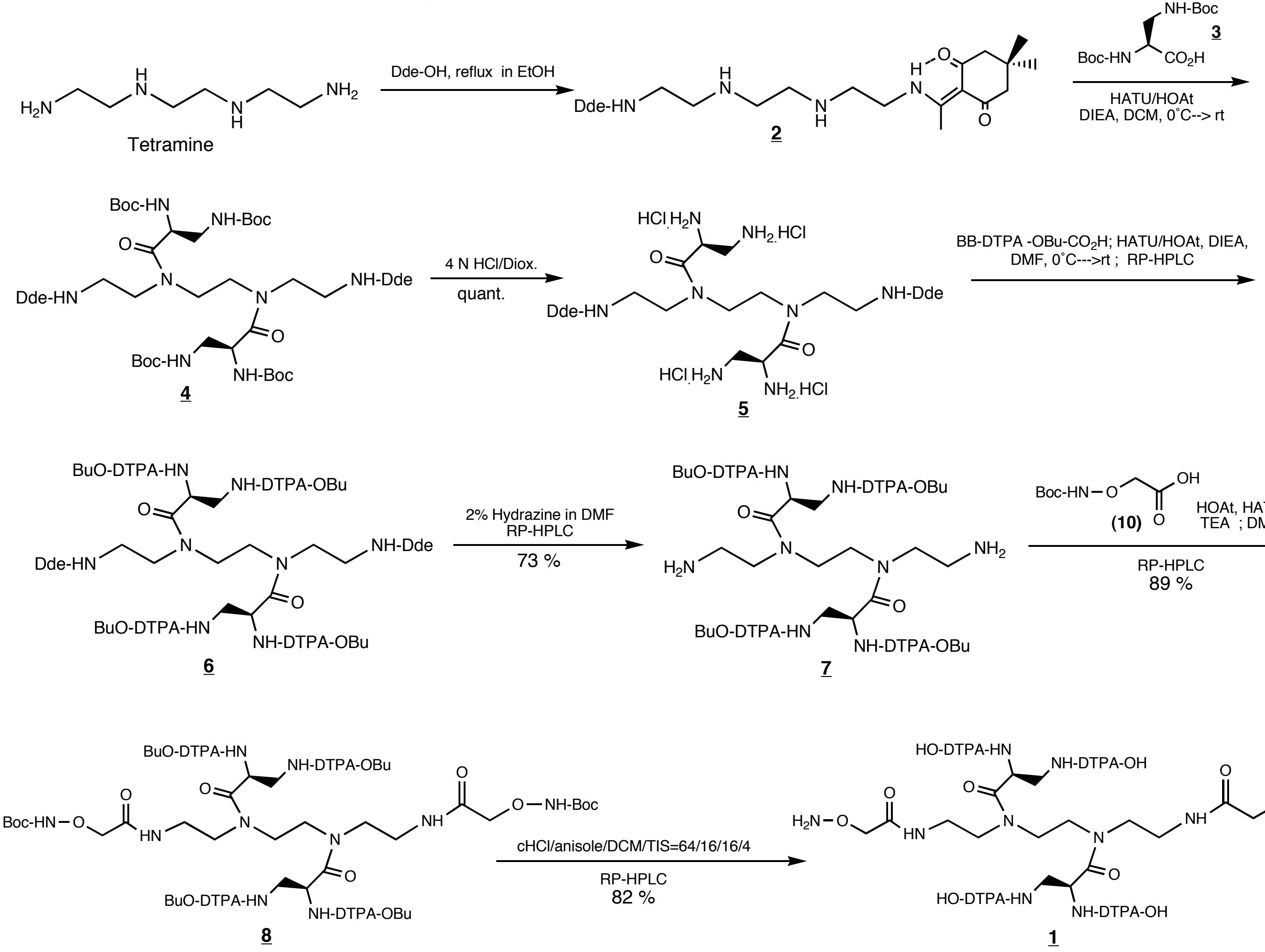
The synthesis of compound **1** is outlined in the scheme below:

Synthesis of Di(Dde)-tetraamine (2)

The selective protection of the primary amines in the presence of secondary amines was achieved with the protecting group Dde-OH (2-acetyldimedone). This reaction is highly selective presumably due to the stabilization provided by a strong intramolecular H-bond.

Triethylenetetraamine (Fluka) (1.50mL, 10 mmol) was taken in a dry 100 mL r.b flask along with Dde-OH (NovaBiochem) (4.0 g, 22 mmol) in 30 mL dry EtOH (200 Proof). The clear pale yellow solution was refluxed for 16 h. After the completion of the reaction (as judged by TLC for the disappearance of tetraamine, traces of Dde-OH remained), the reaction mixture was concentrated *in vacuo* to a dry residue. The residue was dissolved in 250 mL ether and 2 N HCl solution was added until the pH of the aqueous layer was approximately 1. The yellow ether layer containing unreacted Dde-OH was separated. The pale yellow acidic aqueous layer was separated and basified with 50% NaOH solution (exothermic, done in ice bath) to a pH of 12. This aqueous

Synthesis of Compound 1



layer was then back extracted into EtOAc by washing it with EtOAc (3 x 200 mL). The EtOAc layers were pooled, washed with brine (150 mL) and dried (Na_2SO_4) and filtered. The filtrate was evaporated *in vacuo* to yield a pale yellow solid (2.9 g) which was purified on a flash silica column using EtOAc/MeOH (2:3) to EtOAc/(MeOH with 1% NH_4OH) (2:3) to yield pure pale yellow solid as the desired product (2.3 g, 49 %).

