1. General methods and materials.

Peptides were synthesized on an automated peptide synthesizer, Advanced ChemTech ACT-348 Ω. The cyclic peptides, the tetrakis(DTPE) peptides and the tetrakis(DTPA) peptides were analyzed using an Agilent 1100 HPLC coupled with Agilent Series 1100MSD mass detector (Agilent, Santa Clara, CA). The fully deprotected peptides, the tetrakis(DTPE) peptide and the tetrakis(DTPA) peptide conjugates were purified by preparative HPLC (Varian, Palo Alto, CA). Gadolinium concentrations were determined using an Agilent ICP-MS 7500.

Two methods were used for LC-MS analysis:

Analytical method A (for analysis of the protected peptides and the tetrakis(DTPE) peptides): Kromasil 100-3.5 C4 column, 50 × 4.6 mm. Mobile phase A was 0.05% TFA in water, mobile phase B was 0.05% TFA in acetonitrile. The gradient was initiated at 20% B and ramped to 95% B in 7 min, hold for 1 min and re-equilibration back to 20% from 8.6 min. The total analyzed time was 10 min. Flow rate was 0.8 mL/min.

Analytical method B (for analysis of the deprotected peptides and the tetrakis(DTPA) peptides): Kromasil C18 column, 50 × 4.6 mm, 3.5 μ. Mobile phase A was 0.05% TFA in water, mobile phase B was 0.05% TFA in acetonitrile. The gradient was initiated at 5% B and ramped to 75% B in 7 min, followed by a wash to 95% B within 1 min and re-equilibration back to 5% from 8.6 min. The total analyzed time was 10 min. Flow rate was 0.8 mL/min.

Two methods were used for the preparative HPLC purification:

Preparative method A (for purification of the tetrakis(DTPE) peptides): Vydac protein C4 column, 250 x 20 mm. The mobile phase A was 1% TFA in water, the mobile phase B was 1% TFA in acetonitrile. The gradient was initiated at 20% B for 8 min., then 20-95 % B over 30 min, hold for 4 min and re-equilibration at 20% for 3 min. Flow rate was 20 mL/min.

Preparative method B (for purification of the deprotected peptides and the tetrakis(DTPA) peptides): Kromasil C18 column, 250×20 mm. The mobile phase A was 1% TFA in water, the mobile phase B was 1% TFA in acetonitrile. The gradient was initiated at 2% B for 9 min., then 2-25% B within 1 min and 25-60% B over 30 min, followed by a 4 min ramp to 95% B for 4 min and re-equilibration at 2% for 7 min. Flow rate was 20 mL/min.

ICP-MS method: All samples were diluted with 0.1% Triton X-100 in 5% Nitric Acid containing 20 ppb of Tm (as internal standard). The ratio of Gd(157)/Tm(169) was used to quantify the gadolinium concentration. A linear calibration curve ranging from 0.5 ppb to 1000 ppb was generated daily.

Fmoc-Bip-OH was purchased from RSP amino Acids, LLC (Shirley, MA). All other amino acids were purchased from Novabiochem (San Diego, CA), which include Fmoc-Tyr(tBu)-OH, Fmoc-Cys(Acm)-

OH, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Thr(tBu)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gly-OH. 1,3-Bis-(aminomethyl)-benzene trityl NovaSyn TGT resin and NovaSyn TGR resin were purchased from Novabiochem (San Diego, CA). Fmoc-A(Bhoc)aeg-OH, Fmoc-C(Bhoc)aeg-OH, Fmoc-G(Bhoc)aeg-OH and Fmoc-T-aeg-OH were purchased from PerSeptive Biosystems (Framingham, MA). All solvents were purchased from J.T. Baker (Philipsburg, NJ). All other reagents were purchased from Sigma-Aldrich (Milwaukee, WI) and used without further purification unless otherwise stated.

2. Synthesis

2-1. Synthesis of cyclic peptides: General procedures.

Standard Fmoc chemistry was used to elongate the peptide on the resin. The Fmoc was removed with a solution of 20% piperidine in DMF. Each amino acid dissolved in a 0.2 M solution of 1-hydroxybenzotriazole (HOBt) in NMP (as a 0.2M solution) was coupled to the peptide using a 0.2 M solution of diisopropylcarbodiimide (DIC) in NMP. Double coupling cycle was used for each amino acid and a 5-fold excess of the amino acids and the coupling reagents was used per coupling to synthesize the peptide on the resin. After each deprotection or coupling steps the resin was washed alternatively three times with DMF and MeOH. The completed linear peptide on resin was washed with DCM and dried under nitrogen.

