Electronic Supplementary Material (ESI) for Dalton Transactions.

Supplementary Information for

Towards an MMP-2-activated molecular agent for cancer imaging

S. Cowell,[a,b] L. Carroll,[b] I. Lavdas,[b] E. O. Aboagye,[b] R. Vilar[a]

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Contents

1. General experimental procedures, materials and Instrumentation

Reaction conditions: Reagents were bought from commercial sources and were used as received unless stated otherwise. Where anhydrous conditions were used, reactions were carried out using Schlenk line techniques under a nitrogen atmosphere. MMP-2 and MMP-9 enzymes were purchased from Sigma-Aldrich.

Solvents: Mili-Q water was used for HPLC and biological applications. HPLC-grade methanol was bought from VWR. HPLC-grade acetonitrile and FA, along with deuterated solvents for use in NMR spectroscopy were bought from Sigma-Aldrich. Acetonitrile was purchased from Sigma Aldrich and was dried over potassium carbonate (5% w/v, dried in oven) for 24 hours, which was followed by stirring over 3Å molecular sieve powder for 24 hours (5% w/v, activated by heating under vacuum) under nitrogen. Alternatively, anhydrous acetonitrile was purchased from Sigma Aldrich and used as received under nitrogen. Anhydrous DMF was purchased from Sigma Aldrich and used as received under nitrogen. All other solvents were purchased from Sigma Aldrich and used without further purification unless otherwise stated.

^{1}H NMR spectra: Were recorded at 400 MHz on a Bruker AV 400 instrument at 298K. Chemical shifts (δ_H) are given in parts per million (ppm) and referenced to any residual solvent peaks (^{1}H: $CDCl_3$ 7.26; CD_3OD 3.34; D6-DMSO 2.54), coupling constants are in Hz.

^{19}F NMR spectra, T_1 and T_2 values: Recorded on Bruker DRX-400 MHz, at 400MHz and 298K, T_1 and T_2 experiments carried out by Pete Haycock. Samples dissolved in DMSO (1mM) for relaxation time measurements. T_1 measurements were carried out using T_1 weighted inversion recovery (t1ir), T_2 measurements were carried out using Carr Purcell Meiboom Gill (CPMG) pulse sequence. For comparison studies samples were dissolved in water/D_2O or DMSO-d_6 and TFA was used as a standard. Chemical shift are reported relative to trichlorofluoromethane.

Mass Spectra (*m/z*): MALDI analysis was carried out on a Micromass MALDI-ToF using a 337 nm nitrogen laser, Operates in reflectron (under 7KDa) or linear (over 7KDa) modes. ESI and Elemental composition was carried out with ES-ToF, Waters LCT Premier/Acquity uPLC. Capillary Voltage: 2000, Cone Voltage: 30, Desolvation Temp: 350 degrees, Source Temp 120 degrees, desolvation gas 400 L/hr, Cone gas 10 L/hr. Only major peaks reported.

Reversed phase HPLC: Analysis and purification of peptides was carried out using a Waters HPLC system comprised of a 600 pump and 2487 UV detector using a Luna 5u C18 column; 5 µM (particle size); 100A (pore diameter); 30 mm inner diameter and 75 mm length. This system was also used for fluorescent HPLC with the addition of a Waters 470 scanning fluorescence detector, using a Waters u Bondapak; C18; 10µM (particle size); 125A (pore diameter); 7.8 mm inner diameter and 300 mm length column. Analysis of biological assays and semi-prep could also be carried out on Agilent technologies 1200 series system which comprised a G1379B vacuum degasser, and a G1312A Bin pump. The column used was a Waters u Bondapak; C18; 10 µM (particle size); 125A (pore diameter); 7.8 mm inner diameter and 300 mm length. For radiochemical studies analysis was carried out on HP1100 series system comprising of a G1322A degasser, GA1312A bin pump, G1514A UV detector and a Flowcount BFC 200 radiodetector. The column used was a Gemini 5u; C18; 5 µM; 110A (pore diameter); 4.6 mm inner diameter and 150 mm length.

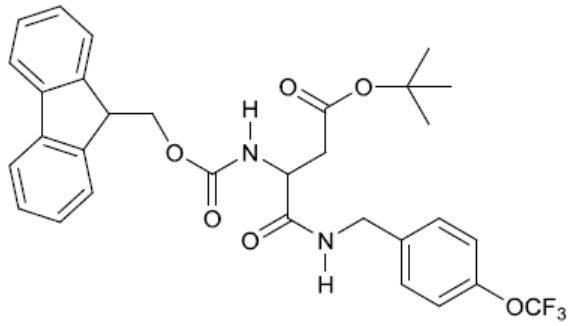
LCMS: Analysis and purification were carried out on a Waters HPLC system using a Waters 2767 autosampler for samples injection and collection, a Waters 515 HPLC pump for delivery of the mobile phase to the source, a Waters 3100 mass spectrometer with ESI and Waters 2998 Photodiode Array (detection at 200-600 nm). The columns used were XBridge C18 columns (Waters, 4.6 mm D × 100 mm L analytical, 19 mm D × 100 mmL preparative). Both preparative and analytical analysis using 15 or 18 minute run times in water with 5-98%, 30-98% methanol or 50-98% or methanol.

MRS: MRS was performed in a 4.7T horizontal bore Direct Drive Varian MRI system (Palo Alto, CA). A 20-mm diameter surface coil was used in transmit/receive (T/R) mode. Samples were in 200µl of methanol (1mM or 2mM) in 250µl eppendorf tubes and placed in the middle of the coil loop. Pulse-and-acquire experiment: TR=800 ms, receiver bandwidth= 5 kHz, N_A=50 or 512 (T_A= 40 sec and 6 min respectively).

Gallium generator: ^{68}Ga was synthesized using a benchtop generator supplied by Ekhert & Ziegler containing a ^{68}Ge source. Elution of ^{68}Ga was carried out using 0.1 M HCL producing activity in approximately 2mls.

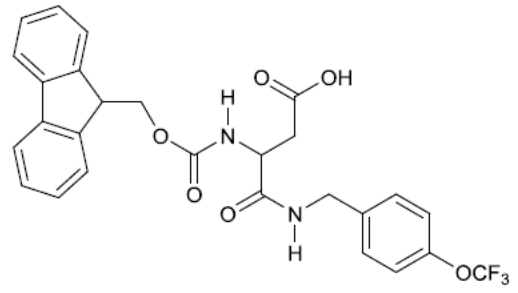
2. Peptide Synthesis

Fmoc-Asp[(trifluoromethoxy)benzylamide]-OtBu



This compound was prepared by modification of a previously reported procedure:[1] Fmoc-Asp-O*t*Bu-OH (430 mg, 1.0 mmol, 1.0 eq.), HBTU (379 mg, 1.0 mmol, 1.0 eq) and DIPEA (0.26 ml, 1.26 mmol, 1.2 eq) were dissolved in dry DMF (20 mL) at 0°C under N_2, then (trifluoromethoxy)benzylamine (200mg, 1.05 mmol, 1.0 eq.) was added at 0 °C. The mixture was stirred at 0 °C for 2 h under N_2, then warmed to RT and stirred for 1 h. The solvent was removed under vacuum, and the crude product was diluted with ethyl acetate and washed with 4% $NaHCO_3$ aq., 10% citric acid, and brine. The organic layer was dried over $MgSO_4$ and removed under vacuum to give crude Fmoc-L-Asp-[(trifluoromethoxy)benzylamide] as a beige solid (552 mg, 0.94 mmol, yield 90%); ^{1}H NMR ($CDCl_3$): δ 1.46 ppm (s, 9H, ROCC***H*****$_3$**), 2.60 ppm (dd, 1H, ^{3}J=6.8Hz, ^{2}J=17.2 Hz, R_3*CC***H_2**COOR), 3.01 ppm (dd, 1H, ^{3}J=4 Hz, ^{2}J=17.6 Hz, R_3*CC***H_2**COOR), 4.21 ppm (t, 1H, ^{3}J=6.6 Hz, R_2C***H***CH_2R), 4.46 ppm (m, 4H, C***H*****$_2$**), 4.58 ppm (broad s, 1H, *C***H**R_3), 5.96 ppm (broad d, 1H, ^{3}J=8.0 Hz, N***H***), 6.81 ppm (broad s, 1H, N***H***), 7.17-7.79 ppm (m, 12H, aromatic protons); ^{19}F NMR ($CDCl_3$): δ - 59.5 ppm (s, ROC**F$_3$**); MS(ESI$^+$) m/z: 585 $[M+H]^+$, 607 $[M+Na]^+$.

Fmoc-Asp[(trifluoromethoxy)benzylamide]-OH



This compound was prepared by small modifications of a previously reported procedure:[1] Fmoc-Asp[*p*-(trifluoromethoxy)benzylamide]-O*t*Bu (552 mg, 0.94 mmol, 1.0 eq) was dissolved in TFA (15 mL) and stirred at RT for 2 h. TFA was then removed under vacuum to yield the crude product. Recrystallization from ethyl acetate and *n*-hexane (8:1) afforded compound (289 mg, 0.55 mmol, yield 58%); ^{1}H NMR (D_6-DMSO): δ 2.55 ppm (dd, 2H, ^{3}J= 8.8 Hz, ^{2}J=14.8 Hz, R_3*CC***H_2**COOR), 2.72 ppm (dd, 2H, ^{3}J=5.2 Hz, ^{2}J=16.0 Hz, R_3*CC***H_2**COOR), 4.27 ppm (m, 5H,: 2 CH_2, R_2C***H***CH_2R), 4.41 ppm (m, 1H, *C***H**R_3), 7.25-7.92 ppm (m, 12H, aromatic protons); ^{19}F NMR

(DMSO): δ -59.6 ppm (s, ROC**F3**); MS (ESI+) m/z: 529.2 $[M+H]^+$, 551.1 $[M+Na]^+$; HRMS (elemental composition) $[M+H]^+$: Mass calculated for $C_{27}H_{24}N_2O_6F_3$ 529.1586, found 529.1577.

Standard protocol – Manual Fmoc solid phase peptide synthesis

Rink amide resin (40 µmol, 1.0 eq or 20 µmol, 1.0 eq) was prepared for synthesis by being swollen with 2ml of DMF for 30 minutes before being rinsed with DMF/CH_2Cl_2/DMF. The resin was then Fmoc deprotected by shaking resin bound compound with a 2 ml mixture of DMF/piperidine (80:20) for 5 minutes carried out 3 times for each deprotection. The resin was then washed with DMF/CH_2Cl2/DMF ready for the first coupling reaction. Coupling reactions were carried out using 3 separate protocols depending on the amino acid/compound:

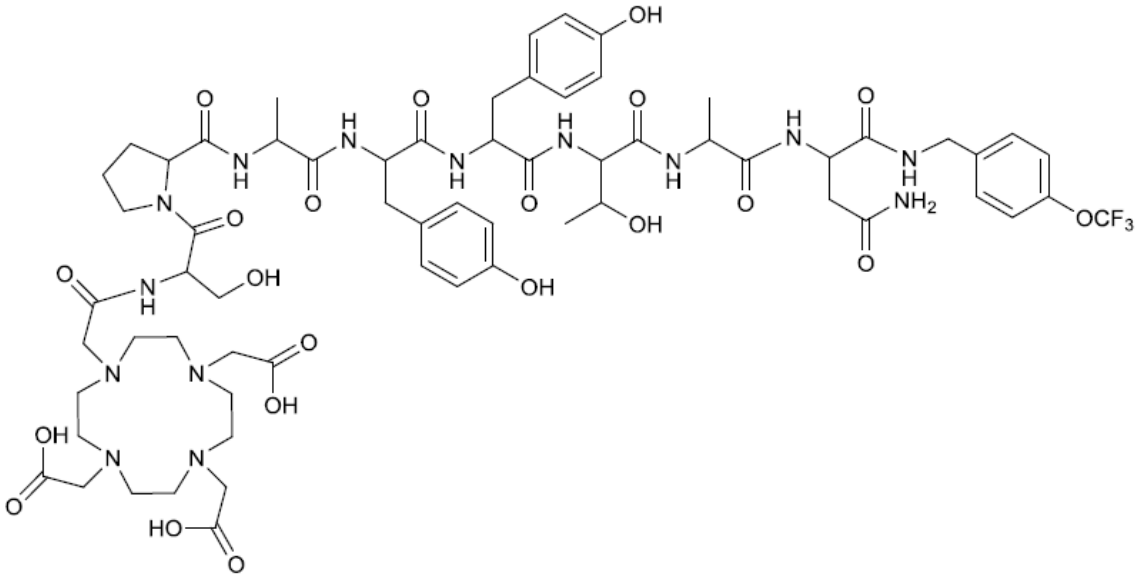
Coupling A: Used for standard Fmoc protected amino acids. Amino acid (5.0 eq) and DIPEA (10 eq) in DMF were mixed together before adding HBTU coupling reagent (4.9 eq) in DMF for 5 minutes preactivation, this was then added to the resin for 30 minutes. This process was repeated (to ensure efficient couplings) with a DMF/CH_2Cl_2/DMF wash between and after couplings.

Coupling B: Used for unusual amino acids or other compounds which can be introduced into the peptide chain; Fmoc-Asp[(trifluoromethoxy) benzyl amide]-OH, 6-(Fmoc-amino)hexanoic acid and *N*-Fmoc-*N*''-succinyl-4,7,10-trioxa-1,13-tridecanediamine. Amino acid (2.5 eq) and DIPEA (5 eq) in DMF were mixed together before adding HATU coupling reagent (2.5 eq) in DMF for 5 minutes preactivation, this was then added with the resin for 30 minutes. Process was repeated (to ensure efficient couplings) with a DMF/CH_2Cl_2/DMF wash between and after couplings.

Coupling C: Used only for 1,4,7-Tris(tert-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane-10-acetic acid (1.5 eq) which was mixed with DIPEA (3 eq) in DMF before addition of HATU (1.5 eq in DMF) for 5 minutes pre-activation, this mixture was then added to the resin for 60 minutes. This process was repeated (to ensure efficient couplings) with a DMF/CH_2Cl_2/DMF wash between and after couplings.

Between each amino acid coupling step Fmoc deprotection took place, as previously described. After all the amino acids/compounds had been sequentially coupled together, the resin was washed with DMF/DCM/MeOH/dietylether and left to dry overnight under vacuum. Peptides were then cleaved from the resin by shaking with 95% TFA, 2.5% water and 2.5% triisopropylsilane for 3 hours. Cold diethyl ether (15 ml) was added to the TFA peptide mixture forming a solid peptide which was collected as a pellet via centrifugation, this pellet was washed with cold diethyl ether (15 ml), after a second centrifugation solvent was drained from the pellet and it was left to dry under vacuum overnight. Peptide could then be dissolved ready for purification *via* prep HPLC on Waters HPLC with Luna C18 column.

DOTA-SPAYYTAD-(trifluoromethoxy)benzylamide, 1



DOTA-SPAYYTAD- (trifluoromethoxy)benzylamide was synthesised using standard protocol for manual Fmoc solid phase peptide synthesis on a Rink amide resin on a 40 µM scale. Purification was carried out by prep HPLC (Water 0.1% FA and 0-90% Acetonitrile 0.1% FA over 0-20 minutes, peak at 12.3 Minutes, UV at 210 nm and 254 nm) lyophilisation yielded a white solid (≥90% purity by HPLC, 34 mg, 59% yield); ^{19}F NMR (Methanol-d): δ -59.2 ppm (s, ROC**F$_3$**)**;** MS (ESI$_+$) m/z: 723.3 and 723.8 $[M+2H]_{2+}/2$, 1445.6 $[M]_+$, 1446.6 $[M+H]_+$, 1467.6 $[M+Na]_+$; MS (MALDI$_+$) m/z: 1445.5 $[M]_+$, 1467.4 $[M+Na]_+$.