TLC: EtOAc: MeOH (1% NH_4OH) = 1:3, R_f=0.22

^{1}H-NMR (300 MHz, $CDCl_3$): d 1.02 (s, 6H, $(CH_3)_2$), 2.34 (s, 4H, Dde-CH_2 x 2), 2.56 (s, 3H, vin CH_3), 2.79 (s, 2H, CH_2 of tetraamine), 2.92-2.94 (t, 2H, CH_2 of tetraamine, *J*=6.2 Hz), 3.48-3.54 (q, 2H, CH_2 of tetraamine, *J*=5.7 Hz)

^{13}C-NMR (75 MHz, CDCl$_3$): d 18.5, 28.5, 30.3, 43.6, 47.9, 48.9, 53.1, 105.5, 108.2, 173.4, 198.2

Analytical HPLC (220 nm): RT=10.14 min on C-4 RP-HPLC (MetaChem, 5 µm): 5% ACN to 80% ACN over 22 min, 8 min hold at 80% ACN, run time 42 min, flow 1.0 mL/min.

MS: m/z 475.2 $(M+H)^+$

Synthesis of DiDde-tetraamine di-Dpr(Boc)$_2$ (4)

Boc-Dpr(Boc)-OH.DCHA (4.85 g, 10 mmol) was suspended in 60 mL EtOAc in a separatory funnel. 1.2 equiv. (12.0 mL, 12 mmol) of ice-cold 2M H_2SO_4 was added to it and the flask shaken till the mixture formed two clear layers. The EtOAc layer was separated and set aside. To the aqueous layer was added 20 mL of cold water and extracted further with EtOAc (2 x 50 mL). The EtOAc layers were combined, washed with brine, dried over anhydrous $MgSO_4$ and filtered. The filtrate was evaporated *in vacuo* and the solid obtained was dried under high-vac pump to yield 3.23 g of the desired Boc-Dpr(Boc)-OH free acid **(3),** as white crystalline solid.

^{1}H-NMR (300 MHz, DMSO-d$_6$): d 1.35 (s, 18H, 2 x Boc CH3), 3.30 (s, 2H, b-CH$_2$ of Dpr), 3.96-3.98 (m, 1H, a-H of Dpr), 6.87-6.90 (d, 1H, NH, *J*=7.8 Hz).

Boc-Dpr(Boc)-OH as free acid (1.52 g, 5.0 mmol) was dissolved in 15 mL anhydrous DCM at 0˚C (ice-bath) under nitrogen atmosphere. To it was added HOAt (0.68 g, 5.0 mmol) and DIEA (0.35 mL, 2 mmol) and the clear solution was allowed to stir at 0˚C (monitored by TLC for disappearance of the acid and formation of the active-ester). After 15 min, the DiDde-tetraamine **(2)** was added, followed by the addition of HATU (1.90 g, 5.0 mmol) and another 2 mmol of TEA. Anhydrous DMF (5 mL) was added to insure solubility. The reaction was stirred for 36 h until completion as judged by TLC analyses. The solvents were evaporated *in vacuo* and the residue was taken up in 150 mL EtOAc. The organic layer was washed with 1 N HCl (2 x 80 mL), saturated $NaHCO_3$ (1 x 80 mL), brine (1 x 100 mL), and then dried over anhydrous Na_2SO_4, filtered and evaporated *in* *vacuo* to give a white solid (1.92 g). This solid was further purified by flash silica column chromatography using EtOAc (5 % MeOH) to give the desired product as a white solid (1.3 g, 62 %).

TLC: EtOAc (5% MeOH)= R_f=0.35

^{1}H-NMR (300 MHz, $CDCl_3$): d 1.01 (s, 6H, $(CH_3)_2$), 1.35 (s,18H, 2X Boc's), 2.34 (s, 4H, 2 x Dde-CH_2), 2.58 (s, 3H, vin CH_3), 3.30 (s, 2H, b-CH2 of Dpr), 3.44-3.67 (bm, 6H, CH_2's of tetraamine), 3.85-4.10 (m, 1H, a-H's of Dpr), 5.44-5.52 (d, 2H, NH), 5.74 (bs, 1H, NH).

Analytical HPLC (220 nm): RT=23.56 min on C-4 RP-HPLC (MetaChem, 5 µm) 5 % ACN to 80 % ACN over 22 min, 8 min hold at 80% ACN, run time 42 min, flow 1.0 mL/min.

MS: m/z 1047.7 $(M+H)^+$

Synthesis of Di-Dde-tetraamine-diDpr.HCl salt (5)

DiDde-tetraamine-diDpr$(Boc)_2$ **(4)** was dissolved in 40 mL 4 N HCl/Dioxane in an ice-bath until a clear solution was obtained. After 20 min, a white lumpy solid formed in the flask which was allowed to stir for another 10 h until the starting material was completely consumed as evidenced by RP-HPLC. The suspension was triturated with cold ether and evaporated *in vacuo* to obtain a white solid. The solid was dried under high-vacuum to yield the desired product as the HCl salt (1.09 g, quant).