The linear peptide on resin was placed into a manual peptide synthesis vessel and swollen by repeated washes with DCM and DMF. To the peptide on resin was added a solution of $Tl(TFA)_3$ (2.2 eq.) in DMF. The vessel was shaken at room temperature for 4 h. The solvent was removed by filtration and the resin was washed several times with DMF, MeOH and DCM to give the cyclic peptide on resin.

2-2. The fully deprotected peptides (Figure S1 & Scheme S1)

The peptides, **NH_2-Pep, A-Pep, C-Pep, G-Pep, and T-Pep** were synthesized by using NovaSyn TGR resin. The cyclic peptides were cleaved from the resin using the following cleavage cocktail: TFA/TIS/anisole/H_2O 85:5:5:5 (5 mL per 100 μmoles of peptide). The solution of fully deprotected peptide was then concentrated to a tenth of its initial volume and the peptide was precipitated with cold ether (20 mL). The crude peptide was isolated after centrifugation and purified by reverse-phase preparative HPLC on a C18 column (eluent: 1% TFA/water/acetonitrile). The fractions of pure peptide were pooled and lyophilized to give the final peptide moiety. The cyclic peptide was analyzed by LC-MS.
NH_2-Pep. Molecular Weight for $C_{64}H_{80}N_{12}O_{18}S_2$: 1369.52. MS (ESI) m/z: Calculated: 1370.52 $(M+H)^+$; Observed: 1369.4.

A-Pep. Molecular Weight for $C_{75}H_{93}N_{19}O_{20}S_2$: 1644.79. MS (ESI) m/z: Calculated: 1645.79 $(M+H)^+$; Observed: 1646.2.

C-Pep. Molecular Weight for $C_{74}H_{93}N_{17}O_{21}S_2$: 1620.76. MS (ESI) m/z: Calculated: 1621.76$(M+H)^+$; Observed: 1621.3.

G-Pep. Molecular Weight for $C_{75}H_{93}N_{19}O_{21}S_2$: 1660.79. MS (ESI) m/z: Calculated: 1661.79 $(M+H)^+$; Observed: 1662.6.

T-Pep. Molecular Weight for $C_{75}H_{94}N_{16}O_{22}S_2$: 1635.77. MS (ESI) m/z: Calculated: 1636.77 $(M+H)^+$; Observed: 1636.3.

2-3. Tetrakis(DTPA)-T-Pep (Scheme S2).

T-Pep-mXD. The protected peptide was synthesized by using 1, 3-bis-(aminomethyl)-benzene trityl NovaSyn TGT resin (0.63 mmol/g). The peptide resin was treated with 1% TFA/DCM (10-15 mL/g resin) for 5 min. The solvent was filtered into a vessel containing 10% pyridine/methanol (1.2 eq. to TFA), and thus, the filtrate was immediately neutralized. This treatment was repeated with further portions of 1% TFA/DCM until, based on the LC-MS or HPLC analysis, no further peptide remained on the resin. The neutralized filtrates were kept in separate vessels and analyzed by LC-MS or HPLC. The fractions containing a significant amount of peptide were pooled into a round bottom flask. The solvents were removed under reduced pressure and the mixture was treated with ether for 1 h to produce a pale yellow precipitate. The precipitate was washed twice with diethyl ether and several times with de-ionized water until the wash become neutral. The crude peptide was dissolved in pyridine and the solvent was removed under reduced pressure to give the crude product as a pale yellow solid. Molecular Weight for $C_{103}H_{143}N_{17}O_{22}S_2$: 2035.47. MS (ESI) m/z: Calculated: 2036.47 $(M+H)^+$; Observed: 2036.9.

bis(DTPE)-Acid. Bis(DTPE)-Acid was synthesized according to the reported procedure (See Reference S-1).