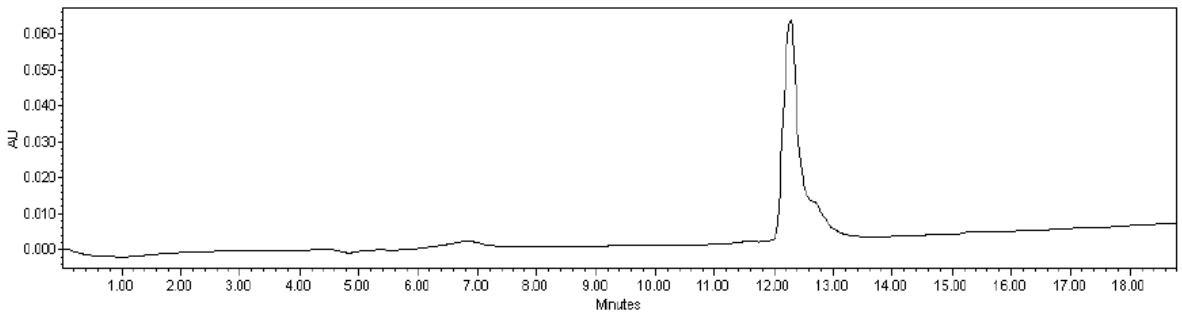
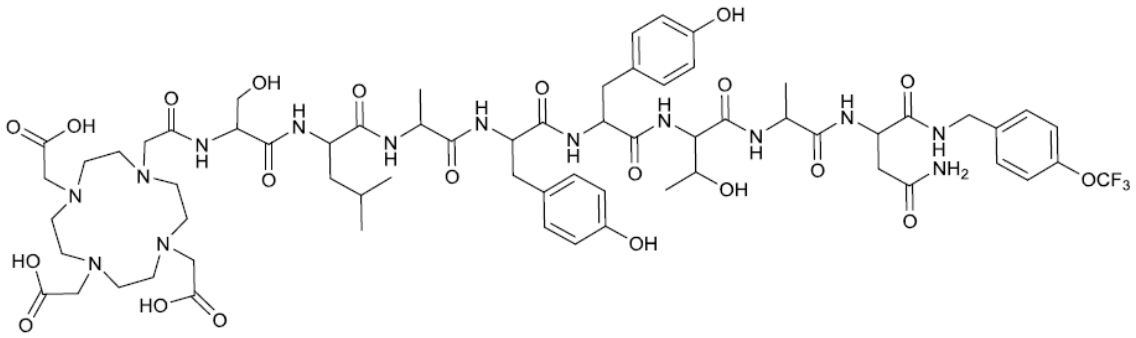


Figure 1 – Purified HPLC trace of **1**

DOTA-SLAYYTAD-(trifluoromethoxy), 2



DOTA-SLAYYTAD-(trifluoromethoxy)benzylamide was synthesised using standard protocol for manual Fmoc solid phase peptide synthesis on a Rink amide resin on a 40 µM scale. Purification carried out by prep HPLC (Water 0.1% FA and 0-90% Acetonitrile 0.1% FA over 0-20 minutes, peak at 12.7 minutes, UV at 210 nm and 254 nm) lyophilisation yielded a white solid (≥95% purity by HPLC, 23.5 mg, 40% yield)**;** ^{19}F NMR (Methanol-d): δ -59.1ppm (s, ROC**F**$_3$)**;** MS(ESI$_+$)m/z: 731.2 and 731.7 $[M+2H]_{2+}/2$, 1461.5 $[M+H]_+$ **;** MS (MALDI$_+$) m/z: 1461.7 $[M+H]_+$, 1483.6 $[M+Na]_+$.

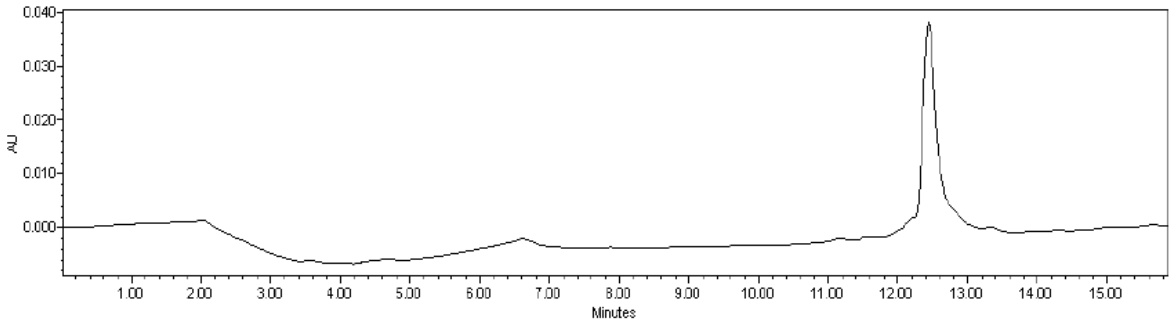
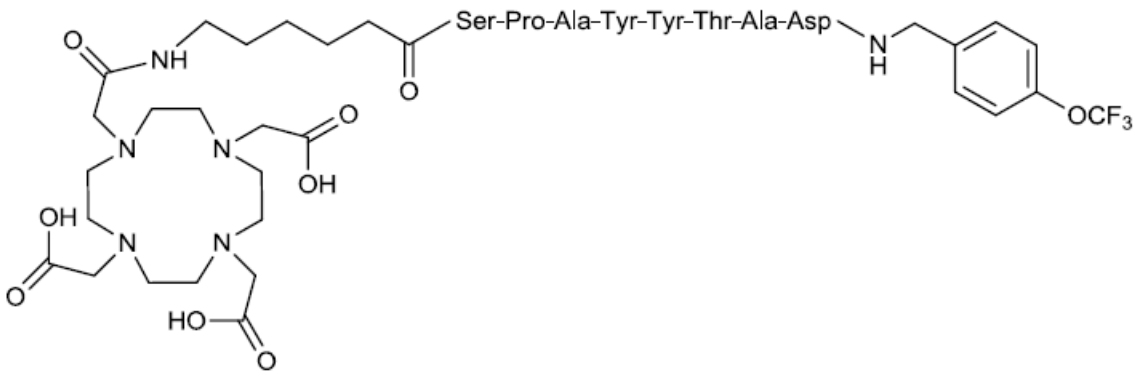


Figure 2 – Purified HPLC trace of **2**

DOTA-Ahx-SPAYYTAD-Tfb, 3



DOTA-Ahx-SPAYYTAD-(trifluoromethoxy)benzylamide was synthesised using standard protocol for manual Fmoc solid phase peptide synthesis on a Rink amide resin on a 20 µM scale. Purification carried out by prep HPLC (Water 0.1% FA and 0- 90% Acetonitrile 0.1% FA over 0-20 minutes, peak at 12.3 minutes, UV at 210 nm and 254 nm) lyophilisation yielded a white solid (≥90% purity by HPLC, 10 mg, 32% yield); ^{19}F NMR (DMSO-d$_6$): δ -59.6 ppm (s, ROC**F$_3$**); MS (MALDI$_+$) m/z: 1558.9 [M]$_+$, 1559.9 [M+H]$_+$, 1597.9 [M+K]$_+$.

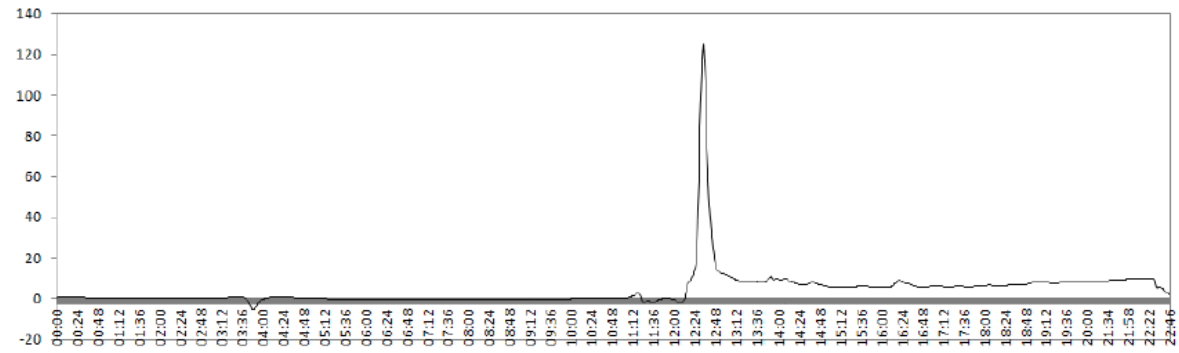
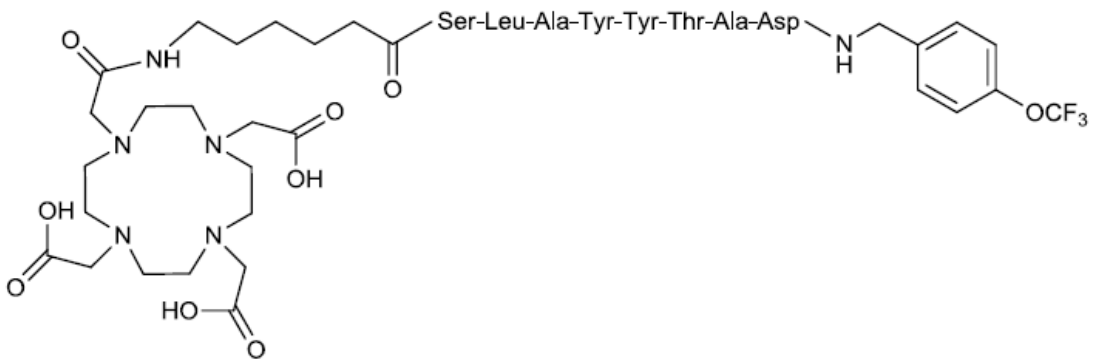


Figure 3 – Purified HPLC trace of **3**

DOTA-Ahx-SLAYYTAD-Tfb, 4



DOTA-Ahx-SLAYYTAD-(trifluoromethoxy)benzylamide was synthesised using standard protocol for manual Fmoc solid phase peptide synthesis on a Rink amide resin on a 20 µM scale. Purification carried out by prep HPLC (Water 0.1% FA and 0- 90% Acetonitrile 0.1% FA over 0-20 minutes, peak at 13.1 minutes, UV at 210 nm and 254 nm) lyophilisation yielded a white solid (≥90% purity by HPLC, 8.5 mg, 27% yield); ^{19}F NMR (DMSO-d_6): δ -60.0 ppm (s, ROC**F$_3$**); MS (MALDI$_+$) m/z: 1575.3 $[M]_+$, 1576.3 $[M+H]_+$.

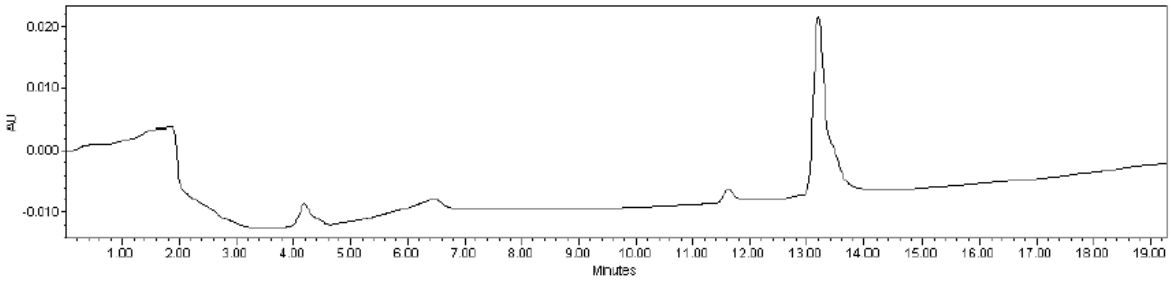
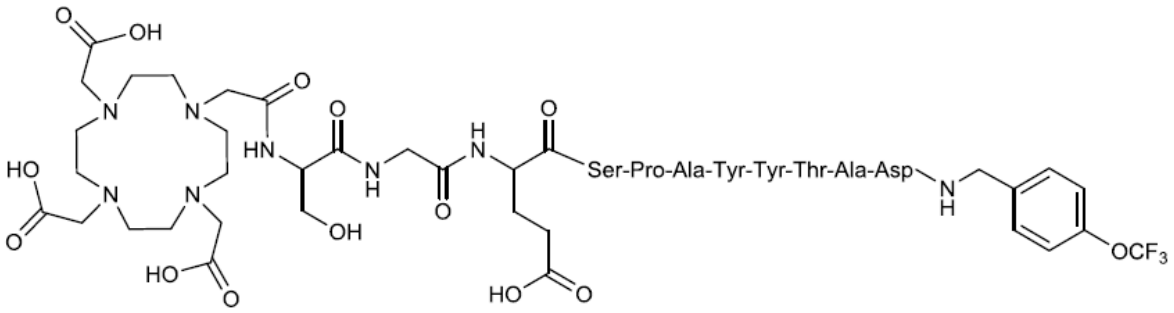


Figure 4 – Purified HPLC trace of **4**

DOTA-SGESPAYYTAD-Tfb, 5



DOTA-SGESPAYYTAD-(trifluoromethoxy)benzylamide was synthesised using standard protocol for manual Fmoc solid phase peptide synthesis on a Rink amide resin on a 20 µM scale. Purification carried out by prep HPLC (Water 0.1% FA and 0- 90% Acetonitrile 0.1% FA over 0-20 minutes, UV monitored at 210 nm and 254 nm, peak at 12.6 minutes) lyophilisation yielded a white solid (≥95% purity by HPLC, 14.5 mg, 42% yield); ^{19}F NMR (DMSO-d_6): δ -60.0 ppm (s, ROC**F**$_3$); MS (MALDI$_+$) m/z: 1719.2 $[M]_+$, 1758.2 $[M+K]_+$.