TLC: EtOAc: MeOH (1% NH_4OH) =1:3, R_f=0.10

Analytical HPLC (220 nm): RT=9.36 min on C-4 RP-HPLC (MetaChem, 5 µm) 5 % ACN to 80 % ACN over 22 min, 8 min hold at 80 % ACN, run time 42 min, flow 1.0 mL/min.

MS: m/z 647.3 $(M+H)^+$

Synthesis of DiDde-diDpr-tetra-BB-DTPA-OtBu (6)

BB-DTPA-O^{t}Bu (Hauser, CO) (2.16 g, 3.0 mmol) was dissolved in 10 mL anhydrous DMF in a dry r.b flask at 0˚C (ice-bath) under nitrogen atmosphere. To it was added HOAt (0.41 g, 3.0 mmol) and DIEA (0.52 mL, 3 mmol) and the clear solution was allowed to stir at 0˚C (monitored for active-ester formation **by???**). The Di-Dde-tetraamine-diDpr salt **(5)** (0.40 g, 0.50 mmol) was separately dissolved in 3 mL anhydrous DMF and DIEA (0.13 mL, 0.75 mmol) was added to it to yield a clear solution. This free amine solution was slowly dripped into the activated ester solution over a period of 5 min. Finally HATU (1.14 g, 3.0 mmol) was added to the mixture along with the remainder DIEA (0.09 mL). The yellow colored solution was stirred at 0˚C and allowed to warm to room temp overnight. The reaction was stirred for 36 h. The solvents were removed *in vacuo* and the residue taken up in 150 mL EtOAc. The organic layer was washed with 1 N HCl (2 x 80 mL), saturated $NaHCO_3$ (2 x 80 mL), brine (1 x 100 mL), dried over anhydrous Na_2SO_4, filtered and evaporated *in vacuo* to give a pale yellow colored solid (3.15 g). The crude product was further purified on the Prep RP-HPLC [C-4, flow 12 ml/min, 220 nm: 0 to 1 min, 50% B; 1 to 31 min, 50 to 100 %B; stay at 100% B till 39 min. Buffer, A=95% H_2O (5% ACN); Buffer, B=ACN (No TFA)]. The fractions containing the desired compound were pooled and lyophilized to give a white solid (1.01g, 58 %).

Analytical HPLC (220 nm): RT=25.22 min on C-4 RP-HPLC (MetaChem, 5 µm) 5 % ACN to 80 % ACN over 22 min, 8 min hold at 80 % ACN, run time 42 min, flow 1.0 mL/min.

Synthesis of Tetraamine-tetra-BB-DTPA-O^{t}Bu (7)

The driving force for the deprotection with hydrazine is the formation of 3,6,6-trimethyl-4-oxo-4,5,6,7-tetrahydro-1H-indazole (A_{max} = 300 nm, can be easily monitored by HPLC). DiDde-tetraamine-diDpr-tetra-BB-DTPA-O^{t}Bu **(6)** (0.38 g, 0.11 mmol) was dissolved in 8 mL, 2 % v/v hydrazine in DMF and stirred for 10 min at room temp (monitored by HPLC). The reaction mixture was concentrated *in vacuo* and the residue taken up in CH_3CN and purified on Prep-RP-HPLC [C-4, flow 20 ml/min, 220 nm, 0 to 8 min 40% B; 8 to 12 min 40 to

85 %B; 12 to 16 min, 85% to 100 %B; stay at 100% B until 18 min. Buffer, A=95% H_2O (5% ACN); Buffer, B=ACN (No TFA)]. The fractions containing the desired compound were pooled and lyophilized to give a white solid (0.25 g, 73 %)

Analytical HPLC (220 nm): RT=24.14 min on C-4 RP-HPLC (MetaChem, 5 µm) 5 % ACN to 80 % ACN over 22 min, 8 min hold at 80 % ACN, run time 42 min, flow 1.0 mL/min.

MS: m/z 1563.5 $(M+2H)^+$; 1042.6 $(M+3H)^+$

Synthesis of Di-Boc-AoA-tetra-BB-DTPA-O^{t}Bu (8)

Boc-aminooxyacetic acid **(10)** (EMD Biosciences) (0.17 g, 0.88 mmol) was dissolved in 5 mL anhydrous DMF solution along with HOAt (0.12 g, 0.88 mmol) at 0˚C under nitrogen atmosphere (choice of reaction solvent is critical as there were difficulties in identifying and isolating the product when the reaction is carried out in either DCM or ACN). Tetraamine-tetra-BB-DTPA-O^{t}Bu **(7)** (1.25 g, 0.40 mmol) was dissolved in 10 mL anhydrous DMF and added dropwise to the activated acid solution over 5 min. TEA (0.23 mL, 1.60 mmol) was added followed by HATU (0.34 g, 0.88 mmol) and stirred overnight and allowed to warm to room temp. After completion, the reaction mixture was taken in 200 mL EtOAc and the organic layer was washed with 0.1 N HCl solution (2 x 100 mL), washed with saturated $NaHCO_3$ solution (2 x 100 mL), brine (150 mL) and dried over anhydrous $MgSO_4$. The solution was filtered and the filtrate evaporated *in vacuo* to obtain a white solid (1.23 g, 88.7 %). The product was sufficiently pure and was taken further without HPLC purification. This compound is extremely hydrophobic and earlier attempts to purify by RP-HPLC on C-4 column resulted in peak tailing and reduced purification yields.