tetrakis(DTPE)-T-Pep-mXD. T-Pep-mXD (0.71 g, 0.349 mmol) and bis(DTPE)-Acid (1.198 g, 0.768 mmol) were dissolved in DCM (60 mL) and DMF (60 mL). Diisopropylethylamine (1.74 mmol, 0.31 mL), diisopropylcarbodiimide (97 mg, 0.768 mmol) and HOBt (134 mg, 0.872 mmol) were added to the mixture. The mixture was stirred at room temperature for two h and monitored using LC/MS. If needed, additional pre-activated bis(DTPE)-acid (activation with diisopropylcarbodiimide, diisopropylethylamine, and HOBt) was added in one portion, and this was repeated two h later. After the reaction was complete**,** solvents were removed under reduced pressure and the crude product was purified by RP-HPLC (Method B) to give the

product as a pale yellow oil (568 mg, 0.279 mmol, 80% yield). Molecular Weight for $C_{255}H_{413}N_{35}O_{68}S_2$: 5121.33. MS (ESI) m/z: Calculated: 2561.67 $[(M+2H)]^{2+}$; Observed: 2562.4.

tetrakis(DTPA)-T-Pep-mXD. tetrakis(DTPE)-T-Pep-mXD (437 mg, 0.085 mmol) was dissolved in DCM (10 mL) and anisole (10 mL) and the solution was stirred at 4 °C for 10 min. To the solution was added concentrated HCl solution (12N, 10 mL) dropwise. The mixture was stirred at 4–12 ^{o}C for 4 h and then to the mixture was added water. The mixture was extracted four times with ether. The aqueous solution was lyophilized to give a crude product which was purified by using RP-HPLC (Method B). The fractions containing the pure product were pooled and lyophilized to give the product as a white foam (142 mg, 0.038 mmol, 45% yield). Molecular Weight for $C_{155}H_{213}N_{35}O_{68}S_2$: 3718.67. MS (ESI) m/z: Calculated: 1240.56 $[(M+3H)/3]^{3+}$; Observed: 1241.3.

2-4. tetrakis(DTPA)-Gly2-Pep-mXD

In a similar procedure as described for the synthesis of tetrakis(DTPA)-T-Pep-mXD, tetrakis(DTPA)-Gly2-Pep-mXD was synthesized.

Gly2-Pep-mXD. Molecular Weight for $C_{96}H_{135}N_{15}O_{20}S_2$: 1883.32. MS (ESI) m/z: Calculated: 1884.32 $(M+H)^{+}$; Observed: 1884.8.

Tetrakis(DTPE)-Gly2-Pep-mXD. Molecular Weight for $C_{222}H_{405}N_{33}O_{66}S_2$: 4969.18. MS (ESI) m/z: Calculated: 1657.39 $[(M+3H)/3]^{3+}$; Observed: 1657.8.

Tetrakis(DTPA)-Gly2-Pep-mXD. Molecular Weight for $C_{148}H_{205}N_{33}O_{66}S_2$: 3566.53. MS (ESI) m/z: Calculated: 1784.27 $[(M+2H)/2]^{2+}$; Observed: 1784.7.

2-5. Gd2-Pep-Gd2

The synthesis was reported in reference S-2.

2-6. General procedure for final compound preparation.

The gadolinium tetramers **Gd2-Gly2-Pep-Gd2** and **Gd2-T-Pep-Gd2** were each prepared by reacting the respective tetrakis(DTPA)-peptide with gadolinium chloride *in situ*. Each tetrakis(DTPA)-peptide was dissolved in a small volume of distilled, deionized water (3 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 $GdCl_3 \cdot 6H_2O$ were added to the tetrakis(DTPA)-peptide solution and the pH adjusted to 6.5 by the addition of NaOH to give an aqueous

solution of the final compound. The exact concentration was determined by ICP-MS and contained no excess gadolinium as detected by xylenol orange, nor measurable amounts of underchelated product as determined by photometric titration.

Gd2-T-Pep-Gd2. Molecular Weight for $C_{155}H_{201}Gd_4N_{35}O_{68}S_2$: 4335.98. MS (ESI) m/z: Calculated: 1446.3 $[(M+3H)/3]^{3+}$; Observed: 1444.6.

Gd2-Gly2-Pep-Gd2. Molecular Weight for $C_{148}H_{193}Gd_4N_{33}O_{66}S_2$: 4183.92. MS (ESI) m/z: Calculated: 1395.6 $[(M+3H)/3]^{3+}$; Observed: 1394.0.