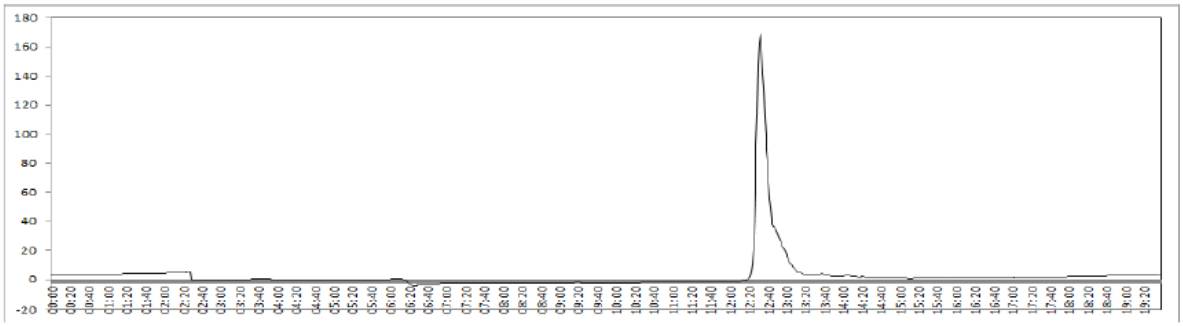
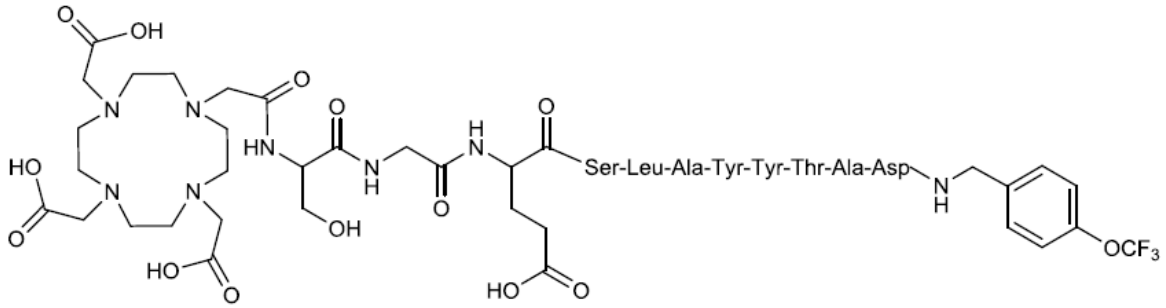


Figure 5 – Purified HPLC trace of **5**

DOTA-SGESLAYYTAD-Tfb, 6



DOTA-SGESLAYYTAD-(trifluoromethoxy)benzylamide was synthesised using standard protocol for manual Fmoc solid phase peptide synthesis on a Rink amide resin on a 20 µM scale. Purification carried out by prep HPLC (Water 0.1% FA and 0- 90% Acetonitrile 0.1% FA over 0-20 minutes, peak at 12.3 minutes, UV at 210 nm and 254 nm) lyophilisation yielded a white solid (≥95% purity by HPLC, 9 mg, 26% yield). ^{19}F NMR (DMSO-d_6): δ -60.0 ppm (s, ROC**F$_3$**); MS (MALDI$^+$) m/z: 1735.1 $[M]^+$, 1736.1 $[M+H]^+$.

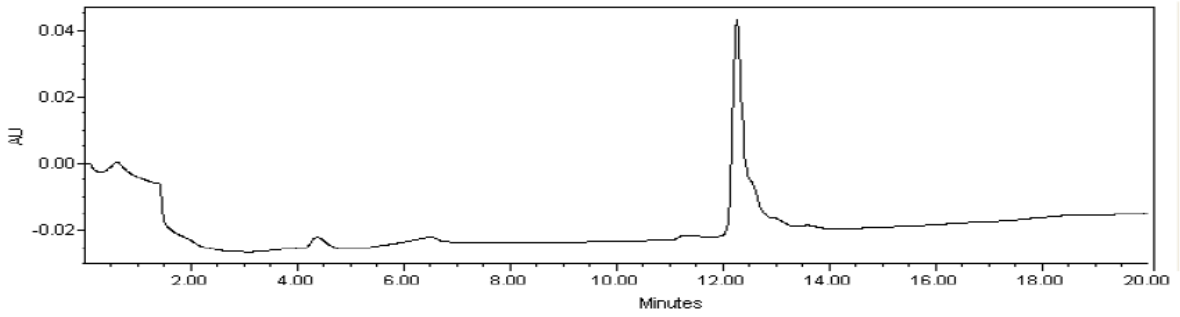
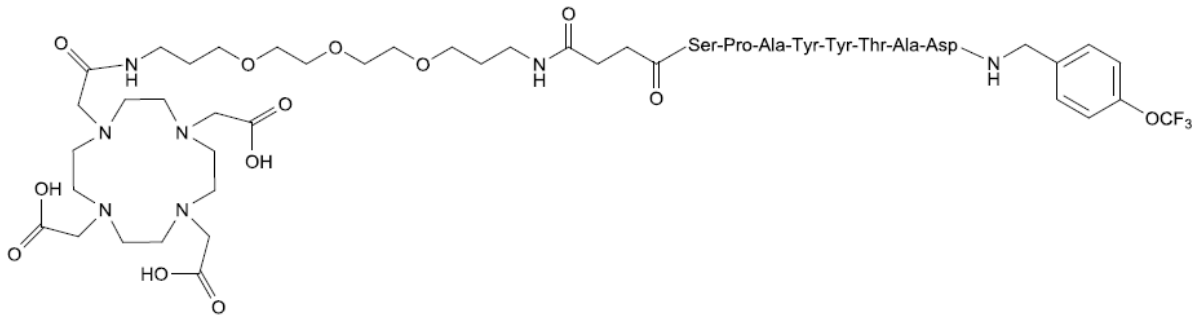


Figure 6 – Purified HPLC trace of **6**

DOTA-PEG$_3$-SPAYYTAD-Tfb, 7



DOTA-PEG$_3$-SPAYYTAD-(trifluoromethoxy)benzylamide was synthesised using standard protocol for manual Fmoc solid phase peptide synthesis on a Rink amide resin on a 20 µM scale. Purification carried out by prep HPLC (Water 0.1% FA and 0- 90% Acetonitrile 0.1% FA over 0-20 minutes, peak at 11.6 minutes, UV at 210 nm and 254 nm) lyophilisation yielded a white solid (≥95% purity by HPLC, 10.5 mg, 30% yield); ^{19}F NMR (DMSO-d$_6$): δ -59.1 ppm (s, ROC**F$_3$**); MS (MALDI$_+$) m/z: 1749.1 $[M]_+$, 1750.1 $[M+H]_+$.

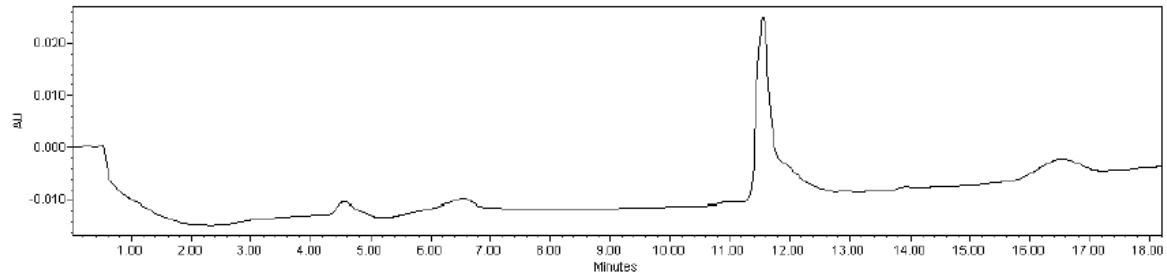
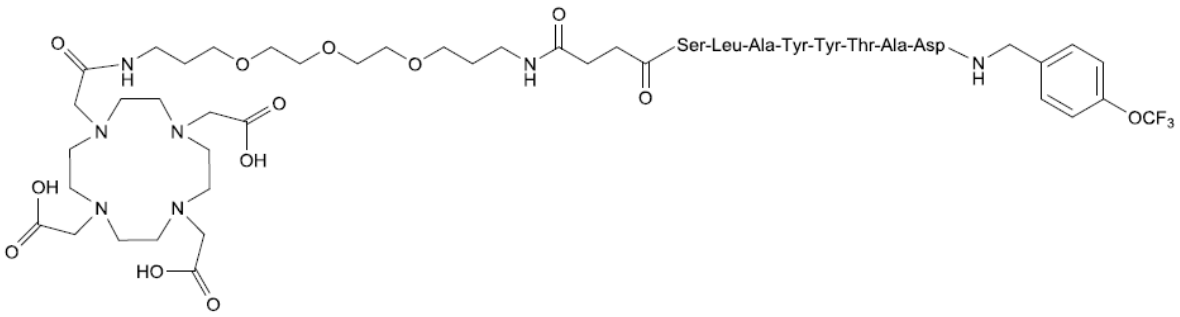


Figure 7 – Purified HPLC trace of **7**

DOTA-PEG$_3$-SLAYYTAD-Tfb, 8



DOTA-PEG$_3$-SLAYYTAD-(trifluoromethoxy)benzylamide was synthesised using standard protocol for manual Fmoc solid phase peptide synthesis on a Rink amide resin on a 20 µM scale. Purification carried out by prep HPLC (Water 0.1% FA and 0- 90% Acetonitrile 0.1% FA over 0-20 minutes, peak at 12.1 minutes, UV at 210 nm and 254 nm) lyophilisation yielded a white solid (≥95% purity by HPLC, 10 mg, 28% yield); ^{19}F NMR (DMSO-d$_6$): δ -60.0 ppm (s, ROC**F$_3$**); MS (MALDI$_+$) m/z: 1764.0 $[M+H]_+$, 1802.0 $[M+K]_+$.

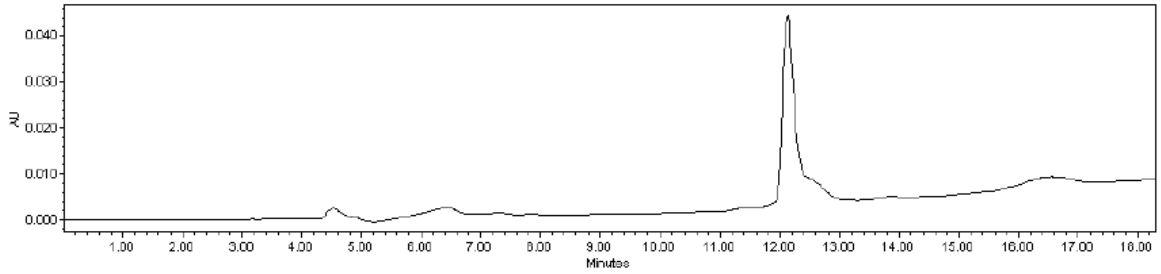
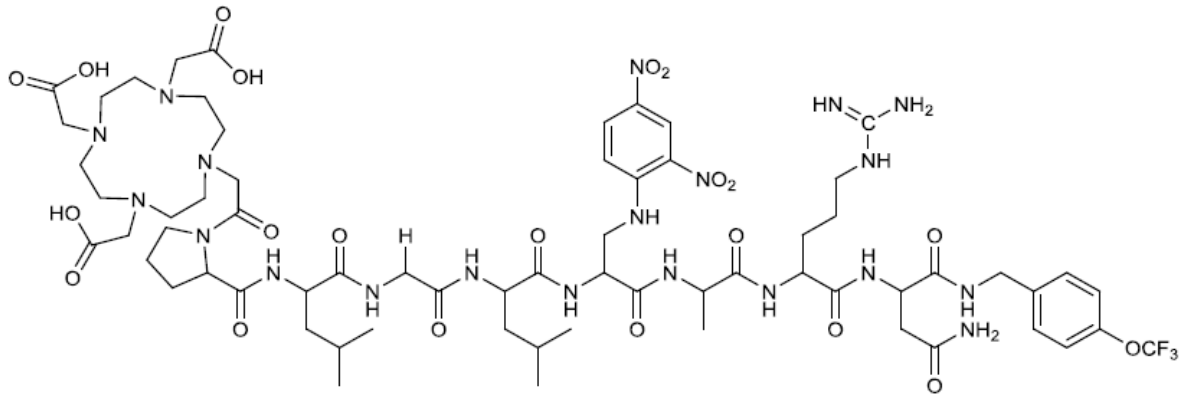


Figure 8 – Purified HPLC trace of **8**

DOTA- PLGL-Dpa-ARD-Tfb, 9



DOTA-PLGL-Dpa-ARD-Tfb was synthesised using standard protocol for manual Fmoc solid phase peptide synthesis on a Rink amide resin on a 40 µM scale. Purification was carried out by prep

HPLC (Water 0.1% FA and 0-90% Acetonitrile 0.1% FA over 1-21 minutes, peak at 8.3 Minutes, UV at 210 nm and 254 nm) lyophilisation yielded a bright yellow solid (≥95% purity by HPLC, 34 mg, 55% yield). ^{19}F NMR (DMSO-d_6): δ -59.4ppm; MS (ESI$_+$) m/z: 1589.7 [M+K]$_+$; MS (MALDI$_+$) m/z: 1551.8 [M+H]$_+$.

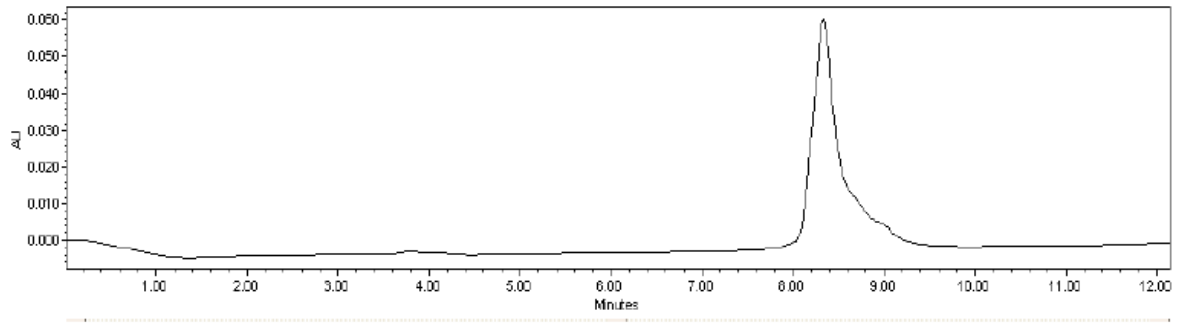
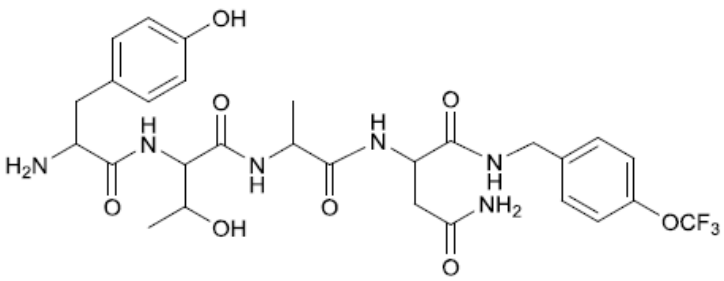


Figure 9 – Purified HPLC trace of **9**

YTAD-(trifluoromethoxy)benzylamide 10



Compound **10** was synthesised using the standard protocol for manual Fmoc solid phase peptide synthesis on a Rink amide resin on a 40 µM scale. Purification carried out by prep HPLC (Water 0.1% FA and 0-90% Acetonitrile 0.1% FA over 20 minutes, peak at 11.35 minutes, UV at 210 nm and 254 nm) lyophilisation yielded a white solid (≥90% purity by HPLC, 10.5 mg, 41 % yield)**.** ^{19}F NMR (Methanol-d): δ -59.9ppm (s,ROC**F$_3$**)**.** MS(ESI$_+$)m/z: 641.3 [M+H]$_+$ 663.2 [M+Na]$_+$**.** MS (MALDI$_+$) m/z: 641.5 [M+H]$^+$.