Analytical HPLC (220 nm): Broad peak at RT=13.82 min on RP-HPLC (C-4, 150 X 4.6mm, MetaChem, 5 µm). 20 % ACN (0.1% TFA) to 100 % ACN (0.1% TFA) over 12 min, hold at 80 % ACN for 4 min, Flow 1.0 mL/min.

MS: m/z 1733.5 $(M+2H)^{2+}$; 1156.1 $(M+3H)^{3+}$

Synthesis of AoA-tetra-BB-DTPA-OH (1)

Di-Boc-AoA-tetra-BB-DTPA-O^{t}Bu **(8)** (2.40 g, 0.69 mmol) was dissolved in 10-12 mL cleavage cocktail comprising of cHCl/anisole/DCM/TIS (64/16/16/4) (using a smaller volume of cleavage cocktail was preferential since the removal of large amounts of cHCl by lyophilization is a problem). Water was found to be an essential scavenger in the cleavage cocktail as several unknown adducts were isolated when this deprotection was carried out in anhy. HCl in dioxane. The mixture was stirred in ice-bath for 3 h. A milky viscous suspension was obtained. The reaction was monitored for the disappearance of the starting material. The product was not retained on the column and eluted with the solvent front. After complete deprotection the reaction mixture was diluted with 20 mL water and extracted with ether (2 x 60 mL). The ether layer was separated and the aqueous layer lyophilized and further purified on a Prep RP-HPLC [C-18 VyDac, 250 x 22 mm, Flow 10 ml/min, 220 nm (0.2 AUFS): 0 to 6 min, 0 % B; 6 to 31 min, 0 to 50 % B; 31 to 33 min, 50 to 100 % B. Buffer, A=95 % 5 mM NH_4OAc (5 % ACN); Buffer, B=95 % ACN (5 %, 5 mM NH_4OAc)]. The product **(9)** was isolated from the first fractions which eluted off the column. It was lyophilized to a white solid (1.26 g, 82.2 %) and stored in the freezer till further use.

Analytical HPLC (220 nm): RT=1.80 min on RP-HPLC (C-4, 250 x 4.6 mm, YMC-Pack, 5 μm). Buffer: 0.13 M sodium phosphate (NaP_i), 0.2 mM EDTA, pH 6.5, Flow 1.5 mL/min.

MS: m/z 1071.9 $(M+2H)^{2+}$; 715.2 $(M+3H)^{3+}$

Synthesis of compound 2: Oxidation of SLPCDYYGTC-Bip-dD-NH$_2$

The peptide c[SLPCDYYGTCBip-dD-NH_2] (0.29 g, 0.20 mmol) was dissolved in 10 mL of 20 mM NaP_i buffer (pH 6.8) at room temperature (20 mM solution). 0.13 g of sodium metaperiodate was separately dissolved in 15 mL of 20 mM NaP_i buffer (pH 6.8) to yield a 40 mM solution. 10 mL of the $NaIO_4$ solution (2 equiv.) was slowly added to the peptide solution and stirred for 30-45 min. The oxidation was stopped by the addition of ethylene glycol (2 equiv. over periodate). The reaction mixture was applied to a C-18 Sep-Pak cartridge previously washed with MeOH and equilibrated with 0.1% TFA. The Sep-Pak was washed with 10 % CH_3CN containing 0.1% TFA to remove the excess reagent and the product was then eluted with 80 % CH_3CN containing 0.1% TFA. The desired fractions were pooled and purified further on RP-HPLC. The product, α-N-glyoxylyl-c[LPCDYYGTCBip-dD-NH_2] bearing fractions were pooled and lyophilized to a white powder (0.25 g, 88 %) and stored at -20 C till further use.

Analytical HPLC (220 nm): RT=27.93 min on RP-HPLC (C-4, 250 x 4.6 mm, YMC-Pack, 5 μm). Buffer: 0.13 M NaP_i, 0.2 mM EDTA, pH 6.5, Flow 1.5 mL/min.

MS: m/z 1426.1 $(M+H)^+$

Chemoselective Ligation-Final assembly: Synthesis of EP-1084

Fragments (scaffold bearing DTPA template) and peptide carrying either the aldehyde and aminooxy groups *devoid of protecting groups* were synthesized as described above. These fragments spontaneously self-assemble under very mild and specific conditions through the formation of oxime bonds giving stable macromolecules (stable in water at room temp at pH 2-7).

