Supporting Information

Gemma E. Douglas,[a, b] Madhusudan Vyas,[c, d] Iman Kavianinia* [a, b, e]

[a] School of Biological Sciences, The University of Auckland, 3A Symonds Sreet, Auckland 1010, New Zealand; E-mail: i.kavianinia@auckland.ac.nz

[b] School of Chemical Sciences, The University of Auckland, 23 Symonds Street, Auckland 1010, New Zealand

[c] Allevia Radiology, Auckland, New Zealand

[d] Unitec Institute of Technology, Auckland, New Zealand

[e] Maurice Wilkinson Centre for Molecular Biodiscovery, The University of Auckland, 3 Symonds Street, Auckland 1010, New Zealand

Contents

1. General

1.1 Reagents and Materials

All solvents and reagents were purchased and used as reagent grade, without further purification. Solvents were purchased as technical grade while solvents used for RP HPLC were purchased as HPLC grade. *N,N'*-Dimethylformamide (DMF), dichloromethane (CH_2Cl_2), diethyl ether (Et_2O), methanol (MeOH), piperidine, formic acid, and ethanol (EtOH) were purchased from ECP Limited (Auckland, NZ). Acetonitrile (MeCN) was purchased as HPLC grade from ECP Limited (Auckland, NZ). Fmoc-L-amino acids were purchased from GL Biochem (Shanghai, China) unless specified. *O*-(7-aza-1*H*-benzotriazol-1-yl)-*N,N,N'N'*-trimethyluronium hexafluorophosphate (HATU) was purchased from GL Biochem (Shanghai, China). Fmoc-transexamic acid, Fmoc-L-Nal-OH, bis(nitrophenyl) carbonate, H-L-Asp(OAll)-OH, 4-(4-hydroxymethyl-3-methoxyphenoxy)butanoic acid (HMPB), *N,N'*-carbonyldiimidazole (CDI), *N,N'*-disuccinimidyl carbonate (DSC), and triisopropyl silane (TIPS) were purchased from AK Scientific (Union City, CA, USA). 4-Nitrophenyl chloroformate and *N,N'*-diisopropylethylamine (DIPEA) were purchased from Sigma Aldrich (Darmstadt, Germany). H-L-Lys(Fmoc)-OtBu•HCl, Fmoc-D-Lys-OtBu•HCl, 2-(1,4,7,10-tetraazacyclododecane-4,7,10-tris(t-butyl acetate))-pentanedioic acid-1t-butyl ester (DOTAGA), 2-(4,7,10-tris(2-(tert-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)acetic acid (DOTA), Fmoc-D-Tyr(3-I)-OH, and disuccinimidyl suberate were purchased from AMBEED (Buffalo Grove, IL, USA). 2-Chlorotrityl chloride resin was purchased from ChemPep (Miami, FL, USA). *N,N'*-Dimethylpyridin-4-amine (DMAP) was purchased from Aappatec (Louisville, KN, USA). Trifluoroacetic acid (TFA) was purchased from Oakwood Chemicals (Estill, SC, USA). *N,N'*-Diisopropylcarbodiimide (DIC) was purchased from Sigma Aldrich (Burlington, MA, USA). Fmoc-D-Phe-OH was purchased from Auspep (Parkville, VIC, Australia). Fmoc-Dbz-OH was purchased from Combi-Blocks (San Diego, CA, USA). TentaGel® was purchased from RAPP Polymere (Türbingen, Germany). Milli-Q high purity deionised water (MQ H_2O) was used from Satorius Arium® Pro Ultrapure Water System from Sartorius Stedim Biotech (Göttingen, Germany).

1.2 Tandem Mass Spectrometry (LCMS)

Analytical tandem liquid chromatography and mass spectrometry (LCMS) was performed on an Agilent Technologies (Santa Clara, CA, USA) 1260 Infinity LC connected to an Agilent Technologies 6120 quadrupole MSD spectrophotometer. Data analysis was done with OpenLAB software. An Agilent Zorbax 300SB-C3, 3.5 µm, 3.0 × 150 mm column was used with a linear gradient (5-95% B at a flow rate of 0.3 mL per min) where solvent A was MQ H_2O containing 0.1% formic acid and solvent B was MeCN with 0.1% formic acid.