3. DD(E) binding assay.

The DD(E) binding assay was described previously (ref S-2). DD(E) is a soluble fibrin fragment and it was previously shown that this class of peptides binds similarly to both soluble DD(E) and insoluble fibrin gels (ref S-2 and S-3). The assay involves displacement of a fluorescent peptide bound to DD(E) by a new test compound. The fluorescent peptide "Fl" (Fluor-Aca-LPCDYYGTCLD, where Fluor=fluorescein and Aca=aminocaproic acid) has a K_d of 1.3 ± 0.4 μM for binding to DD(E).

Binding of peptides and peptide-chelate conjugates to DD(E) was measured by fluorescent peptide displacement. DD(E) (1.5 μM), **Fl** (1 μM) and competing peptide conjugate (0.1 - 50 μM) were mixed in TBS with 2 mM $CaCl_2$. Fluorescence anisotropy (r_{obs}) (100 μL, n = 3 wells) was measured in a 96 - well microplate (Costar Cat. No. 3915), using a Tecan Polarian 96 - well FP microplate reader (ex = 485 nm; em = 535 nm). In the presence of an inhibitor, an apparent dissociation constant for the fluorescent probe, K_d^{app}, is determined, eq S1 where r_{fr} and r_{bd} refer to the anisotropy of the unbound and DD(E)-bound probe. The inhibition constant, K_i, is related to K_d^{app} by eq S2 where K_d is the true dissociation constant of the fluorescent probe measured in the absence of inhibitor (1.3 μM). K_i values were obtained by least squares fitting of the data.

$$r_{obs} = r_{fr} + \frac{r_{bd} - r_{fr}}{[Fl]_t} \times \frac{\left([DDE]_t + [Fl]_t + K_d^{app}\right) - \sqrt{\left([DDE]_t + [Fl]_t + K_d^{app}\right)^2 - 4[Fl]_t[DDE]_t}}{2} \quad \text{(S1)}$$

$$K_d^{app} = K_d\left(1 + \frac{[inhibitor]_{free}}{K_i}\right) \quad \text{(S2)}$$

4. Relaxivity determination.

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 pH 7.4 Tris (50 mM) buffered saline (TBS), human plasma, 10 mg/mL (30 μM) fibrinogen in TBS, or 30 μM fibrin gel 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 100 μM **Gd2-T-Pep-Gd2** or **Gd2-Gly2-Pep-Gd2** solution in TBS, 50 μM **Gd2-T-Pep-Gd2** or **Gd2-Gly2-Pep-Gd2** in 30 μM fibrin gel, 100 μM **Gd2-T-Pep-Gd2** or **Gd2-Gly2-Pep-Gd2** in human plasma, human plasma alone, or 30 μM fibrin gel without compound. There are known to be 2 equivalent binding sites on fibrin for these peptides (ref S-3), so the NMRD in fibrin gel was recorded under conditions where the [binding sites] > [**Gd2-T-Pep-Gd2**]. Based on the measured binding constants, under these conditions **Gd2-T-Pep-Gd2** is 83% bound to fibrin and **Gd2-Gly2-Pep-Gd2** is 82% bound. Compound concentration was determined from ICP-MS analysis of total Gd content and dividing by 4 Gd/molecule. Relaxivity was computed by subtracting the relaxation rate of the medium (TBS, plasma, or fibrin gel) from the relaxation rate of the Gd solution at each field strength and dividing the difference by the gadolinium concentration in millimolar.

The NMRD data was analyzed in the following manner. First, the contribution to relaxivity due to the inner-sphere water molecule is factored out. We did this by subtracting the relaxivity of a GdTTHA derivative (no inner-sphere water molecules) that we had measured previously at the same field strengths and temperature (ref S-4) to obtain r_1^{IS}. Inner-sphere relaxivity can be described by a two-site exchange model as shown in equation S3, where T_{1m} is the T_1 of the bound water molecule and τ_m is the mean residency time of the bound water. We had previously measured τ_m for Gd2-Pep-Gd2 and found that it was very similar to that of other GdDTPA derivatives (ref S-2). Because the new compounds use the same chelator and are structurally very similar to Gd2-Pep-Gd2, we assume that τ_m is the same for each compound and this is fixed in the analysis (τ_m = 140 ns at 35 °C). The higher field NMRD data ($\nu_H \geq 6$ MHz) were analyzed. At these fields, the contribution to electronic relaxation from the static zero-field splitting (ZFS) is negligible and use of modified Solomon-Bloembergen-Morgan theory is appropriate (ref S-4). For the fibrin-bound relaxivities, we found it necessary to apply the Lipari-Szabo modification of the spectral density term to account for internal motion. At these proton frequencies (6 MHz and up), the contribution to relaxation dependent on the electronic Larmor frequency, ω_s, has dispersed and the relaxation of the inner-sphere water is given by equation S4. The global correlation time has contributions from rotation (τ_R) and electronic relaxation (T_{1e}),