The reaction between α-N-glyoxylyl-c[LPCDYYGTCBip-dD-NH_2] (0.34 g, 0.24 mmol) and the AoA-tetra-BB-DTPA-OH (0.25 g, 0.12 mmol) was initiated by mixing peptide solution (10 mM in 1:1 CH_3CN: 20 mM sodium acetate buffer, pH 4.6) and DTPA scaffold solution (10 mM in 20 mM sodium acetate buffer, pH 4.6) at 22˚C. ACN was added just enough to get a clear solution. (Ligation reaction did not proceed well in MeOH-NaOAc buffer mix) The reaction progress was followed by HPLC and the product was characterized by LC-

MS. The reaction was purified on a Prep RP-HPLC [C-18, 250 x 22 mm, VyDac, flow 15 ml/min, 220 nm]: 0 to 1 min, at 10 % B; 1 to 31 min, 10 to 40 % B; 31 to 33 min, 40 to 80 % B. Buffer, A=10 mM NH_4OAc; Buffer, B=ACN. The product bearing fractions were pooled and lyophilized to obtain a white solid, EP-1084-L (0.495 g, 85.3 %).

Analytical HPLC (220 nm): RT=6.74 min on RP-HPLC (C-4, 150 x 4.6 mm, MetaChem, 5 µm). 20 % ACN (0.1% TFA) to 100 % ACN (0.1% TFA) over 12 min, hold at 80 % ACN for 4 min, Flow 1.0 mL/min.

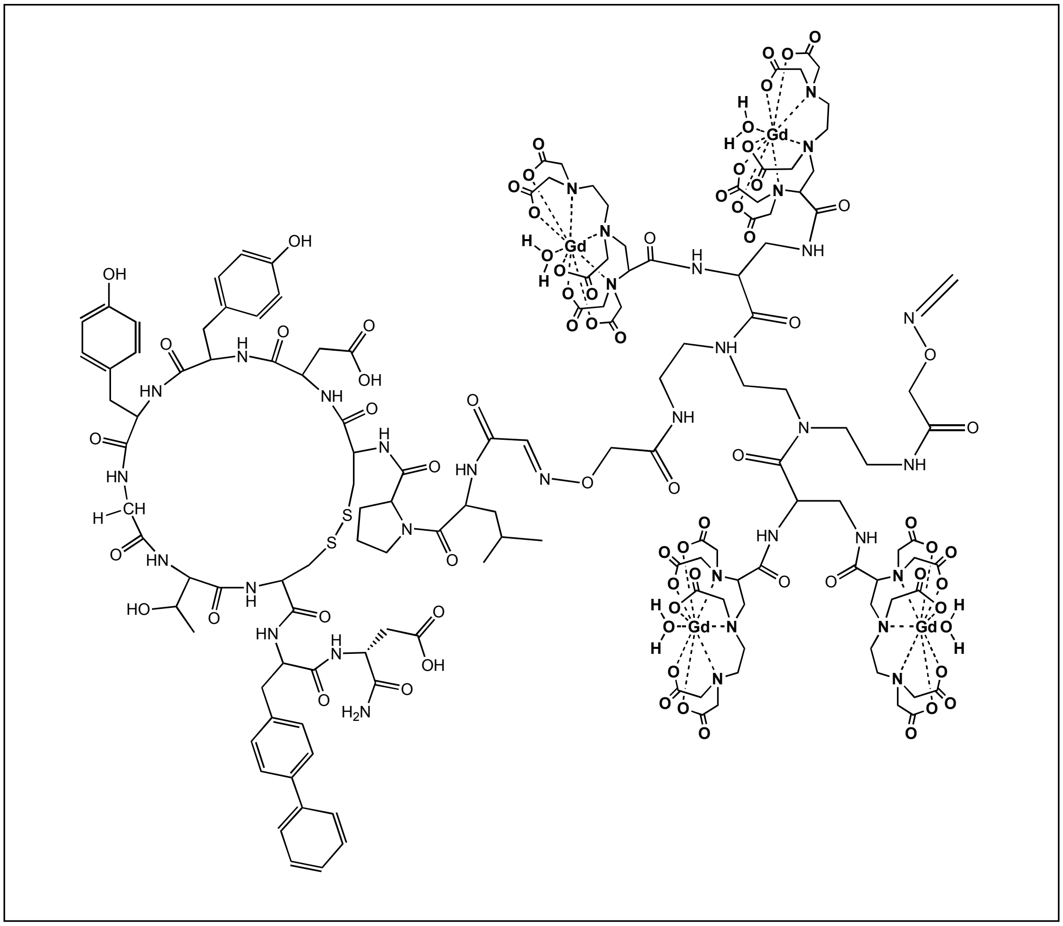
Analytical HPLC (220 nm): RT=26.90 min on RP-HPLC (C-4, 250 x 4.6 mm, YMC-Pack, 5 µm). Buffer: 0.13 M NaP_i, 0.2 mM EDTA, pH 6.5, Flow 1.5 mL/min.