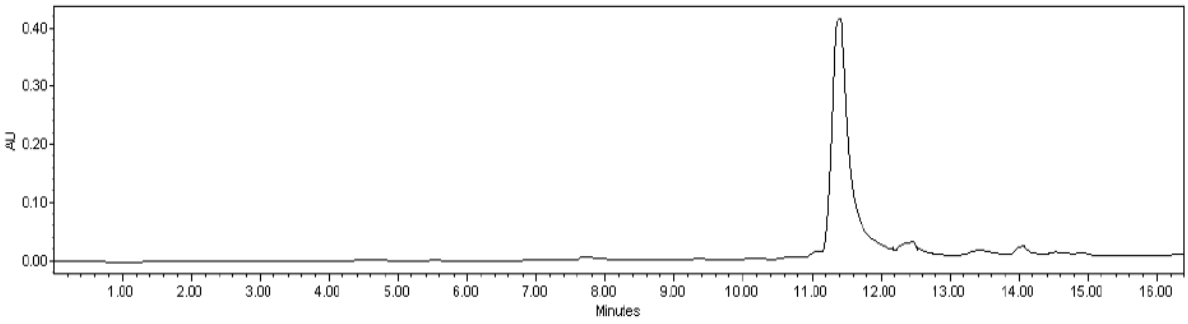
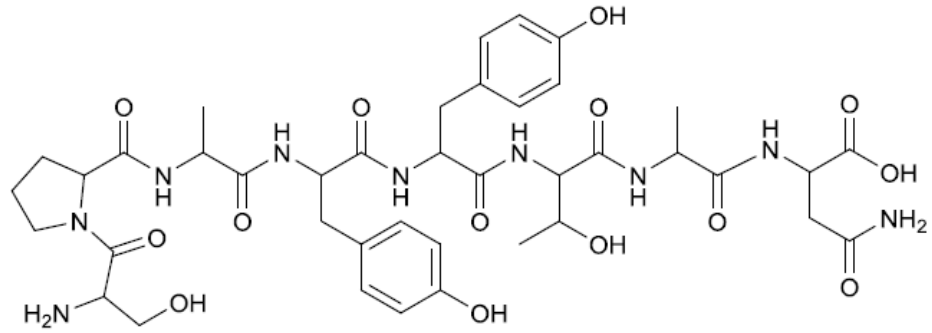


Figure 10 – Purified HPLC trace of **10**

SPAYYTAD 11



SPAYYTAD was synthesised using standard protocol 1 for manual Fmoc solid phase peptide synthesis on a Rink amide resin on a 20 µM scale. Purification was carried out using prep LCMS (water 0.1% FA with 30-98% methanol 0.1% FA over 15 minutes), centrifugal evaporation yielded white solid, yield 31% (5.5 mgs, ≥95% purity by HPLC). MS(ESI+)m/z: 886.6 $[M]_+$, 887.6 $[M+H]_+$, 910.1 $[M+Na]_+$.

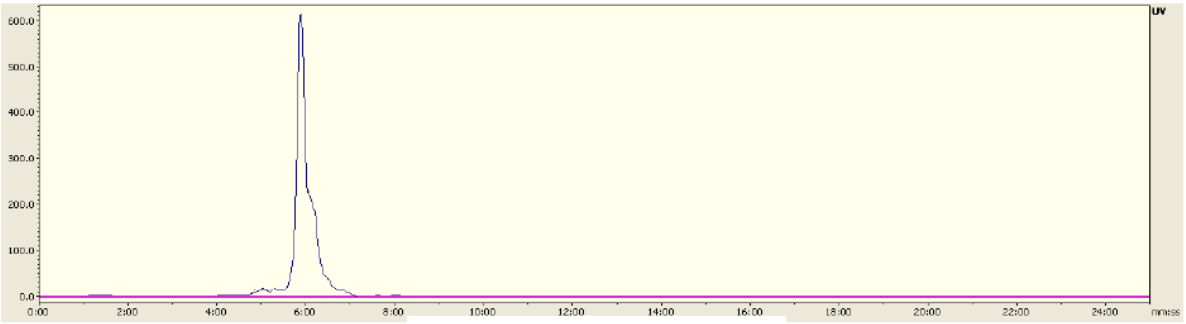
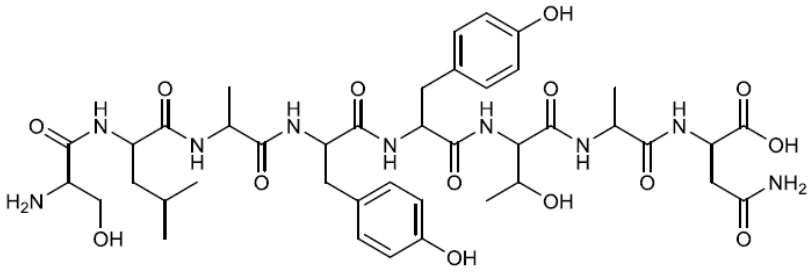


Figure 11 – Purified HPLC trace of **11**

SLAYYTAD 12



SLAYYTAD was synthesised using standard protocol 1 for manual Fmoc solid phase peptide synthesis on a Rink amide resin on a 20 µM scale. Purification was carried out using prep LCMS (water 0.1% FA with 30-98% methanol 0.1% FA over 15 minutes), centrifugal evaporation white solid, yield 28% (5 mgs, ≥ 95% purity by HPLC). MS(ESI+)m/z: 902.7$[M]_+$,903.7 $[M+H]_+$, 926.10 $[M+Na]_+$.

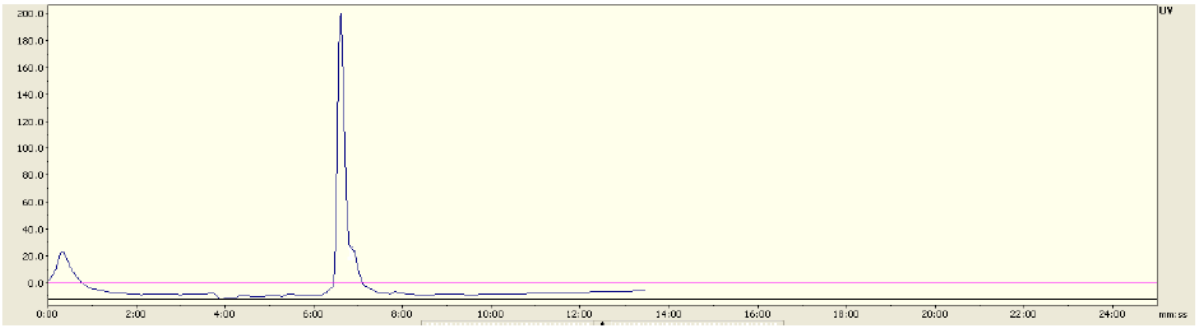


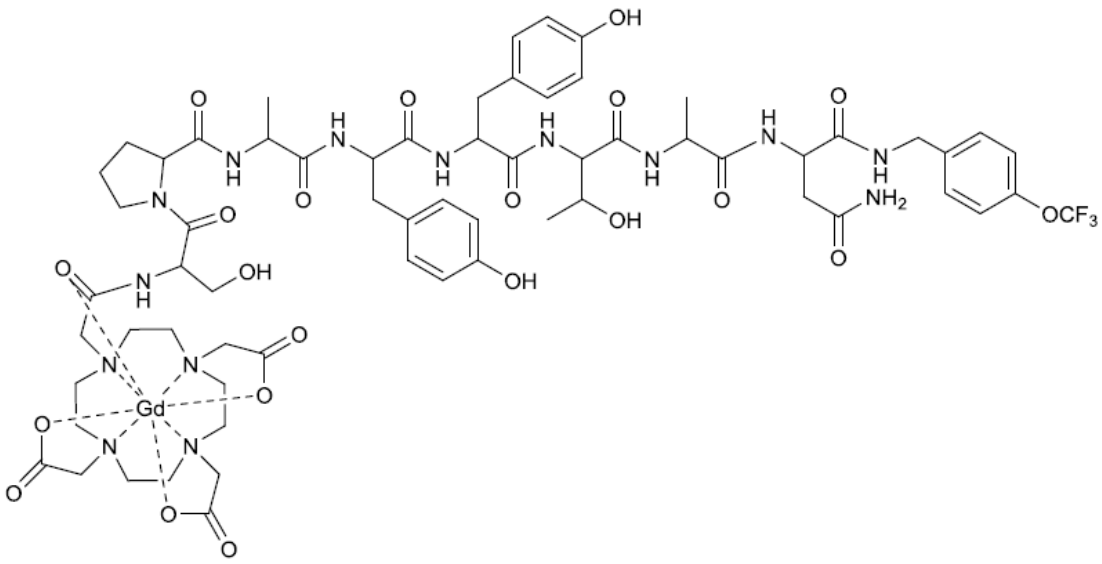
Figure 12 – Purified HPLC trace of **12**

Table 1 – Summary of yields of solid phase synthesised peptides after HPLC purification **1-8**

Peptide	Yield
DOTA-SPAYYTAD-Tfb **1**	59%
DOTA-SLAYYTAD-Tfb **2**	40%
DOTA-Ahx-SPAYYTAD-Tfb **3**	32 %
DOTA-Ahx-SLAYYTAD-Tfb **4**	27%
DOTA-SGE-SPAYYTAD-Tfb **5**	42%
DOTA-SGE-SLAYYTAD-Tfb **6**	26%
DOTA-PEG_3-SPAYYTAD-Tfb **7**	30%
DOTA-PEG_3-SLAYYTAD-Tfb **8**	28%
DOTA- PLGL-Dpa-ARD-Tfb **9**	55%

3. Gadolinium Chelation Reactions

Gd-DOTA-SPAYYTAD-(trifluoromethoxy)benzylamide, [Gd]1



DOTA-SPAYYTAD- (trifluoromethoxy)benzylamide (10 mgs, 6.9 µmols, 1.0 eq) was partially dissolved in 4 mls of MilliQ water then an excess of $GdCl_3 \cdot 6H_2O$ was added as 2 ml of an 6 mM solution in MiliQ water (4.5 mgs, 12 µmols, 1.75 eq), pH adjusted with NaOH to between 6-7 (checking with pH indicator paper) and the mixture was left stirring at room temperature. After 1 hour the pH of the mixture was checked again using indicator paper to ensure pH had not changed and then the reaction was left stirring overnight. The mixture was then purified using reversed phase Prep HPLC (Water 0.1% FA and 0-90% Acetonitrile 0.1% FA over 1-21 minutes, peak at 13.4 Minutes, 210 nm and 254 nm) to give pure Gd-DOTA-SPAYYTAD-(trifluoromethoxy)benzylamide as a fluffy white solid (≥98% purity by HPLC, 9.6 mgs, 87% yield); ^{19}F NMR (DMSO-d_6): δ −59.2 ppm (broadened s, $ROCF_3$); MS (ESI_+) m/z: 800.7 $[M+2H]^{2+}/2$, 801.7 $[M+2H]^{2+}/2$, 1597.5 $[M]_+$, 1598.5 $[M+H]_+$, 1599.5 $[M]_+$, 1600.5 $[M+H]_+$, 1602.5 $[M]_+$, 1603.5 $[M+H]_+$; MS ($MALDI_+$) m/z: 1599.0 $[M+H]_+$, 1600.0 $[M+H]_+$, 1601.0 $[M+H]_+$, 1603.0 $[M+H]_+$, 1620.8 $[M+Na]_+$, 1621.8 $[M+Na]_+$ 1622.8 $[M+Na]_+$, 1624.8 $[M+Na]_+$.

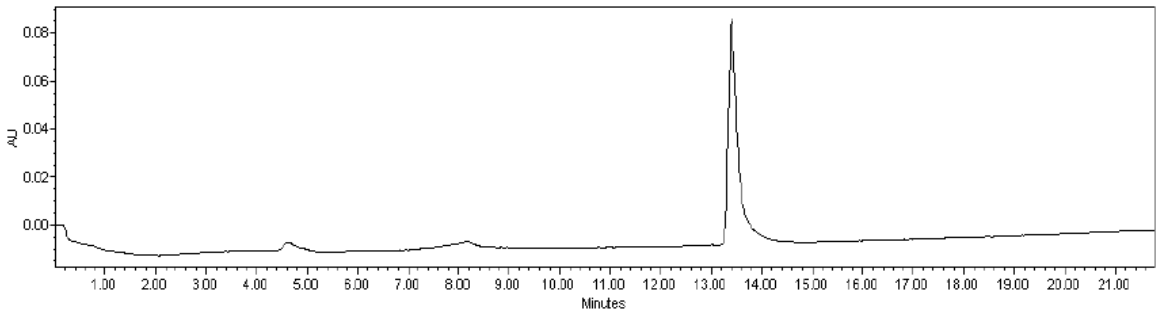
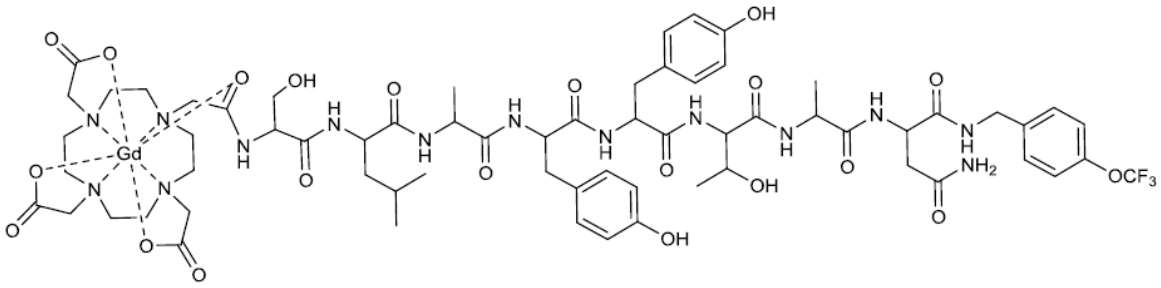


Figure 9 – Purified HPLC trace of **[Gd]1**

Gd-DOTA-SLAYYTAD-(trifluoromethoxy)benzylamide, [Gd]2



Prepared as described for compound **[Gd]1**: starting material DOTA-SLAYYTAD-Tfb (8 mgs, 5.5 µmols, 1eq) yield 70% (6.2 mgs, ≥90% purity by HPLC Water 0.1% FA and 0-90% Acetonitrile 0.1% FA over 1-21 minutes peak at 13.8 minutes UV at 210 nm and 254 nm); ^{19}F NMR (DMSO-d_6): δ -59.6 ppm (broadened s, $ROC\mathbf{F_3}$); MS(ESI_+)m/z: 808.3 $[M+2H]^{2+}/2$, 808.8 $[M+2H]^{2+}/2$, 1613.5 $[M]_+$, 1614.5 $[M+H]_+$, 1615.5 $[M]_+$, 1616.5 $[M+H]_+$, 1617.5 $[M]_+$, 1636.5 $[M+Na]_+$, 1637.5 $[M+Na]_+$, 1638.5 $[M+Na]_+$, 1640 $[M+Na]_+$. MS ($MALDI_+$) m/z: 1613.8 $[M]_+$, 1614.8 $[M]_+$, 1615.9 $[M]_+$, 1616.8 $[M]_+$, 1617.8 $[M]_+$.