equation S5. The field dependence on T_{1e} is given by equation S6. In S4, τ_f is a correlation time that takes into account fast local motion ($1/\tau_f = 1/\tau_c + 1/\tau_l$) and τ_l is a correlation time for fast motion; F^2 is an order parameter (often denoted S^2, but here F is used to avoid confusion with the spin quantum number). ω_H is the Larmor frequency of the proton (rad/s), γ_H is the proton magnetogyric ratio, g_e is the electronic g-factor (g_e = 2 for Gd(III)), μ_B is the Bohr magneton, and μ_0 is the permittivity of vacuum.

$$r_1 = r_1^{IS} + r_1^{OS} = \frac{q/[H_2O]}{T_{1m} + \tau_m} + r_1^{OS} \qquad \text{(S3)}$$

$$\frac{1}{T_{1m}} = \frac{2}{15}\left(\frac{\mu_0}{4\pi}\right)\frac{\gamma_H^2 g_e^2 \mu_B^2 S(S+1)}{r_{GdH}^6}\left[\frac{3F^2\tau_c}{1+\omega_H^2\tau_c^2} + \frac{3(1-F^2)\tau_f}{1+\omega_H^2\tau_f^2}\right] \qquad \text{(S4)}$$

$$\frac{1}{\tau_c} = \frac{1}{\tau_R} + \frac{1}{T_{1e}} + \frac{1}{\tau_m} \qquad \text{(S5)}$$

$$\frac{1}{T_{1e}} = \frac{\Delta_t^2\left[4S(S+1)-3\right]}{25}\left[\frac{\tau_v}{1+\omega_s^2\tau_v^2} + \frac{4\tau_v}{1+4\omega_s^2\tau_v^2}\right] \qquad \text{(S6)}$$

We analyzed the NMRD profiles in each medium (TBS or fibrin) simultaneously. For the compounds in TBS, we found that the curves could be well reproduced by using the same water exchange (τ_m = 140 ns) and electronic relaxation ($\Delta^2 = 10.2 \times 10^{18}$ s^2 and τ_v = 18 ps) as previously determined for Gd2-Pep-Gd2 (ref S-2) and only varying the rotational correlation time τ_R. Furthermore, these profiles in TBS were well fit to an isotropic model. The fitted parameters were τ_R = 428±2, 424±2, 373±2 ps for Gd2-Pep-Gd2, Gd2-T-Pep-Gd2, and Gd2-Gly2-Pep-Gd2, respectively.

The NMRD profiles for the agents bound to fibrin were also analyzed simultaneously. Here, we treated τ_m (fixed at 140 ns) and the global rotational correlation time τ_g as parameters common to each agent (global parameters). The electronic relaxation parameters (Δ^2, τ_v), the local correlation time (τ_l) and the order parameter (F^2) were treated as local parameters. We found that the global rotational correlation time was long, but could not be accurately determined and therefore was fixed at >20 ns. The other four parameters (Δ^2, τ_v, τ_l, and F^2) were iteratively varied to obtain the best fit to the data and these fitted values are listed in Table 3.