1.3 High Performance Liquid Chromatography (HPLC)

Analytical reverse phase high performance liquid chromatography (RP HPLC) was performed on a Waters (Waltham, MA, USA) Alliance analytical HPLC equipped with a Phenomenex (Torrence, CA, USA) Luna® 5 µm, C18(2), 100 Å, 250 × 4.6 mm column. A linear gradient was used (5-95% B at a flow rate of 0.3 mL per min) where solvent A was MQ H_2O containing 0.1% TFA and solvent B was MeCN with 0.1% TFA.

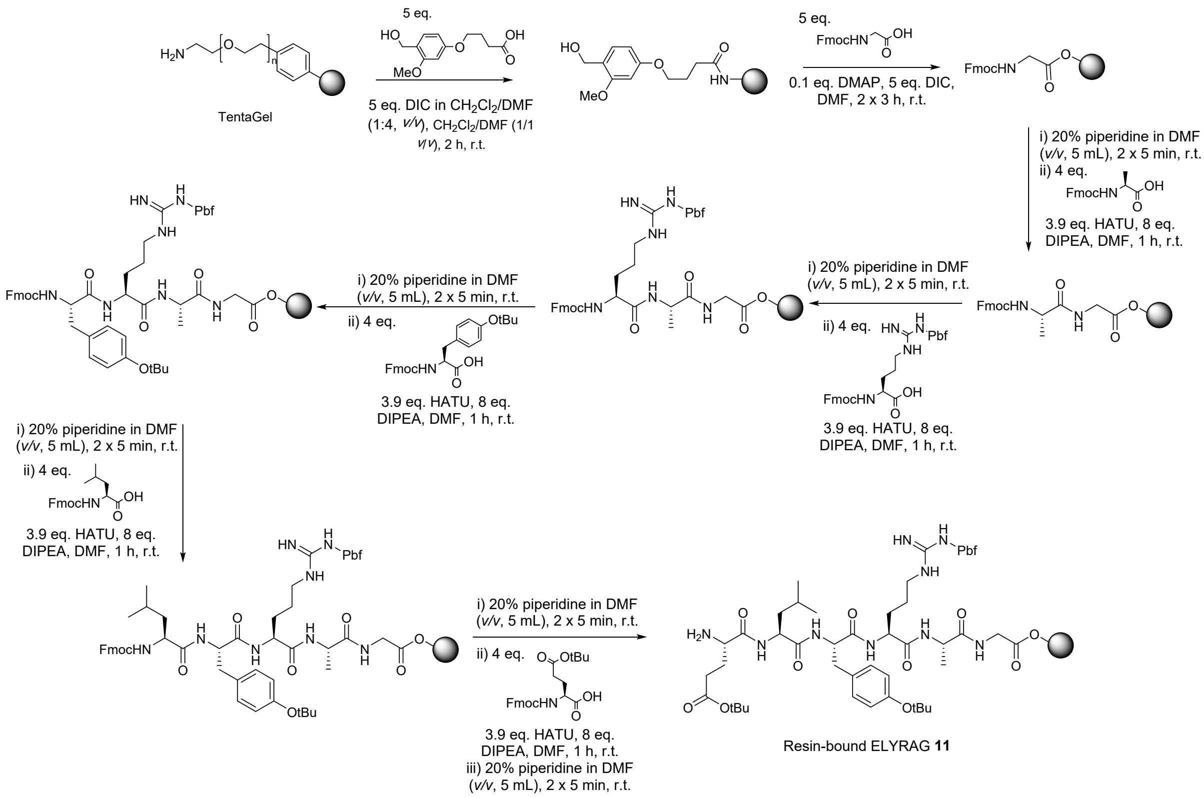
To purify, semi-preparative RP HPLC was performed on a Thermo Fischer Scientific (Waltham, MA, USA) Dionex Ultimate™ 3000 HPLC, equipped with a four channel UV detector at 210, 225, 254, and 280 nm using an Agilent ZORBAX 300SB-C18, 5 µm, 9.4 × 250 mm column. A linear gradient was used (5-95% B at a flow rate of 4 mL per min) where solvent A was MQ H_2O with 0.1% TFA and solvent B was MeCN with 0.1% TFA.

1.4 High Resolution Mass Spectrometry (HRMS)

High resolution mass spectrometry electron ionisation (HRMS EI) was performed on a Thermo Ficher Scientific (Waltham, MA, USA) Orbitrap Exploris 120 by direct infusion method in the positive mode.

2. Experimental Procedures

2.1 Synthesis of ELYRAG



Scheme S1 Synthesis of resin-bound ELYRAG **11**.