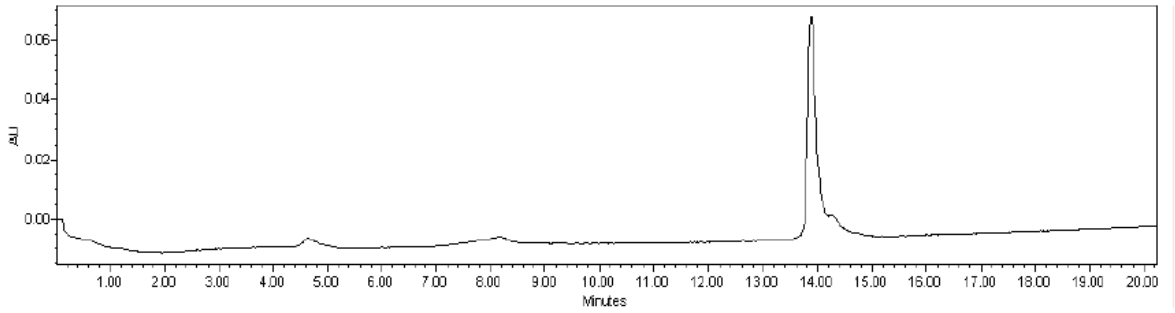
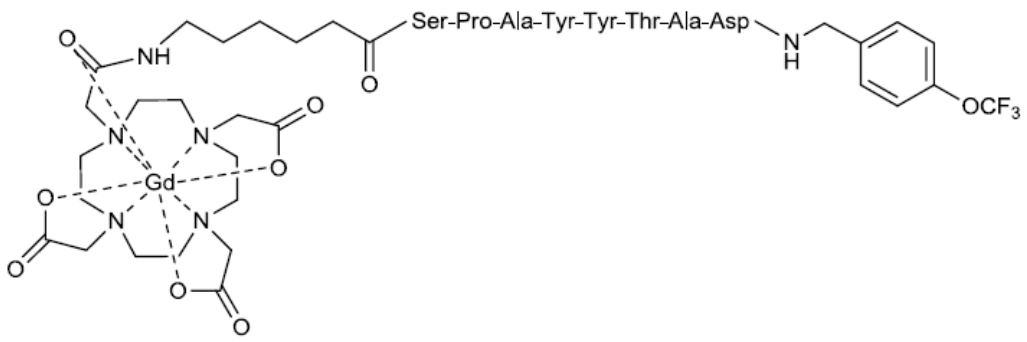


Figure 10 – Purified HPLC trace of **[Gd]2**

Gd-DOTA-Ahx-SPAYYTAD-Tfb, [Gd]3



Prepared as described for compound **[Gd]1**: starting material DOTA-Ahx-SPAYYTAD-Tfb (2.5 mgs, 1.6 µmols, 1 eq) to afford **[Gd]3** as a white solid in a yield of 69% (1.9 mgs, ≥98% purity by HPLC Water 0.1% FA and 0- 90% Acetonitrile 0.1% FA over 1-21 minutes, UV monitored at 210 nm and 254 nm, peak at 13.6 minutes); ^{19}F NMR (DMSO-d_6): δ -59.9 ppm (broadened s, ROC**F$_3$**);

MS (MALDI+) m/z: 1712.0 $[M+H]^+$, 1713.0 $[M+H]^+$, 1714.0 $[M+H]^+$, 1716.0 $[M+H]^+$, 1734.0 $[M+Na]^+$, 1735.0 $[M+Na]^+$, 1736.0 $[M+Na]^+$, 1738.0 $[M+Na]^+$.

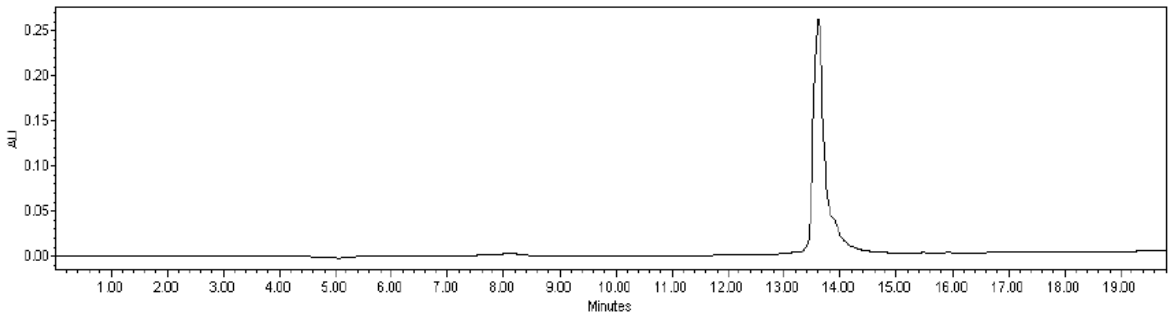
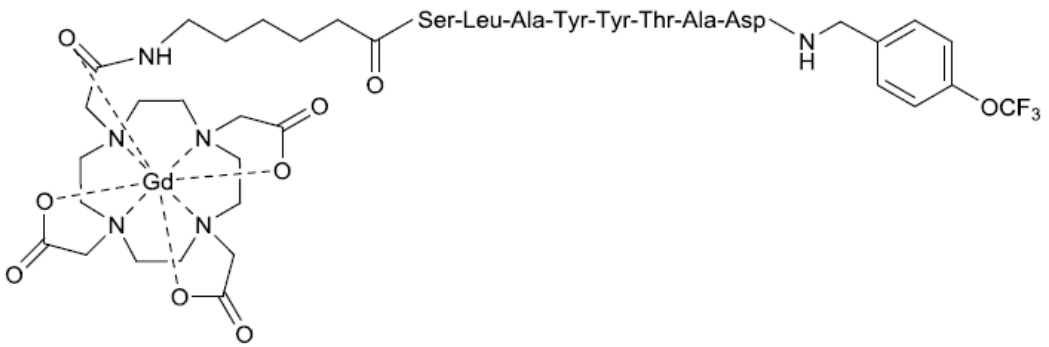


Figure 11 – Purified HPLC trace of **[Gd]3**

Gd-DOTA-Ahx-SLAYYTAD-Tfb, [Gd]4



Prepared as described for compound **[Gd]1**: starting material DOTA-Ahx-SLAYYTAD-Tfb (3.5 mgs, 2.2 µmols, 1 eq) to afford **[Gd]4** as a white solid in a yield of 78% (3 mgs, ≥98% purity by HPLC Water with 0.1% FA and 0-90% Acetonitrile with 0.1% FA over 1-21 minutes, UV monitored at

210 nm and 254 nm, peak at 14.1 minutes); ^{19}F NMR (DMSO-d$_6$): δ -59.3 ppm (broadened s, ROC**F$_3$**); MS (MALDI$_+$) m/z: 1727.1 $[M]_+$, 1728.1 $[M]_+$, 1729.0 $[M]_+$,1730.0 $[M]_+$,1732.1 $[M]_+$.

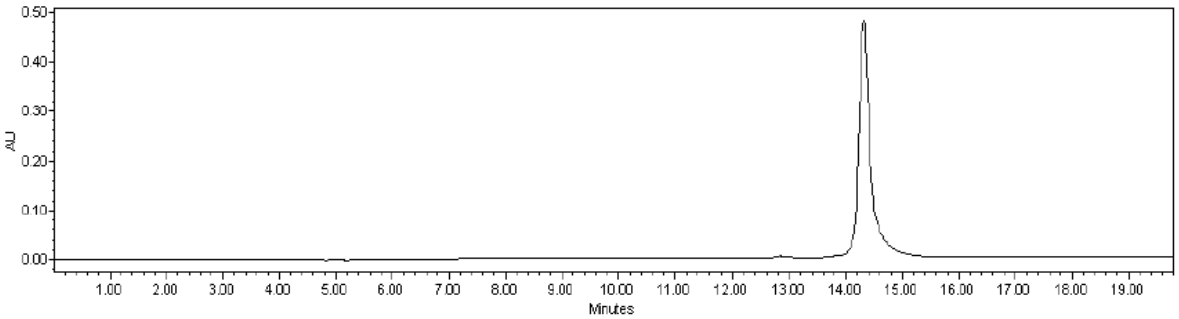
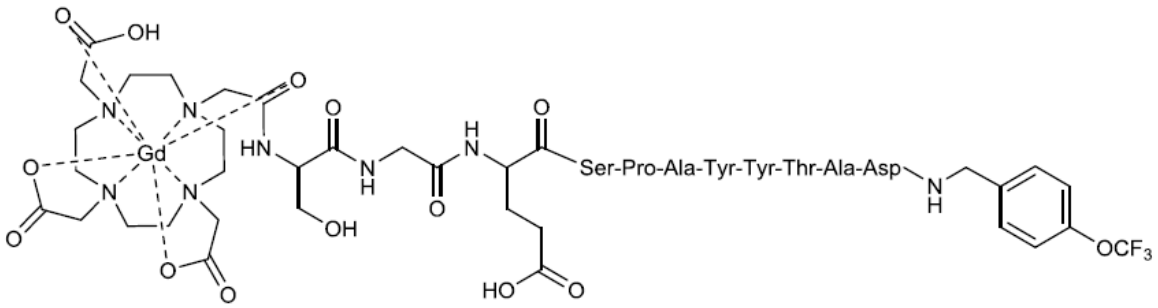


Figure 12 – Purified HPLC trace of **[Gd]4**

Gd-DOTA-SGESPAYYTAD-Tfb, [Gd]5



Prepared as described for compound **[Gd]1**: starting material DOTA-SGESPAYYTAD-Tfb (3.5 mgs, 2 µmols, 1 eq) to afford **[Gd]5** as a white solid in a yield of 52% (2 mgs, ≥90% purity by HPLC: Water (0.1% FA) and 0-90% Acetonitrile (0.1% FA) over 1-21 minutes, UV monitored at 210 nm

and 254 nm, peak at 13.5 minutes); ^{19}F NMR (DMSO-d_6): δ -59.5 ppm (broadened s, ROC**F$_3$**); MS (MALDI$_+$) m/z: 1873.0 $[M]_+$, 1874.0 $[M]_+$,1875.0 $[M]_+$,1876.0 $[M]_+$, 1910.1 $[M+K]_+$, 1911.0 $[M+K]_+$, 1912.0 $[M+K]_+$, 1913.0 $[M+K]_+$, 1915.0 $[M+K]_+$.

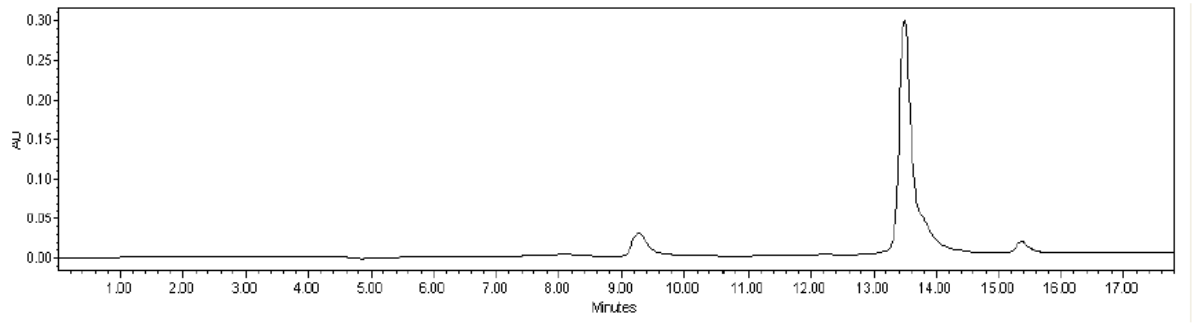
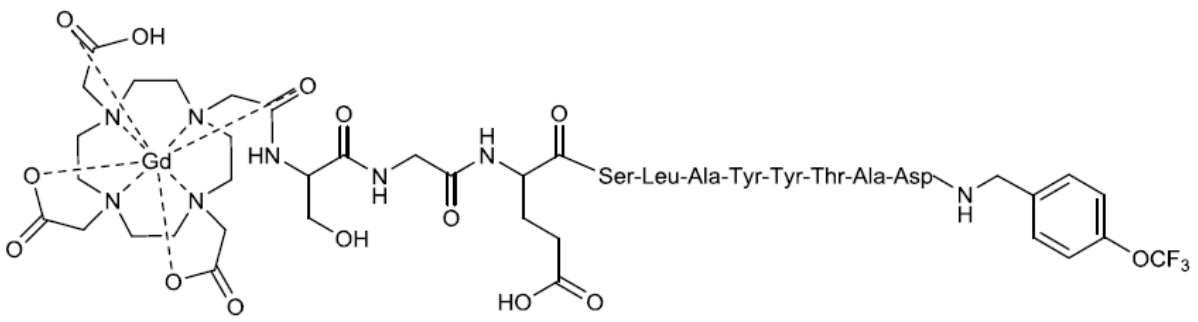


Figure 13 – Purified HPLC trace of **[Gd]5**

Gd-DOTA-SGESLAYYTAD-Tfb, [Gd]6



Prepared as described for compound **[Gd]1**: starting material DOTA-SGESPAYYTAD-Tfb (3.5mgs, 2µmols, 1eq) to afford **[Gd]6** as a white solid in a yield of 79% (3mgs, ≥95% purity by HPLC

Water with 0.1% FA and 0-90% Acetonitrile with 0.1% FA over 1-21 minutes, UV monitored at 210nm and 254nm, peak at 14.0 minutes); ^{19}F NMR (DMSO-d_6): δ -59.5ppm (broadened s, ROC**F**$_3$); MS (MALDI$_+$) m/z: 1889.2 $[M]_+$, 1890.3 $[M]_+$,1910.3 $[M+Na]_+$,1911.2 $[M+Na]_+$, 1912.2 $[M+Na]_+$, 1914.2 $[M+Na]_+$.

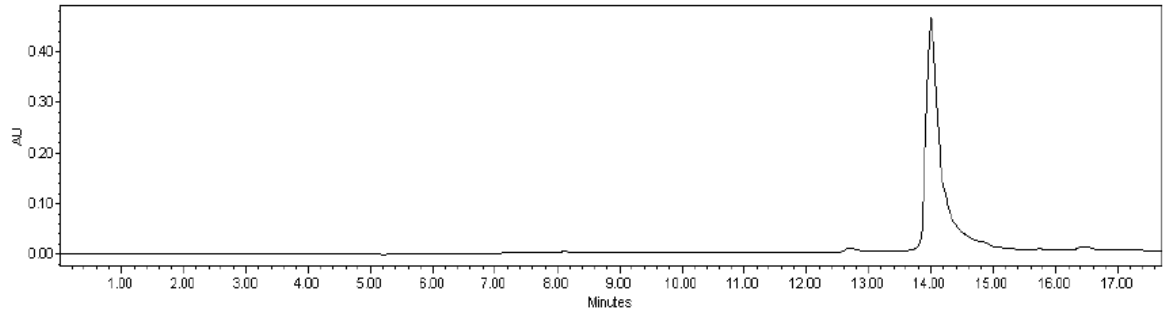
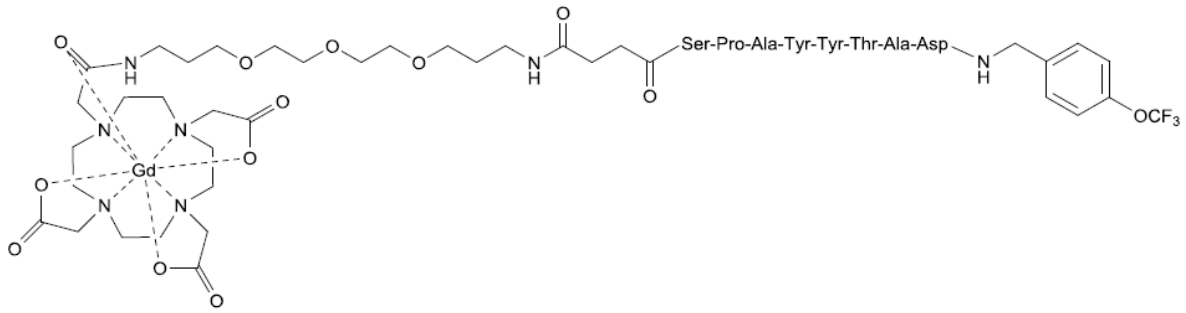


Figure 14 – Purified HPLC trace of **[Gd]6**

Gd-DOTA-PEG$_3$-SPAYYTAD-Tfb, [Gd]7



Prepared as described for compound **[Gd]1**: starting material DOTA-PEG$_3$-SPAYYTAD-Tfb (2.5mgs, 1.4µmols, 1eq) to afford **[Gd]7** as a white solid of in a yield of 62% (1.7mgs, ≥98%

purity by HPLC Water with 0.1% FA and 0-90% Acetonitrile with 0.1% FA over 1-21 minutes, UV monitored at 210nm and 254nm, peak at 13.5 minutes); ^{19}F NMR (DMSO-d_6): δ -59.6ppm (broadened s, ROC**F$_3$**); MS (MALDI$_+$) m/z: 1902.1 $[M]_+$, 1903.1 $[M]_+$,1904.2 $[M]_+$,1906.1 $[M]_+$.