5. References

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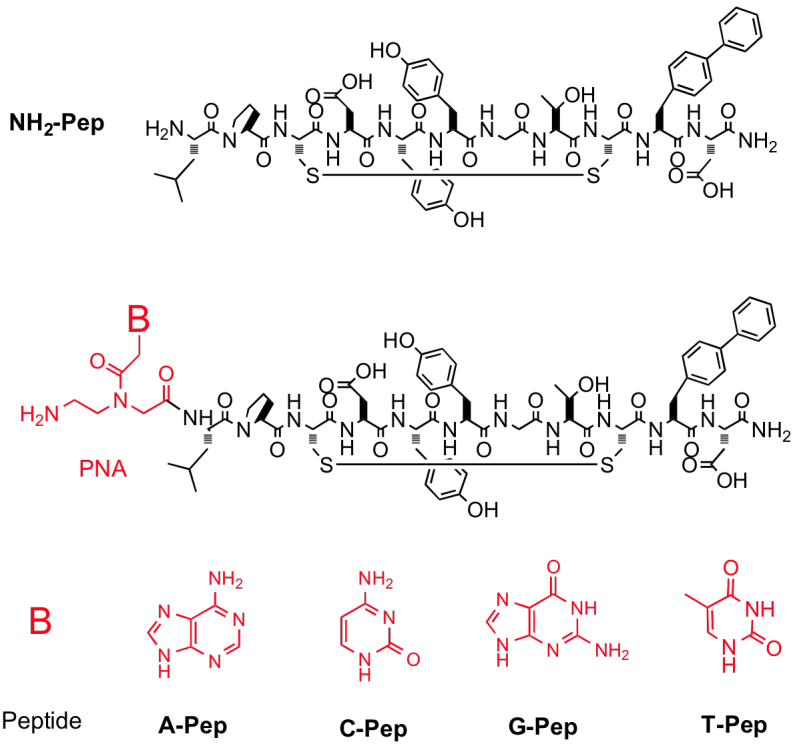