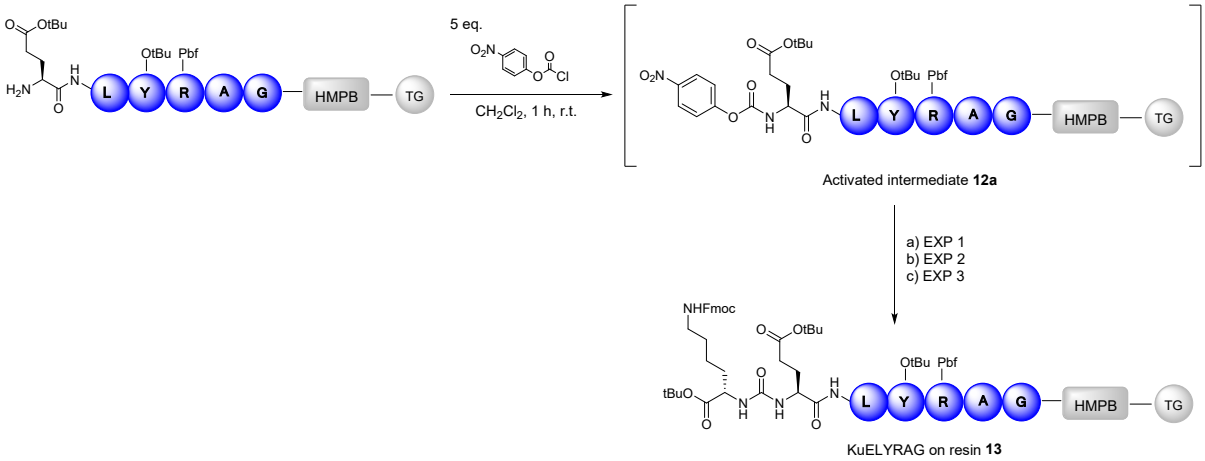
To TentaGel® (1 eq., 0.1 mmol, 385 mg, 0.26 mmol/g), preswollen in CH_2Cl_2/DMF (1:1, *v/v*, 5 mL, 30 min), HMPB linker (5 eq., 0.5 mmol, 120 mg) and DIC (5 eq., 0.5 mmol, 63 mg, 77 µL) dissolved in CH_2Cl_2/DMF (1:4, *v/v*, 5 mL) was added. The mixture was gently agitated at r.t. for 2 h. Reaction completion was monitored with a Kaiser test and, if positive, the procedure was repeated with freshly prepared reagents. After completion, the resin was drained and washed with DMF (3 x 5 mL).

To the HMPB-loaded resin, preswollen in DMF (5 mL, 30 min), a solution of Fmoc-Gly-OH (5 eq., 0.5 mmol, 213 mg), DMAP (0.1 eq., 0.01 mmol, 1 mg), and DIC (5 eq., 0.5 mmol, 63 mg, 77 µL) in DMF (5 mL) was added. The reaction was gently agitated at r.t. for 3 h. Then the resin was filtered and washed with DMF (3 x 5 mL) and the procedure was repeated with freshly prepared reagents and gently agitated for another 3 h. After this, the resin was filtered then washed with DMF (3 x 5 mL).

The Fmoc was removed from the glycine-immobilised resin by treating the resin with 20% piperidine in DMF (*v/v*, 5 mL, 2 x 5 min, r.t.). To the resin, preswollen in DMF (5 mL, 30 min), a solution of Fmoc-L-Ala-OH (4 eq., 0.4 mmol, 124.5 mg), HATU (3.9 eq., 0.39 mmol, 148.3 mg), and DIPEA (8 eq., 0.8 mmol, 103.4 mg, 140 µL) in DMF (5 mL) was added and gently agitated at r.t. for 1 h. The resin was filtered then washed with DMF (3 x 5 mL). These Fmoc removal and coupling steps were repeated for Fmoc-L-Arg(Pbf)-OH (4 eq., 0.4 mmol, 259.5 mg), Fmoc-L-Tyr(OtBu)-OH (4 eq., 0.4 mmol, 161.4 mg), Fmoc-L-Leu-OH (4 eq., 0.4 mmol, 141.4 mg), and Fmoc-L-Glu(OtBu)-OH (4 eq., 0.4 mmol, 170.2 mg). Fmoc was removed from ELYRAG-immobilised resin by treatment with 20% piperidine in DMF (*v/v*, 5 mL, 2 x 5 min, r.t.).