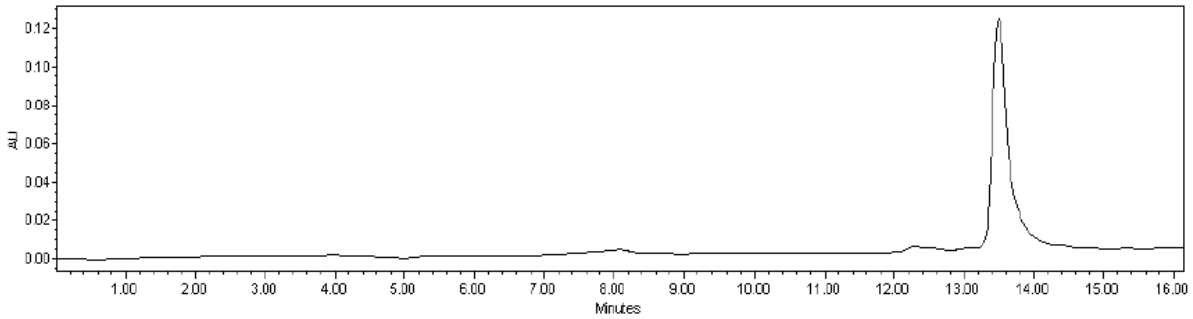
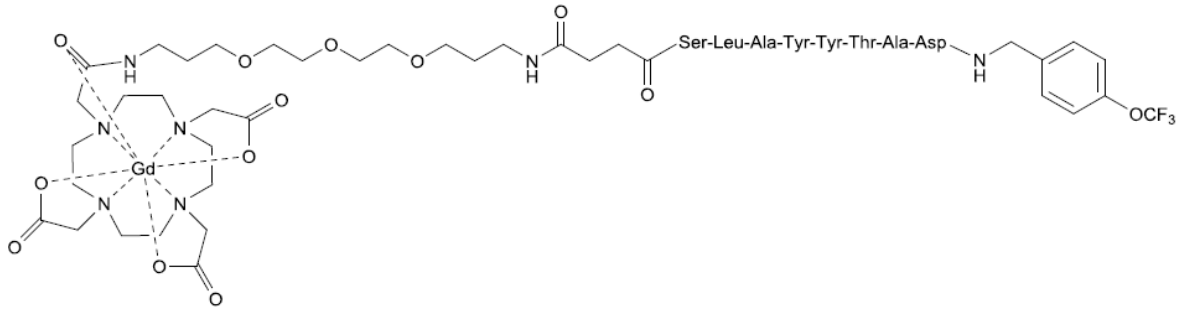


Figure 15 – Purified HPLC trace of **[Gd]7**

Gd-DOTA-PEG$_3$-SLAYYTAD-Tfb, [Gd]8



Prepared as described for compound **[Gd]1**: starting material DOTA-PEG$_3$-SLAYYTAD-Tfb (3mgs, 1.7µmols, 1eq) to afford **[Gd]8** as a white solid in a yield of 85% (2.8mgs, ≥98% purity by HPLC,

Water with 0.1% FA and 0- 90% Acetonitrile with 0.1% FA over 1-21 minutes, UV monitored at 210nm and 254nm, peak at 14.2 minutes); ^{19}F NMR (DMSO-d_6): δ -59.6ppm (broadened s, ROC**F$_3$**); MS (MALDI$^+$) m/z: 1916.1 $[M]^+$, 1917.2 $[M]^+$,1918.2 $[M]^+$,1920.1 $[M]^+$, 1938.1 $[M+K]^+$, 1939.1 $[M+K]^+$, 1941.1$[M+K]^+$, 1943.1 $[M+K]^+$.

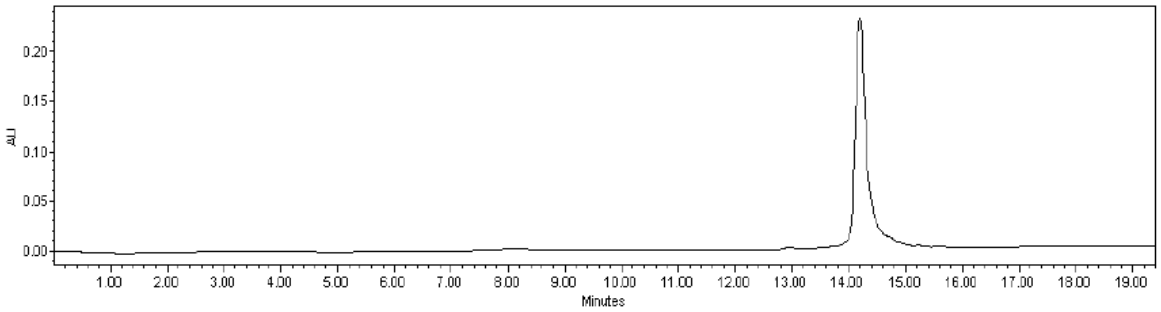


Figure 16 – Purified HPLC trace of **[Gd]8**

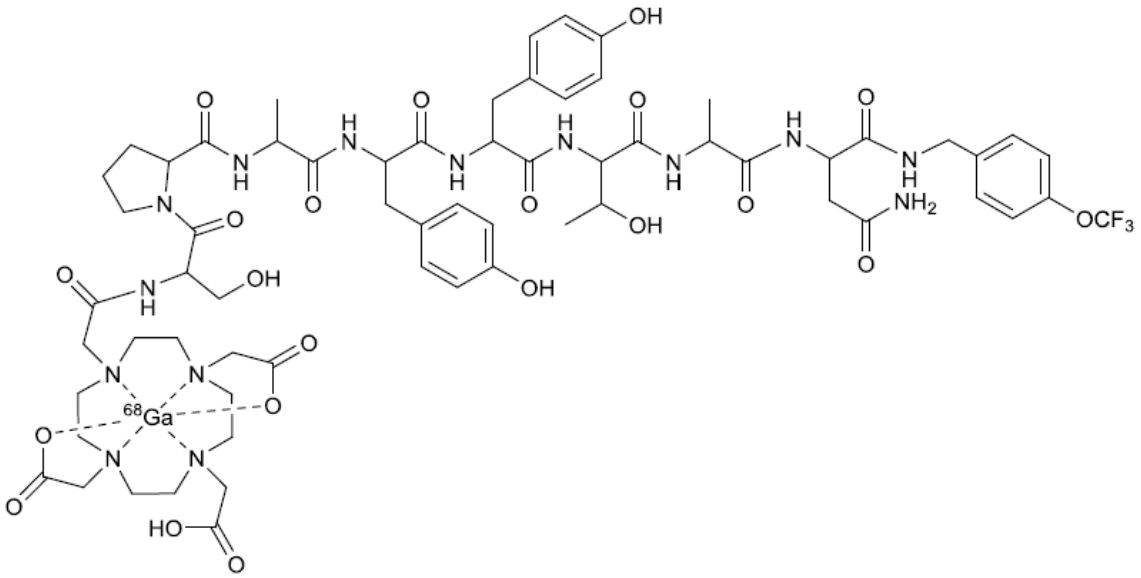
4. ^{68}Ga Radiolabelling

General protocol: Peptide ligand dissolved to 700 µM in DMSO, 50 µl of peptide ligand solution was mixed with 1 ml of NaOAc buffer (pH 5, 0.2 M) in a Wheaton vial. ^{68}Ga was eluted from the generator in 2 ml of 0.1M HCL and 1 ml of this was added to the peptide solution. Reaction mixture was heated at 90°C for 10 minutes and then reaction mixture was removed from the heat. Radio-HPLC was carried out to determine radiochemical yields of compound versus free gallium (which elutes with the solvent front at 2 minutes). Remaining gallium could be removed from sample via solid phase extraction, this was carried out on a *t*C18 light cartridge (prepared by flushing with 5 mls of ethanol and the 10 mls of water), sample was loaded onto cartridge in reaction mixture with any free ^{68}Ga passing straight through, sample was then washed with water and could be removed from the cartridge with 0.8 ml of ethanol collected in 100 µl aliquots, the majority of the radiolabelled sample obtained between aliquots 2 and 3. Analytical

radio-HPLC was carried out again to confirm purity. If being used for assay conditions, samples 2 and 3 were combined and diluted with 300 ml of MMP-2 assay buffer (50 mM Tricine, 10 mM $CaCl_2$, 150 mM NaCl, 0.5% Brij-35; pH 7.5).

Radio-HPLC conditions: Gradient, water (0.1% FA) with 0 - 90% acetonitrile (0.1%FA) over 20 minutes. All HPLCs were carried out using a C18 Gemini column, as described in General Procedures.

^{68}Ga-DOTA-SPAYYTAD-Tfb, [^{68}Ga]1



Decay corrected yield: 98.0±0.8% (n=4)

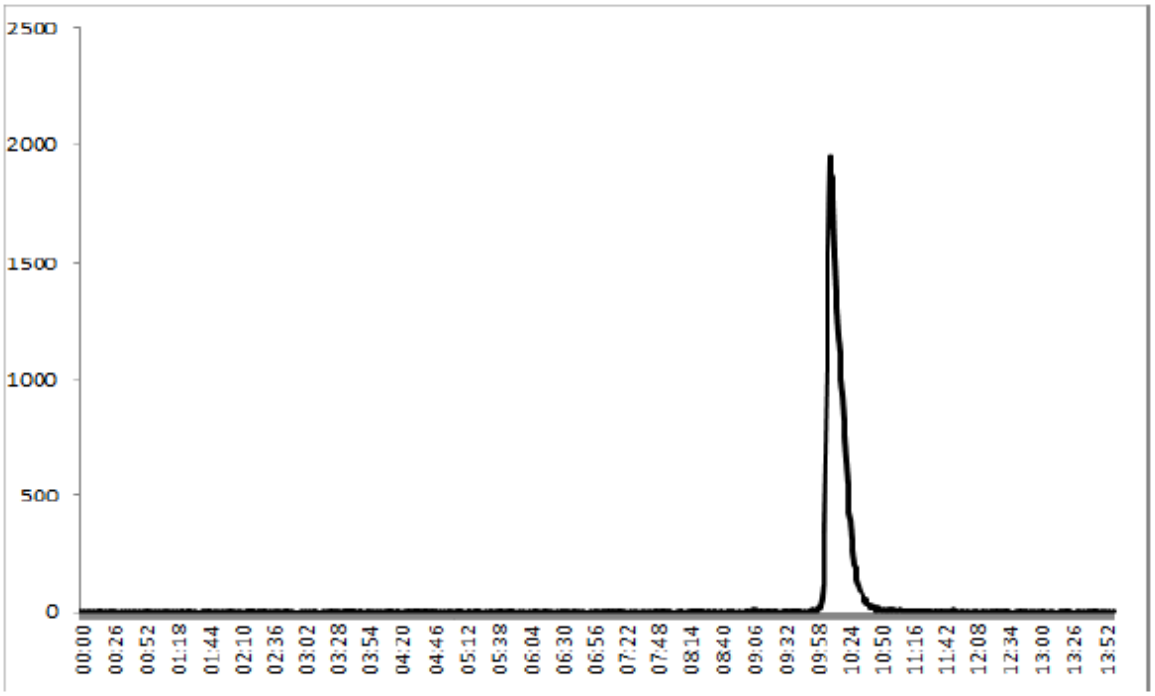
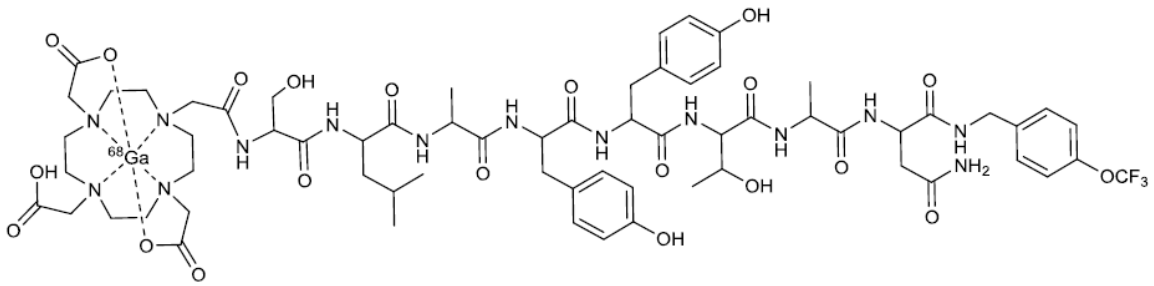


Figure 17 – Purified radioHPLC trace of **[^{68}Ga]1**

^{68}Ga-DOTA-SLAYYTAD-Tfb, [^{68}Ga]2



Decay corrected yield: 97.0±1.4% yield (n=4)

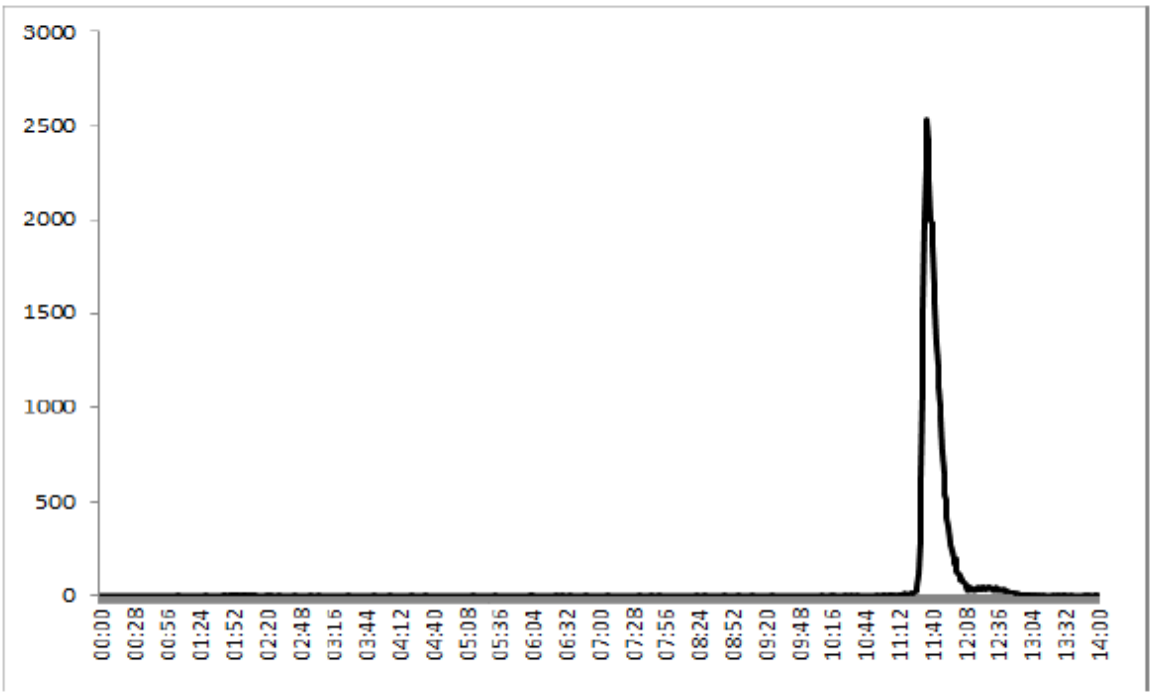
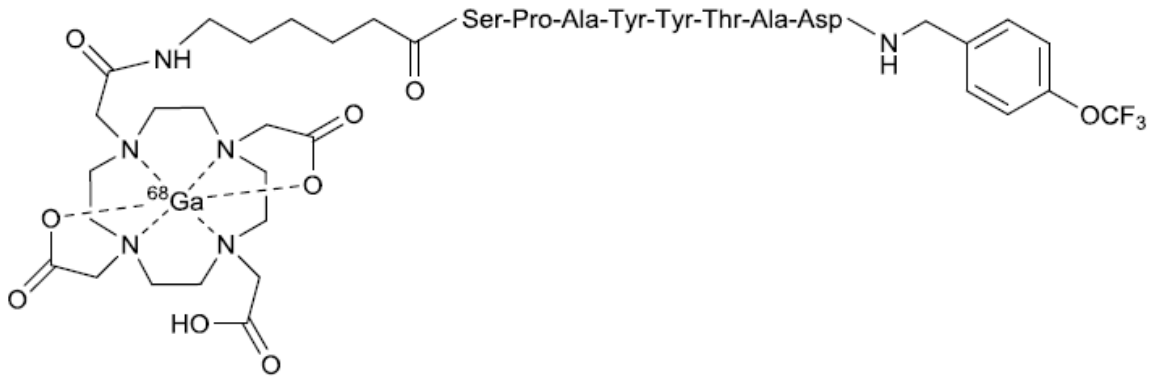