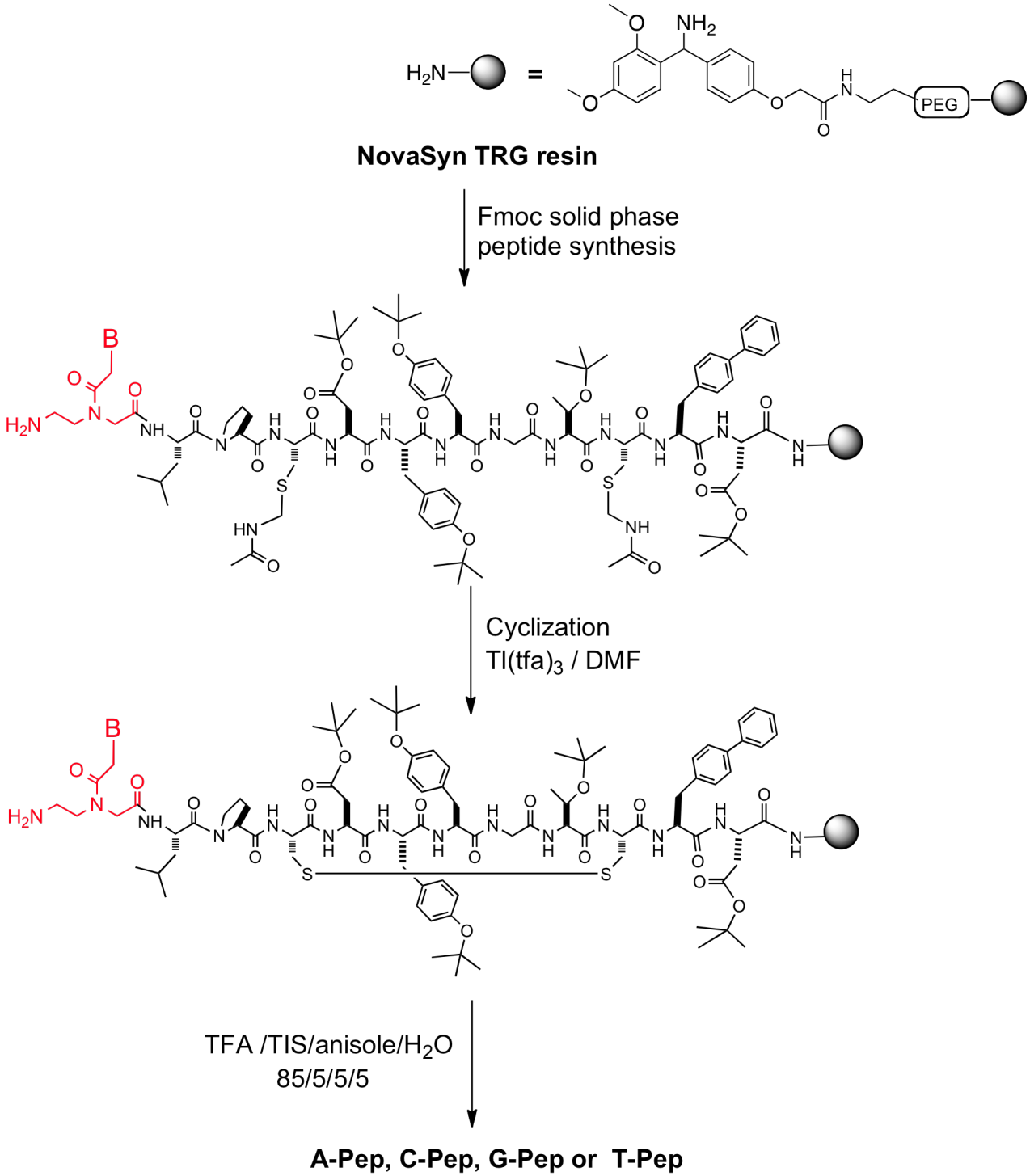
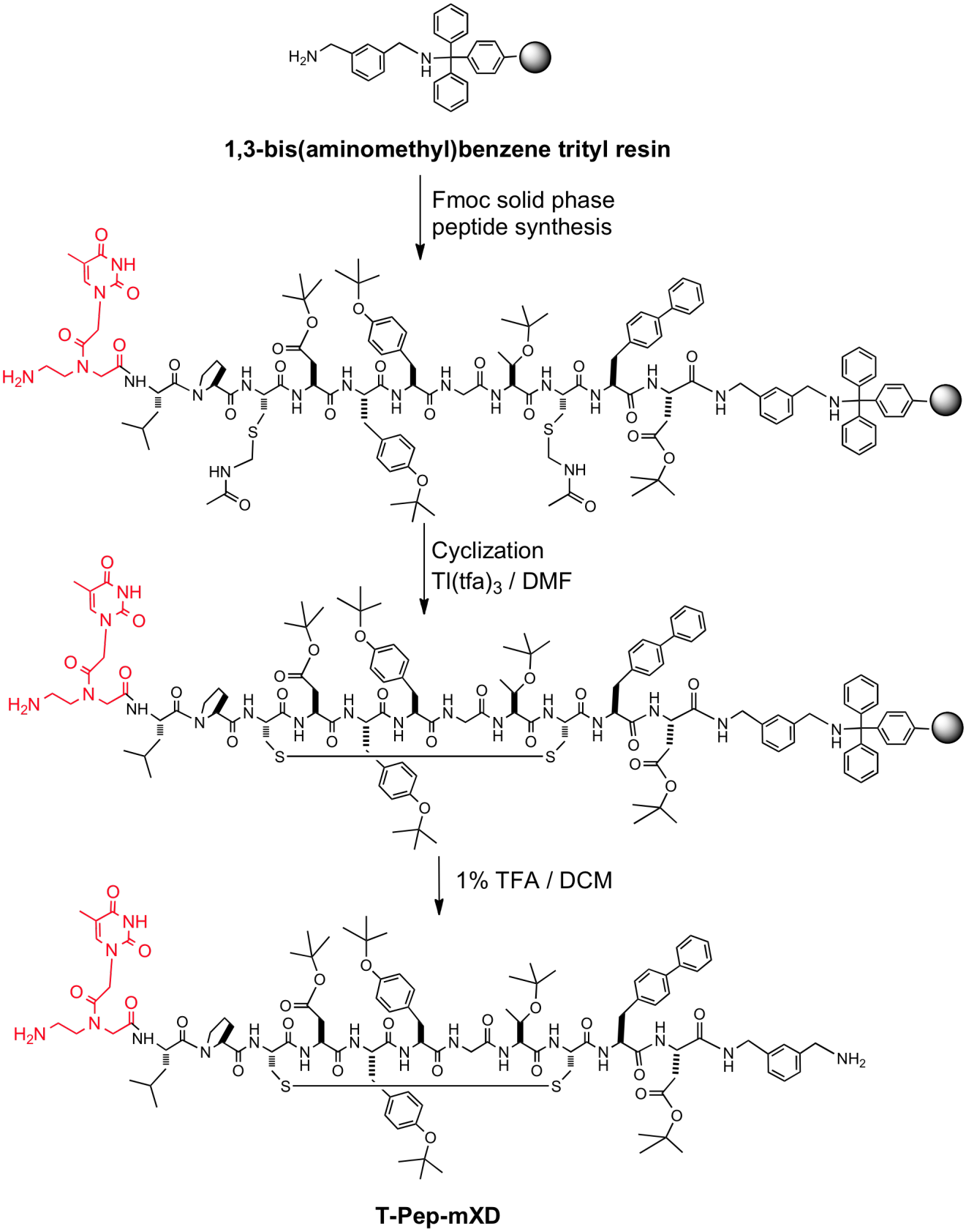