2.2 KuE Synthesis Optimisation - Comparative Study

2.2.1 EXP 1-3



Scheme S2 a) 4 eq. H-L-Lys(Fmoc)-OtBu, DMF, 1 h, r.t.; b) 4 eq. H-L-Lys(Fmoc)-OtBu, 4 eq. DIPEA, DMF, 1 h, r.t.; c) 4 eq. H-L-Lys(Fmoc)-OtBu, 4 eq. DIPEA, DMF, 4 h, r.t.

ELYRAG-immobilised resin was preswollen in DMF (5 mL, 30 min). A solution of 4-nitrophenyl chloroformate (5 eq., 0.5 mmol, 100.8 mg) in CH_2Cl_2 (5 mL) was added to the resin and gently agitated at r.t. for 1 h. The resin was filtered and excess reagent was washed away with CH_2Cl_2 (1 x 5 mL) then DMF (2 x 5 mL).

EXP 1

A solution of H-L-Lys(Fmoc)-OtBu•HCl (4 eq., 0.4 mmol, 184.4 mg) in DMF (5 mL) was added to the resin and gently agitated at r.t. for 1 h.

EXP 2

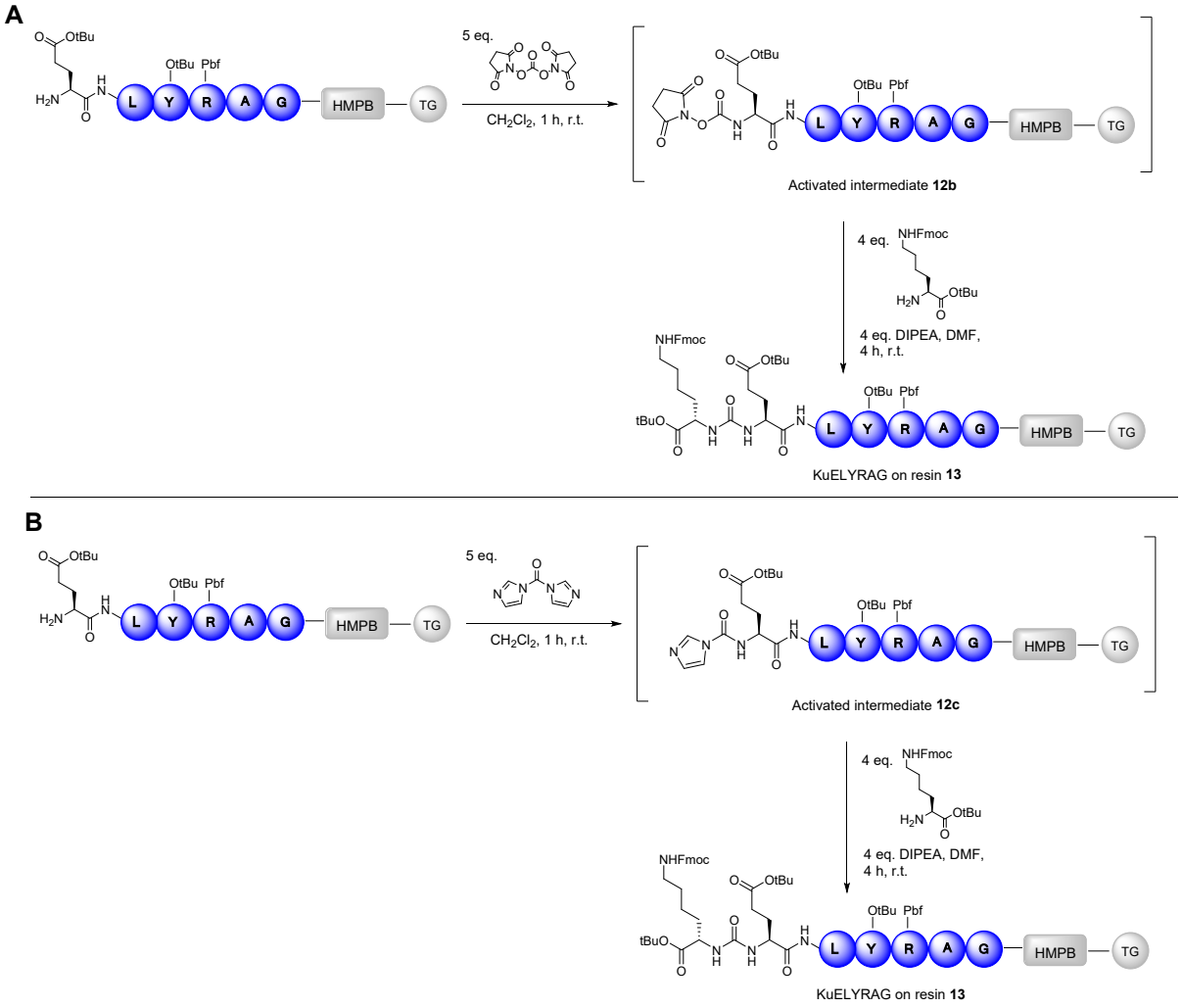
A solution of H-L-Lys(Fmoc)-OH•HCl (4 eq., 0.4 mmol, 184.4 mg) and DIPEA (4 eq., 0.4 mmol, 51.7 mg, 70 µL) in DMF (5 mL) was added to the resin and gently agitated at r.t. for 1 h.