Figure 18 – Purified radioHPLC trace of **[^{68}Ga]2**

^{68}Ga-DOTA-Ahx-SPAYYTAD-Tfb, [^{68}Ga]3



Decay corrected yield: 95.7±2.5% (n=3)

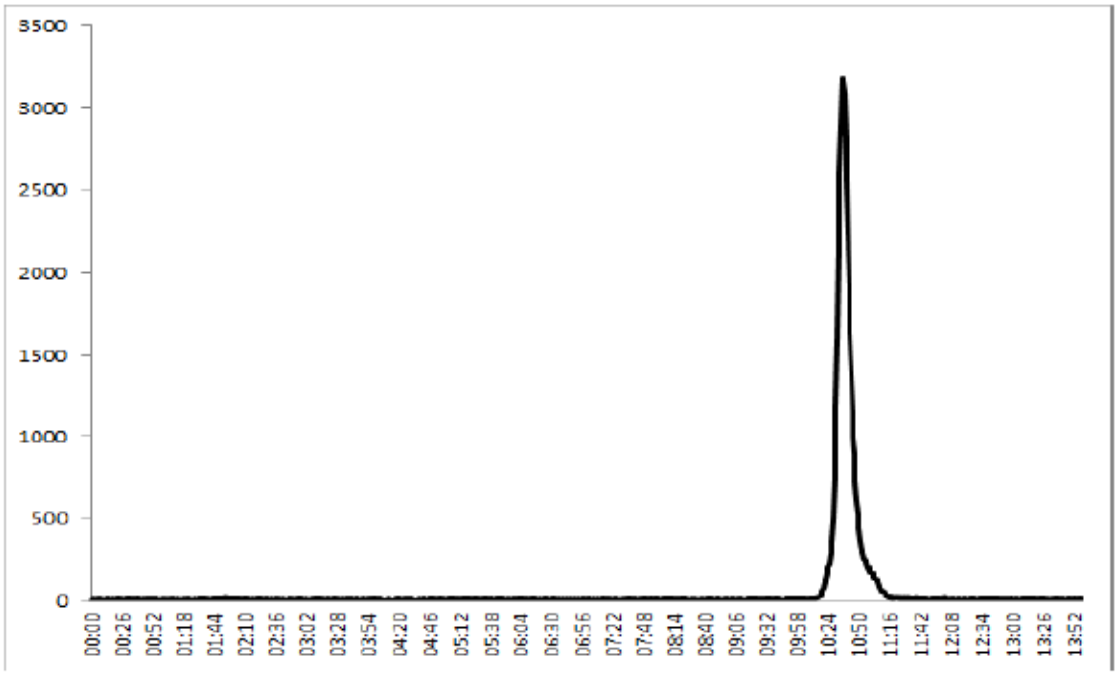
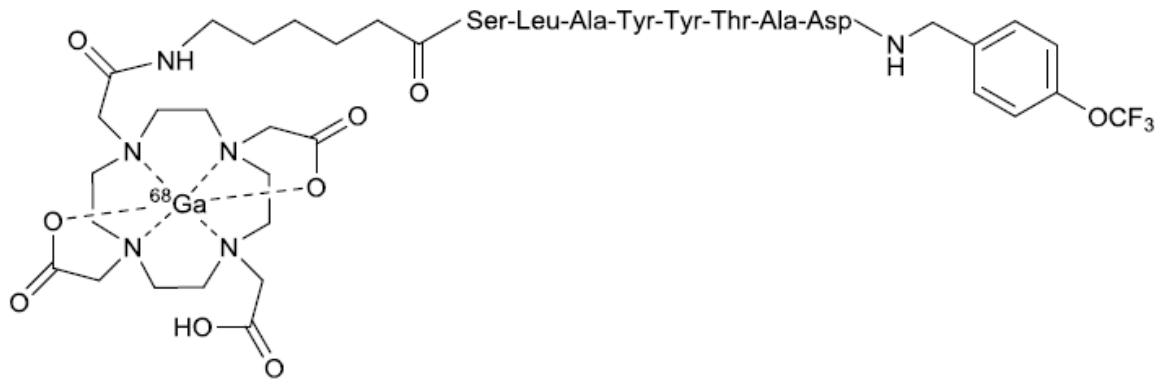


Figure 19 – Purified radioHPLC trace of **[^{68}Ga]3**

^{68}Ga-DOTA-Ahx-SLAYYTAD-Tfb, [^{68}Ga]4



Decay corrected yield: 97.3±0.5% (n=3)

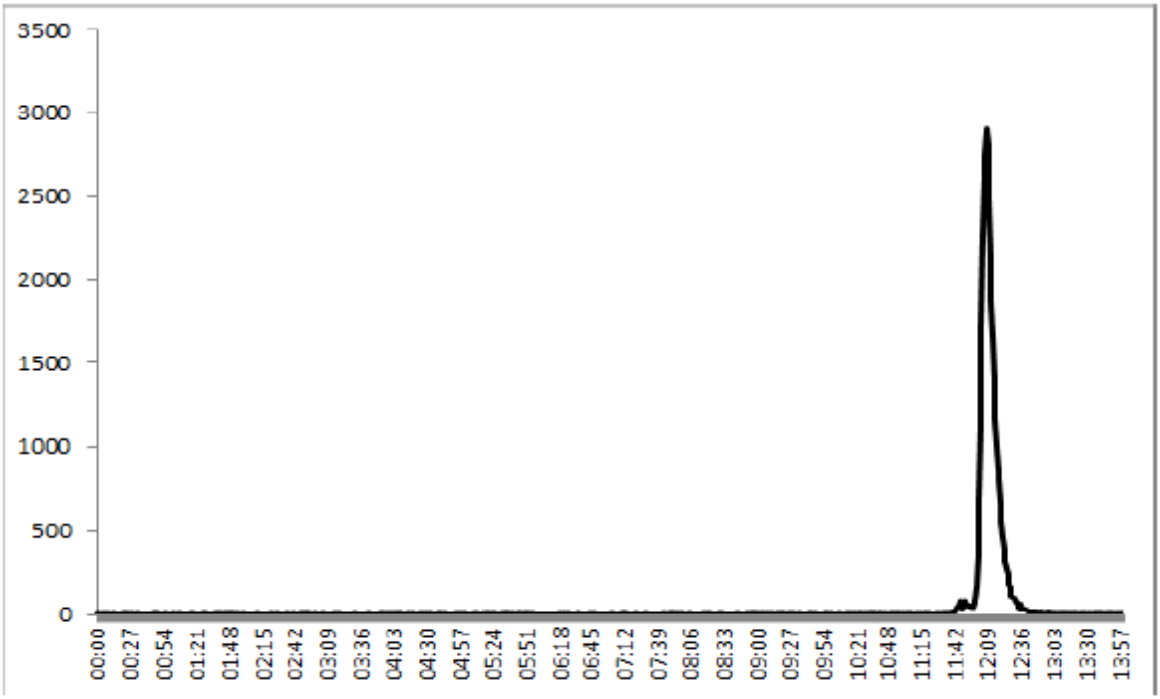
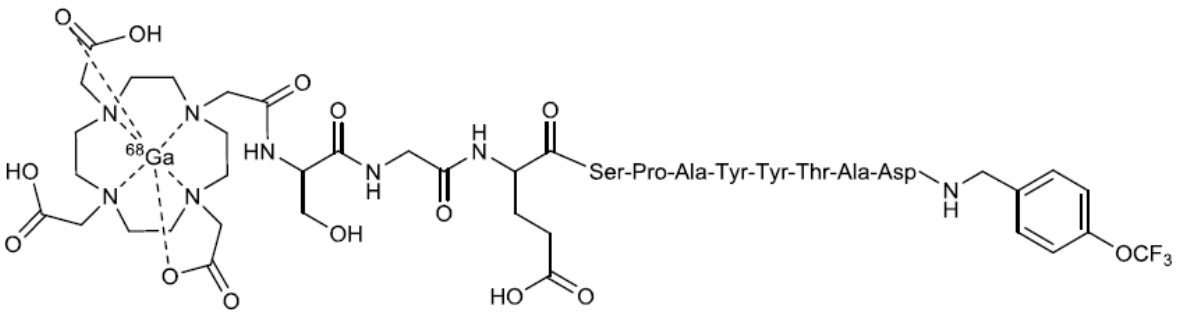


Figure 20 – Purified radioHPLC trace of **[^{68}Ga]4**

^{68}Ga-DOTA-SGESPAYYTAD-Tfb, [^{68}Ga]5



Decay corrected yield: 95.0±4.6% (n=3)

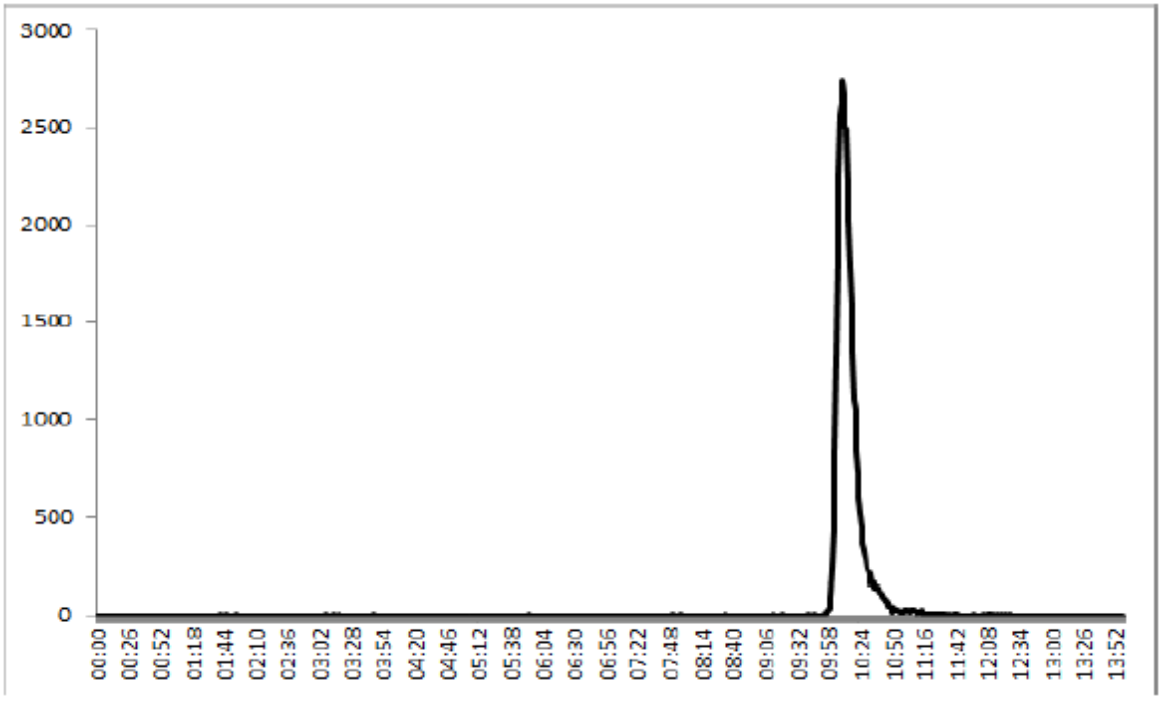
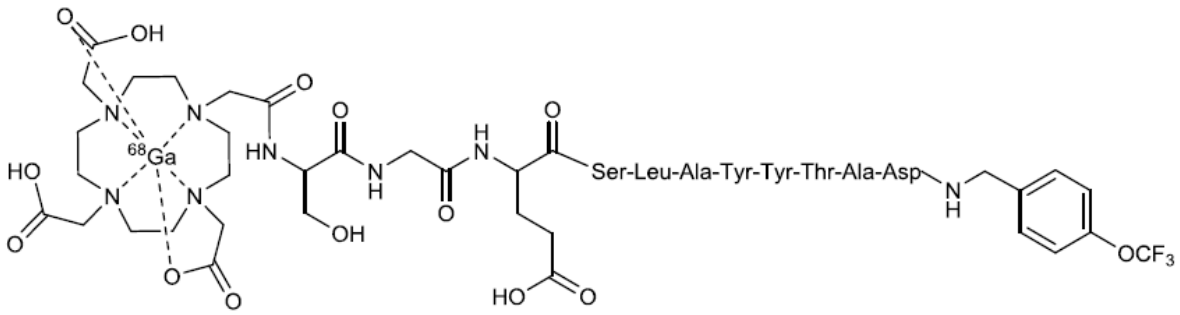


Figure 21 – Purified radioHPLC trace of **[^{68}Ga]5**

^{68}Ga-DOTA-SGESLAYYTAD-Tfb, [^{68}Ga]6



Decay corrected yield: 96.0±3.0% (n=3)