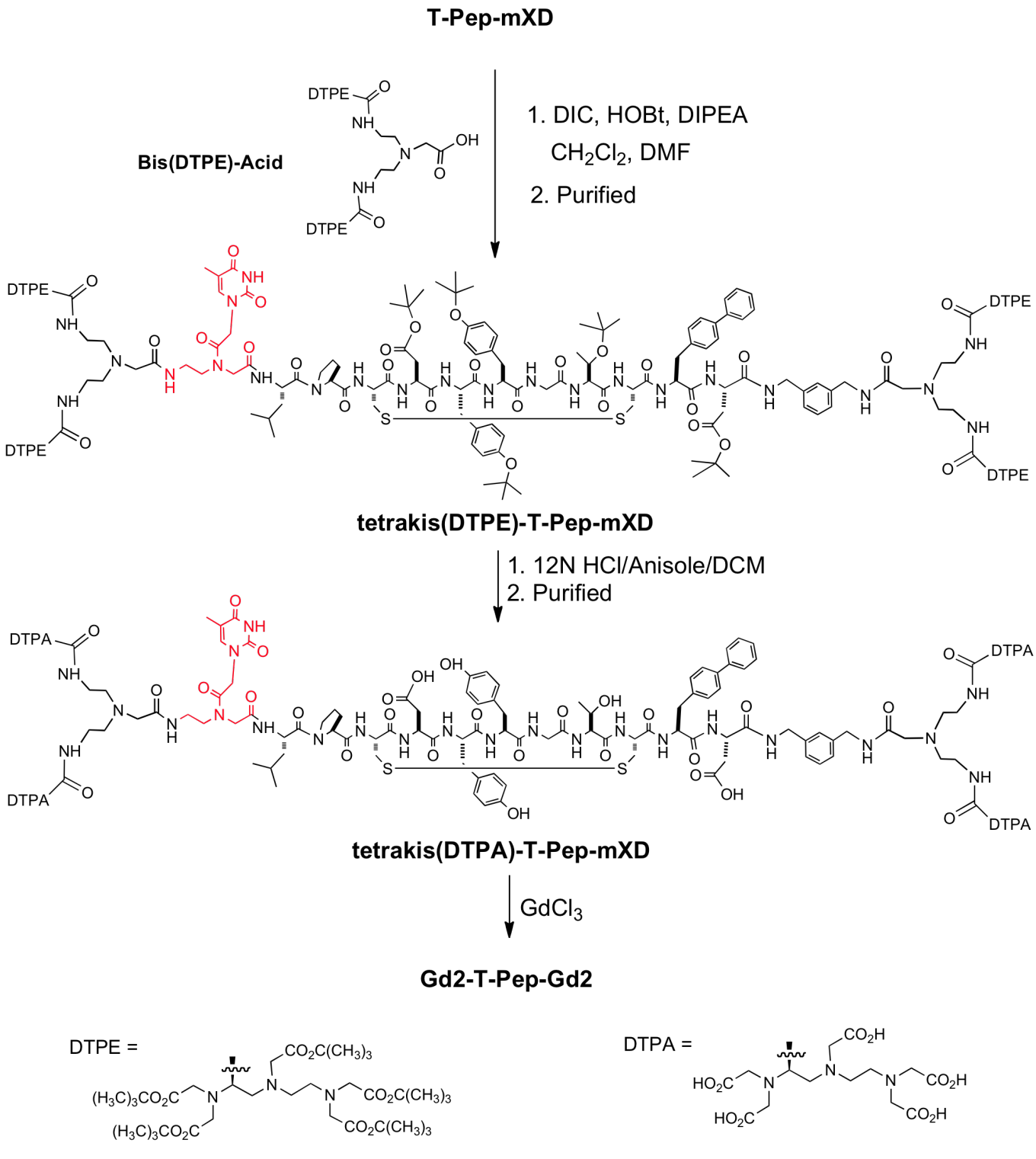
Figure S1. The peptides.



Scheme S1. Synthesis of Peptides.



Scheme S2a. Synthesis of Gd2-T-Pep-Gd2.



Scheme S2b. Synthesis of Gd2-T-Pep-Gd2.