MS: m/z 1653.7 $(M+3H)^{3+}$; 1240.6 $(M+4H)^{4+}$

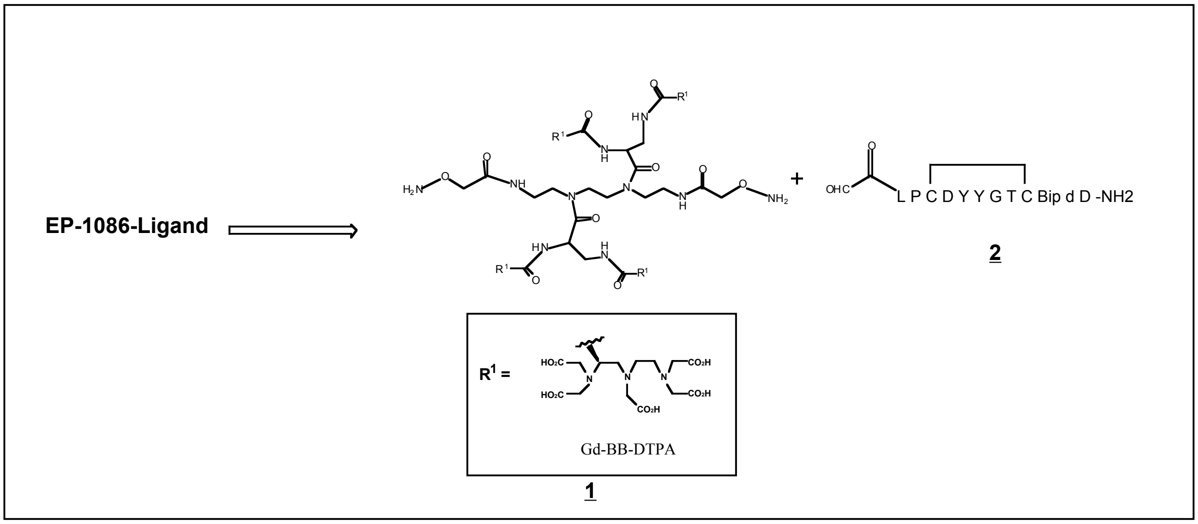
This compound (EP-1084-L) was reacted with gadolinium chloride *in situ* to give EP-1084. EP-1084-L (200 mg, 40 µmol theoretical) was dissolved in distilled, deionized water (6 mL), and the pH was adjusted to 6.5 with NaOH. The exact ligand concentration of the solution was determined by photometric titration with standardized gadolinium chloride in 0.02 M xylenol orange (pH 4.9, acetate buffer, monitor at 572 nm). There is a marked increase in absorbance once the endpoint has been reached. Four equivalents of $GdCl_3 \bullet 6H_2O$ (42.6 mg, 115 μmol) was added to the solution of EP-1084-L and the pH adjusted to 6.5 by the addition of NaOH to give an aqueous solution of EP-1084. This EP-1084 solution was 4.42 mM as determined by ICP-MS and contained no excess gadolinium as detected by xylenol orange, nor measurable amounts of underchelated product by photometric titration.

Synthesis of mono-peptide tetramer: EP-1086

EP-1086



The synthesis of EP-1086 ligand is outlined in the scheme below:



EP-1086 ligand was synthesized using the oxime ligation chemistry between the hydroxyl amine groups in **1** and the aldehyde group of the peptide **2**, α-N-glyoxylyl-c[LPCDYYGTCBip-dD-NH_2] in the presence of trace formaldehyde. The high reactivity of the aminooxy group towards carbonyl containing compounds such as common aldehydes and ketones to form stable oximes is well documented (**Ref**: Rapid Commun. Mass Spectrom. 14, 2158-2164 (2000). The bisaminooxy derivative **1** upon routine work-up and handling conditions at room temp reacted with formaldehyde at one terminal to form a stable oxime and blocking it from further ligation reactions. This monoaminooxy acetyl derivative was isolated by RP-HPLC and further reacted with α-N-glyoxylyl-c[LPCDYYGTCBip-dD-NH2] (0.091 mmol of each) under chemoselective ligation conditions in 20 mM NaOAc, pH 4.6 at 22 C). The reaction progress was followed by HPLC and the product was characterized by LC-MS. The reaction mixture was purified on a Prep RP-HPLC [C-18, 250 x 22 mm, VyDac, flow 15 ml/min, 220 nm: 0 to 1 min, at 10 % B; 1 to 31 min, 10 to 40 % B; 31 to 33 min, 40 to 80 % B. Buffer, A=10 mM NH_4OAc; Buffer, B=ACN. The product bearing fractions were pooled and lyophilized to obtain a white solid (0.27 g, 80 %).

Analytical HPLC (220 nm): RT=5.91 min on RP-HPLC (C-4, 150 x 4.6 mm, MetaChem, 5 µm). 20 % ACN (0.1% TFA) to 100 % ACN (0.1% TFA) over 12 min, hold at 80 % ACN for 4 min, flow 1.0 mL/min.

Analytical HPLC (220 nm): RT=25.2 min on RP-HPLC (C-4, 250 x 4.6 mm, YMC-Pack, 5 µm). Buffer: 0.13 M NaP_i, 0.2 mM EDTA, pH 6.5, Flow 1.5 mL/min.