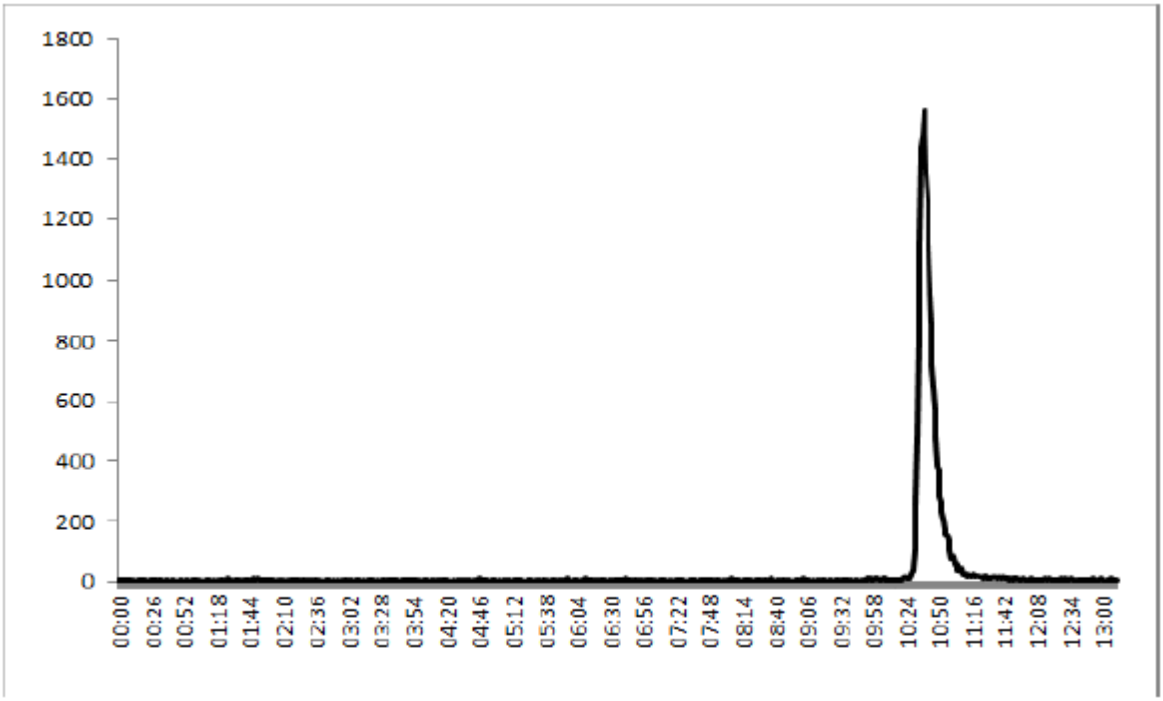
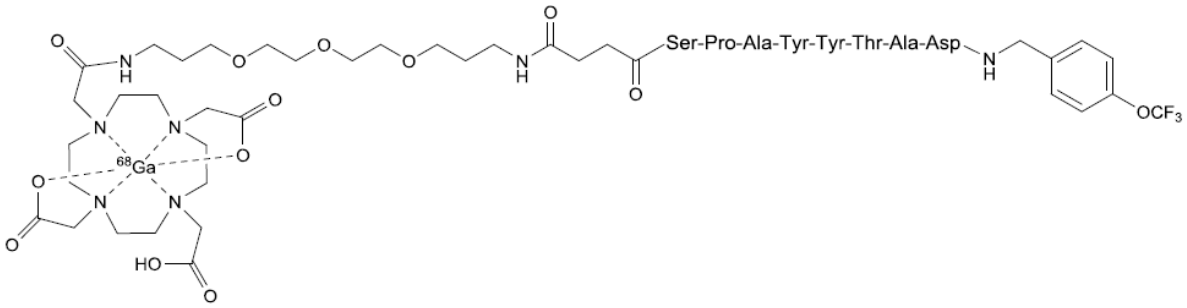


Figure 22 – Purified radioHPLC trace of **[^{68}Ga]6**

^{68}Ga-DOTA-PEG$_3$-SPAYYTAD-Tfb, [^{68}Ga]7



Decay corrected yield: 95.3±3.5% (n=3)

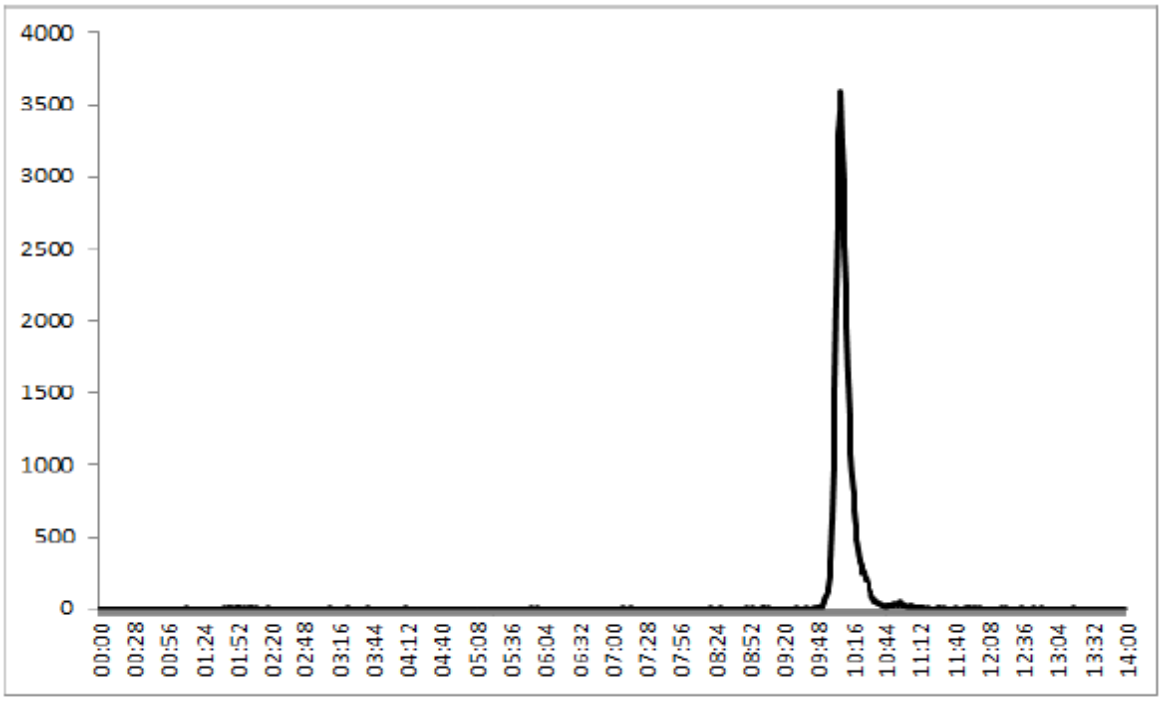
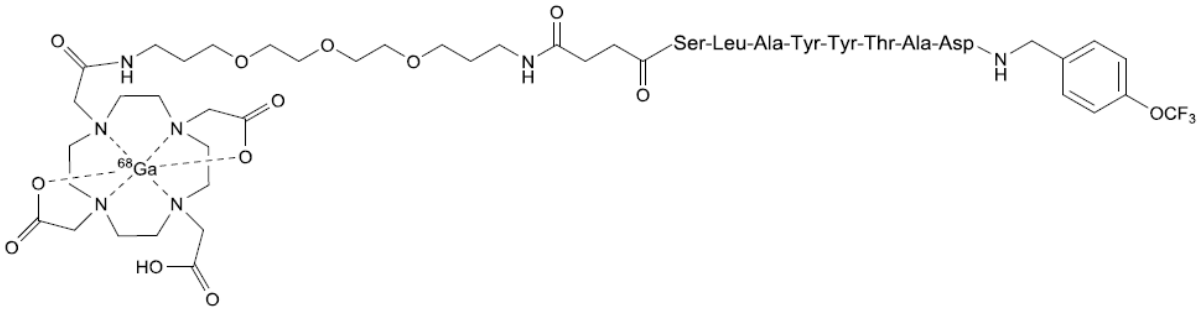


Figure 23 – Purified radioHPLC trace of **[^{68}Ga]7**

^{68}Ga-DOTA-PEG$_3$-SLAYYTAD-Tfb, [^{68}Ga]8



Decay corrected yield: 92.0±2.0% (n=3)

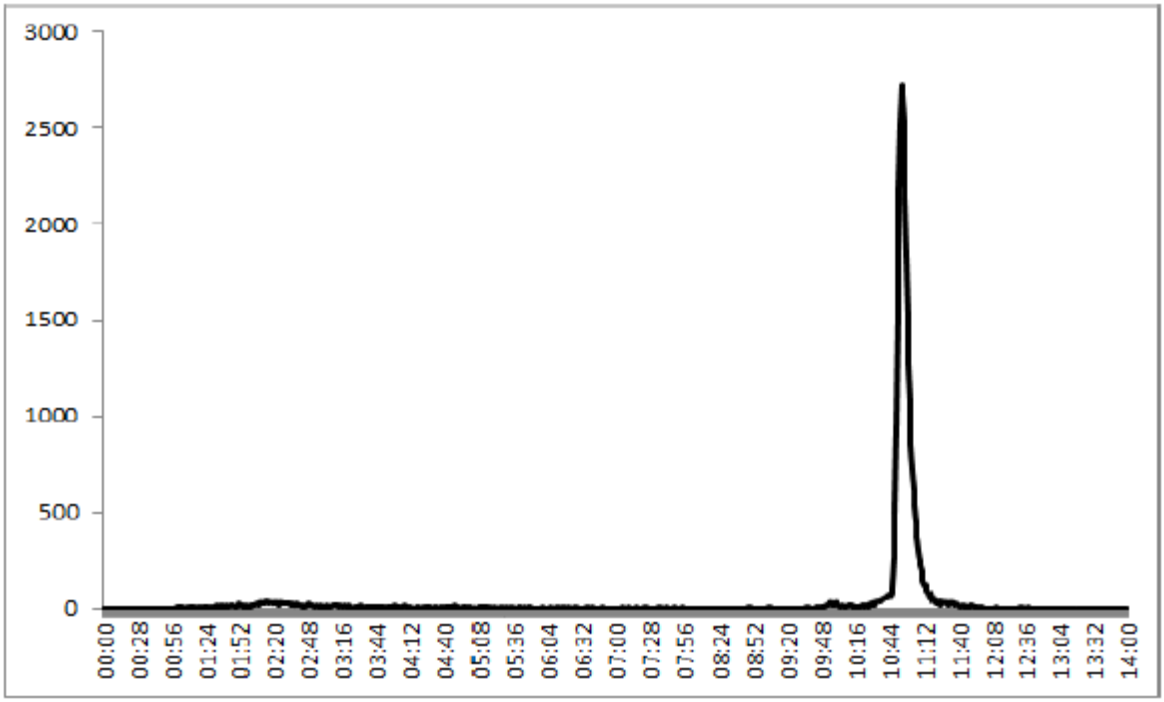


Figure 24 – Purified radioHPLC trace of **[^{68}Ga]8**

Table 2 – Summary of radiochemical yields of synthesised peptides **1-8**

Peptide	Radiochemical Yield (d.c.)
[^{68}Ga]DOTA-SPAYYTAD-Tfb **[^{68}Ga]1**	98%
[^{68}Ga]DOTA-SLAYYTAD-Tfb **[^{68}Ga]2**	97%
[^{68}Ga]DOTA-Ahx-SPAYYTAD-Tfb **[^{68}Ga]3**	96%
[^{68}Ga]DOTA-Ahx-SLAYYTAD-Tfb **[^{68}Ga]4**	97%
[^{68}Ga]DOTA-SGE-SPAYYTAD-Tfb **[^{68}Ga]5**	95%
[^{68}Ga]DOTA-SGE-SLAYYTAD-Tfb **[^{68}Ga]6**	96%
[^{68}Ga]DOTA-PEG_3-SPAYYTAD-Tfb **[^{68}Ga]7**	95%
[^{68}Ga]DOTA-PEG_3-SLAYYTAD-Tfb **[^{68}Ga]8**	92%

5. Enzymatic Assays

MMP assays ^{68}Ga labelling- radioHPLC analysis

Assay buffer: 50 mM Tricine, 10 mM $CaCl_2$, 150 mM NaCl, 0.5% Brij-35; pH 7.5
Radiolabelled peptides: Synthesised and purified as described above into approximately 200 µl of ethanol. This would be diluted by 300 µl of assay buffer.

Method A: MMP-2 activation: MMP-2 dissolved to 694 nM in assay buffer and 2.5 mM APMA (final concentration) was added (from stock solution 100 mM in DMSO) and incubation at 37°C took place for 2 hours. Reaction was carried out in Wheaton vial, 100 µl of MMP-2 (2.8nM final

concentration) and 100 µL radiolabelled peptide compound added (15-40 µci), incubation at 37°C for time points 0, 30, 60 and 90 minutes. After incubation peptide was injected into radio-HPLC to monitor formation of new radioactive compounds.

Method B (standard protocol following optimisation): MMP-2 activation was carried out as described previously; enzyme was diluted to 44 nM. Reaction was carried out in Wheaton vial, 100 µl of MMP-2 (22 nM final concentration) and 100 µL radiolabelled peptide compound added (15-40 µci), incubation at 37°C for time points 0, 30, 60, 90 and 120 minutes. After incubation peptide was injected into radio-HPLC, monitoring formation of new radioactive compounds. Simultaneously stability studies were carried out on all peptides, incubating radiolabelled peptide (15-40 µci) in assay buffer without enzyme for 2.5 hours at 37 °C followed by incubation into radio-HPLC.

MMP-9 and MMP-14 assays were also carried out using Method B. Enzyme activation was carried out as follows. MMP-9 was activated using the aforementioned method for MMP-2 but with a longer incubation time of 24 hours. MMP 14 assays required the addition of ZnCl 5 µM to the assay buffer solution. MMP-14 activation was carried out with MMP-14 dissolved in assay buffer to 166.7 nM (final concentration) and 3.9 nM (final concentration) of trypsin was added, incubation took place at 37 °C for 1 hour. After this time AEBSF 1 mM (final concentration) was added to the mixture and it was left at room temperature for 15 minutes. This could then be diluted to 22 nM ready for assay. MMP-2 activity was confirmed by incubation with control peptide as described above (fluorescent compound Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 purchased from R&D systems, incubation for 1 hour resulted in a 4 fold increase in fluorescence).

Example radioHPLC traces of MMP-2 assay:

^{68}Ga-DOTA- PLGL-Dpa-ARD-Tfb, [^{68}Ga]9

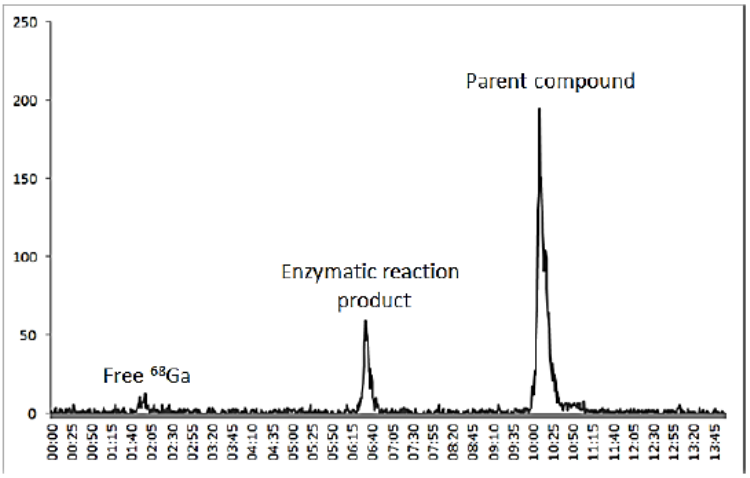


Figure 25 - Radio HPLC trace of ^{68}Ga-DOTA-PLGL-Dnp-ARD-Tfb **[^{68}Ga]9** after 120 minutes incubation with MMP-2

^{19}F NMR enzymatic assays

Assay protocol: MMP-2 activation was carried out as described previously. Enzymatic reaction was carried out by incubating 100 µl of MMP-2 and 100 µl of peptide at 37 °C for 0, 5, 30, 60, 120 minutes and 24 hours, after which time enzymatic reaction was stopped with 200 µl of a phenanthroline solution (20 mM in buffer and D_2O 2:1). Reaction mixture was then submitted for ^{19}F NMR with the peptide compound now at a 50 µM concentration. ^{19}F peak was seen to get sharper over the enzymatic reaction.

For Gd-DOTA-SPAYYTAD-Tfb (**[Gd]1**), concentrations used were as follows: peptide 100 µM and enzyme 45 nM with a final peptide concentration of 50 µM for NMR, peptide 500 µM and enzyme 90 nm with a final peptide concentration of 250 µM for NMR, peptide 500 µM and enzyme 500 nM with a final peptide concentration of 250 µM for NMR.

For Gd-DOTA-SLAYYTAD-Tfb (**[Gd]2**), concentrations used were as follows: peptide 500 µM and enzyme 90 nM with a final peptide concentration of 250 µM for NMR.

6. References

1. S. Mizukami, R. Takikawa, F. Sugihara, Y. Hori, H. Tochio, M. Walchli, M. Shirakawa and K. Kikuchi, *J. Am. Chem. Soc.*, **2007**, *130*, 794-795.