EXP 3

A solution of H-L-Lys(Fmoc)-OtBu•HCl (4 eq., 0.4 mmol, 184.4 mg) in DMF (5 mL) was added to the resin and gently agitated at r.t. for 4 h.

The resin was washed with DMF (2 x 5 mL) then CH_2Cl_2 (1 x 5 mL) and air dried. Some resin beads from each experiment were retrieved and treated with a solution of TFA/triisopropylsilane/water (95:2.5:2.5, *v/v/v*, 0.5 mL, r.t. 1 h) to cleave some product from the resin for analysis.

2.2.2 EXP 4 & 5



Scheme S3 A) EXP 4 using DSC; B) EXP 5 using CDI.

ELYRAG-immobilised resin was preswollen in DMF (5 mL, 30 min).

EXP 4

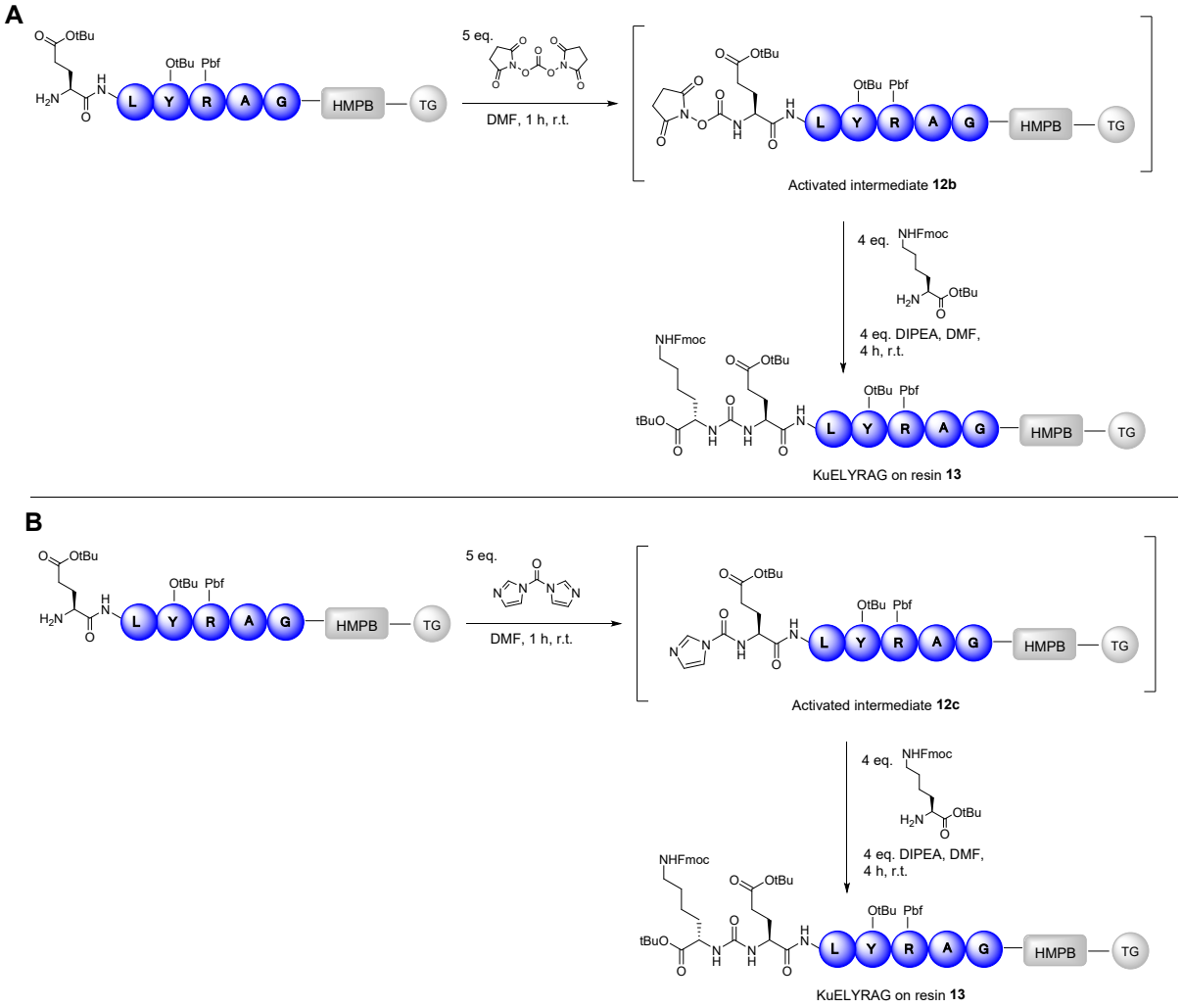
A solution of DSC (5 eq., 0.5 mmol, 128.1 mg) in CH_2Cl_2 (1 mL) was added to the resin and gently agitated at r.t. for 1 h.