MS: m/z 1188.14 $(M+3H)^{3+}$; 891.7 $(M+4H)^{4+}$

This compound (EP-1086-L) was reacted with gadolinium chloride *in situ* to give EP-1086. EP-1086-L (150 mg, 35 µmol theoretical) was dissolved in distilled, deionized water (4 mL), and the pH was adjusted to 6.5 with NaOH. The exact ligand concentration of the solution was determined by photometric titration with standardized gadolinium chloride in 0.02 M xylenol orange (pH 4.9, acetate buffer, monitor at 572 nm). There is a marked increase in absorbance once the endpoint has been reached. Four equivalents of $GdCl_3$•$6H_2O$ (38.4 mg, 103 μmol) was added to the solution of EP-1086-L and the pH adjusted to 6.5 by the addition of NaOH to give an aqueous solution of EP-1086. This EP-1086 solution was 5.73 mM as determined by ICP-MS and contained no excess gadolinium as detected by xylenol orange, nor measurable amounts of underchelated product by photometric titration.

EP-782 annd EP-821 synthesis. These compounds were custom synthesized by Bachem (Torrance, CA) using the t-butyl protected DTPA precursor N,N-Bis[2-[N',N'-bis[(tert-butoxycarbonyl)methyl]-amino]ethyl]-L-aspartic acid that was prepared according to a published method (Amedio, J. C.; Van Wagenen, G,, Jr.; Zavlin, G.; Gyorkos, A.; Peterson, S. A. *Synthetic Communications*, **2000**, *30*, 3755.). The protected peptides with sequences listed in Table 1 were synthesized on resin using standard Fmoc chemistry. The DTPA precursor was coupled to the peptide on using a double coupling procedure with dicyclohexylcarbodiimide and 1-hydroxybenzotriazole (DIC/HOBt) followed by O-(7-azabenzotriazol-1-yl)-N,N,N'N'-tetramethyluronium hexafluorophosphate/1-hydroxy-7-azabenzotriazole/N,N-diisopropylethylamine (HATU/HOAt/DIEA) coupling for 30 min. The fully protected DTPA-peptides were cleaved from the resin, and partially deprotected using a TFA cocktail (94% TFA, 2.5% triethylsilane (TES), 2.5% water, and 1% anisole). The Cys(Acm) groups were unaffected by the TFA treatment. The linear DTPA-peptide conjugates were then purified by C_4 RP-HPLC using a linear gradient of ACN (0.1% TFA) and water (0.1% TFA).

For the deprotection of the Acm groups with the concomitant oxidation of the thiols to the disulfide, the S-protected DTPA-peptide conjugate was dissolved in TFA:anisole (19:1) at 0ºC, and $Tl(CF_3COO)_3$ was added to the solution and stirred for 60 min. TFA/anisole was removed by evaporation under vacuum, and the conjugate was precipitated with excess cold ether. The pellet was collected by centrifugation, dissolved in 5% acetic acid and purified on RP-HPLC (Kromasil C_{18}) using a linear gradient of 0.1% TFA in acetonitrile and 0.1% aqueous TFA. Identify and purity (>95%) were confirmed by RP-HPLC and electrospray MS. The Gd^{3+} complexes were prepared *in situ* as described above for EP-1084 and not isolated.

Fibrin binding. Plasminogen free human fibrinogen (Calbiochem Cat No.341578) was dialyzed against 50 mM Tris, pH 7.4, 150 mM sodium chloride, 5 mM sodium citrate (TBS•citrate). Fibrinogen solution (5 mg/mL based on the absorbance at 280 nm and $\varepsilon_{280} = 1.512\ L\bullet g^{-1}cm^{-1}$) was dispensed (50 µL) into the wells of a 96-well microplate (Immulon-II®). A solution (50 µL) of human thrombin (2 U/mL) and 7 mM $CaCl_2$ in TBS was added to each well to clot the fibrinogen and to yield a final fibrin concentration close to 2.5 mg/mL = 7.3 µM based on fibrinogen MWt = 340kDa. The plates were incubated at 37 ºC and evaporated to dryness overnight. Varying concentrations of peptide conjugate (1-100 µM; 100 µL/well) were dissolved in TBS and incubated in duplicate wells of dried fibrin plates (0.25 mg/well) for 2 hr at 37ºC. The plate was sealed with tape to prevent evaporation and agitated at 300 rpm on a plate shaker. The supernatant in each well was removed by pipet. Peptide conjugate concentrations were measured by ICP-MS.

Relaxivity. Relaxivities were determined at 20 MHz (0.47 T) and 64.5 MHz (1.5 T) using a Bruker NMS 120 Minispec and a modified Varian XL-300, respectively. T_1 was measured with an inversion recovery pulse sequence. Relaxivity was determined from the slope of a plot of $1/T_1$ versus concentration for 0, 10, 15 and 20 μM compound samples (200 μL) in either Tris buffered (50 mM) saline (TBS) or in 10 mg/mL (30 μM) fibrin gels in TBS. The fibrin gel samples were prepared by first mixing appropriate amounts of fibrinogen stock solution (15 – 20 mg/mL), gadolinium-peptide conjugate stock, and TBS to a total volume of 200 μL. To this solution was added 4 μL of a 2 M $CaCl_2$ solution and 2 μL of human thrombin (0.6 units), the resultant solution was vigorously mixed for 3 seconds and then incubated for one hour at 37 °C to allow for complete polymerization of the fibrinogen. The ^1H NMRD profiles were recorded on a field cycling relaxometer at NY Medical College over the frequency range 0.01 to 50 MHz at 35 °C. Twenty-two data point dispersions were recorded for either a 0.1 mM compound solution in TBS, a 50 μM compound in 30 μM fibrin gel, or 30 μM fibrin gel without compound. Compound concentration was determined from ICP-MS analysis of total Gd content and dividing by the number of Gd/molecule. Relaxivity was computed by subtracting the relaxation rate of the medium (TBS or fibrin gel/TBS) from the relaxation rate of the Gd solution at each field strength and dividing the difference by the gadolinium concentration in millimolar.