EXP 5

A solution of CDI (5 eq., 0.5 mmol, 81.1 mg) in CH_2Cl_2 (1 mL) was added to the resin and gently agitated at r.t. for 1 h.

The resin was filtered and excess reagent was washed away with CH_2Cl_2 (1 x 5 mL) then DMF (2 x 5 mL). A solution of H-L-Lys(Fmoc)-OtBu•HCl (4 eq., 0.4 mmol, 184.4 mg) in DMF (5 mL) was added to the resin and gently agitated at r.t. for 4 h. Some resin beads from each experiment were retrieved and treated with a solution of TFA/triisopropylsilane/water (95:2.5:2.5, *v/v/v*, 0.5 mL, r.t., 1 h) to cleave some product from the resin for analysis.

2.2.3 EXP 6 & 7



Scheme S4 A) EXP 6 using DSC in DMF; B) EXP 7 using CDI in DMF.

ELYRAG-immobilised resin was preswollen in DMF (5 mL, 30 min).

EXP 6

A solution of DSC (5 eq., 0.5 mmol, 128.1 mg) in DMF (1 mL) was added to the resin and gently agitated at r.t. for 1 h.

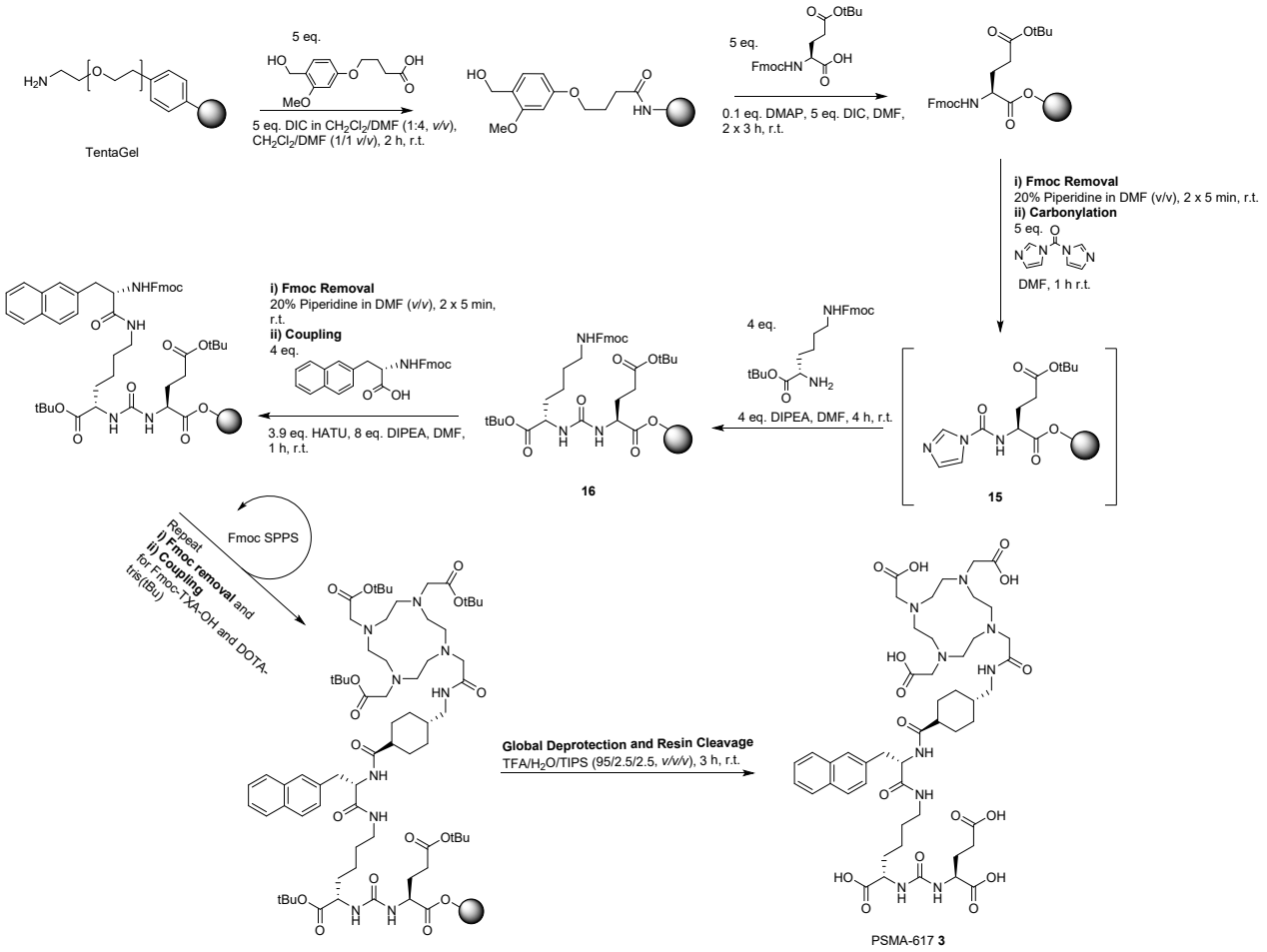
EXP 7

A solution of CDI (5 eq., 0.5 mmol, 81.1 mg) in DMF (1 mL) was added to the resin and gently agitated at r.t. for 1 h.

The resin was filtered and excess reagent was washed away with DMF (3 x 5 mL). A solution of H-L-Lys(Fmoc)-OtBu•HCl (4 eq., 0.4 mmol, 184.4 mg) in DMF (5 mL) was added to the resin and gently agitated at r.t. for 4 h. Some resin beads from each experiment were retrieved