Radiolabeling of EP-1084

In-111 labeled EP-1084 was prepared daily before the animal study. General procedure: To EP-1084, 300 μL of 4.5 mM solution, in a 5 mL glass vial was added $^{111}InCl_3$ to yield a solution containing 80 - 100 μCi of activity. The pH was adjusted to 3.5 by dropwise addition of 1N HCl. The solution was heated at 50 ˚C for 1 hour, allowed to cool, and then filtered through a cation exchange (Na^+ form, Alltech) filter into a clean vial. The pH was adjusted to 7 by dropwise addition 1N NaOH. The resultant solution was then filtered through a 0.2 μm nylon filter (Gelman Acrodisc) into a weighed vial, and the filter rinsed with H_2O. The amount of radioactivity in the solutions was determined using a Capintec CRC7 dose calibrator.

To test for free (unreacted) $^{111}InCl_3$, a solution was prepared containing 15 μL Tris buffer, 5 μL KH_2PO_4, and 5 μL of the 230 mM radiolabeled solution. This solution (5 μL) was spotted onto an ITLC plate (Gelman) at 1 cm above the bottom of the plate. Unreacted $InCl_3$ remains at the origin while the labeled compound elutes with the solvent front. The ITLC plate was developed, cut into three sections (0 – 1 cm, 1 – 3 cm, and 3 – 9 cm) and the sections counted using a Packard Cobra II gamma counter using the counting window 390 - 500 keV. Three empty tubes were measured to determine a background reading. Radiochemical purity was assessed as:

(activity 3-9 cm – background)/(activity in all sections – background) x 100%

Labeled EP-1084 had radiochemical purity > 99%.

Functional activity of I-125 labeled guinea pig fibrinogen

Guinea pig fibrinogen (Sigma) was labeled with I-125 by Amersham Biosciences. An aliquot of ^{125}I-fgn (~1 nCi) was mixed with guinea pig plasma (600 μL). Two 50 μL aliquots were removed, weighed, and the radioactivity measured. To this was added 4 μL of 2 M $CaCl_2$ and 2 μL (0.6 units) of human thrombin and the plasma solution mixed vigorously for 3 seconds. The plasma was allowed to stand for 30 minutes at room temperature. Using an Eppendorf pipet tip, the plasma clot was separated from the serum. Two aliquots of serum (30 μL) were removed, weighed, and counted. The fraction of functional labeled fibrinogen, was calculated as:

functional fgn fraction = 1 – (counts/gram in serun)/(counts/gram in total)

In vivo clot uptake of EP-1084

A 600g guinea pig (Hartley male) was anaesthetized with isofluorane (5% - 0.2%). An incision was made in the abdomen and the inferior vena cava (IVC) isolated and the vessel was allowed to recover for 10 minutes. A 300 μL solution (30 μCi) of guinea pig fibrinogen (Sigma) that had been labeled with I-125 (Amersham Biosciences) was injected via the jugular vein. After 5 minutes, a 1 cm portion of the IVC was clamped and human thrombin (50 mL, 4 units) was injected into the vessel to promote thrombus formation. The lower clamp was opened and closed allowing partial blood flow to the segment. After 2-3 minutes the clamps were removed. The thrombus was allowed to age in the animal for 30 minutes. At this point EP-1084 at a dose of 2 μmol/kg and trace radiolabeled with 70 μCi of ^{111}In, was injected via the jugular vein. Immediately following injection of agent EP-1084, a non-specific control comprising Gd(DTPA) at a dose of 2 μmol/kg mixed with 70 μCi ^{99m}TcDTPA (Cardinal Health) was injected via the jugular vein. After 30 minutes blood (1 mL) was drawn into a citrated vacutainer, the animal sacrificed, and the thrombus removed. The blood and thrombus samples were weighed and counted using a Packard Cobra II gamma counter. The plasma was separated by allowing the blood sample to stand, and fraction of functional fibrinogen was determined as described above. The thrombus was also weighed and counted. Counts arising from ^{125}I were detected from 15 – 70 keV, ^{99m}Tc were detected from 128 - 165 keV, while counts arising from the decay of ^{111}In were detected from 390 -500 keV. Control experiments with only ^{99m}Tc or ^{111}In demonstrated radioactivity arising from ^{99m}Tc was negligible at detection energies used for ^{111}In and vice versa. There was some spill-down of ^{99m}Tc and ^{111}In activity into the iodine channel and the spill-down was non-linear in total ^{99m}Tc or ^{111}In activity. Calibration curves were determined for this spill-down and the I-125 data were corrected accordingly. Spill-down from ^{99m}Tc and ^{111}In accounted for 10 – 25% of the total activity in the I-125 counting window. The radioactive decay data were converted to % initial dose per gram of tissue, %ID/g. The experiment was repeated for a total of three experiments.