and treated with a solution of TFA/triisopropylsilane/water (95:2.5:2.5, *v/v/v*, 0.5 mL, r.t., 1 h) to cleave some product from the resin for analysis.

2.3 Synthesis of PSMA-617 on TentaGel® Resin



Scheme S5 Synthesis of PSMA-617 **3** on TentaGel®.

To TentaGel® (1 eq., 0.1 mmol, 385 mg, 0.26 mmol/g), preswollen in CH_2Cl_2/DMF (1:1, *v/v*, 5 mL, 30 min), HMPB linker (5 eq., 0.5 mmol, 120 mg) and DIC (5 eq., 0.5 mmol, 63 mg, 77 µL) dissolved in CH_2Cl_2/DMF (1:4, *v/v*, 5 mL) was added. The mixture was gently agitated at r.t. for 2 h. Reaction completion was monitored with a Kaiser test and, if positive, the procedure was repeated with freshly prepared reagents. After completion, the resin was drained and washed with DMF (3 x 5 mL).

To the HMPB-loaded resin, preswollen in DMF (5 mL, 30 min), a solution of Fmoc-L-Glu(OtBu)-OH (5 eq., 0.5 mmol, 213 mg), DMAP (0.1 eq., 0.01 mmol, 1 mg), and DIC (5 eq.,

0.5 mmol, 63 mg, 77 µL) in DMF (5 mL) was added. The reaction was gently agitated at r.t. for 3 h. Then the resin was filtered and washed with DMF (3 x 5 mL) and the procedure was repeated with freshly prepared reagents and gently agitated for another 3 h. After this, the resin was filtered then washed with DMF (3 x 5 mL).

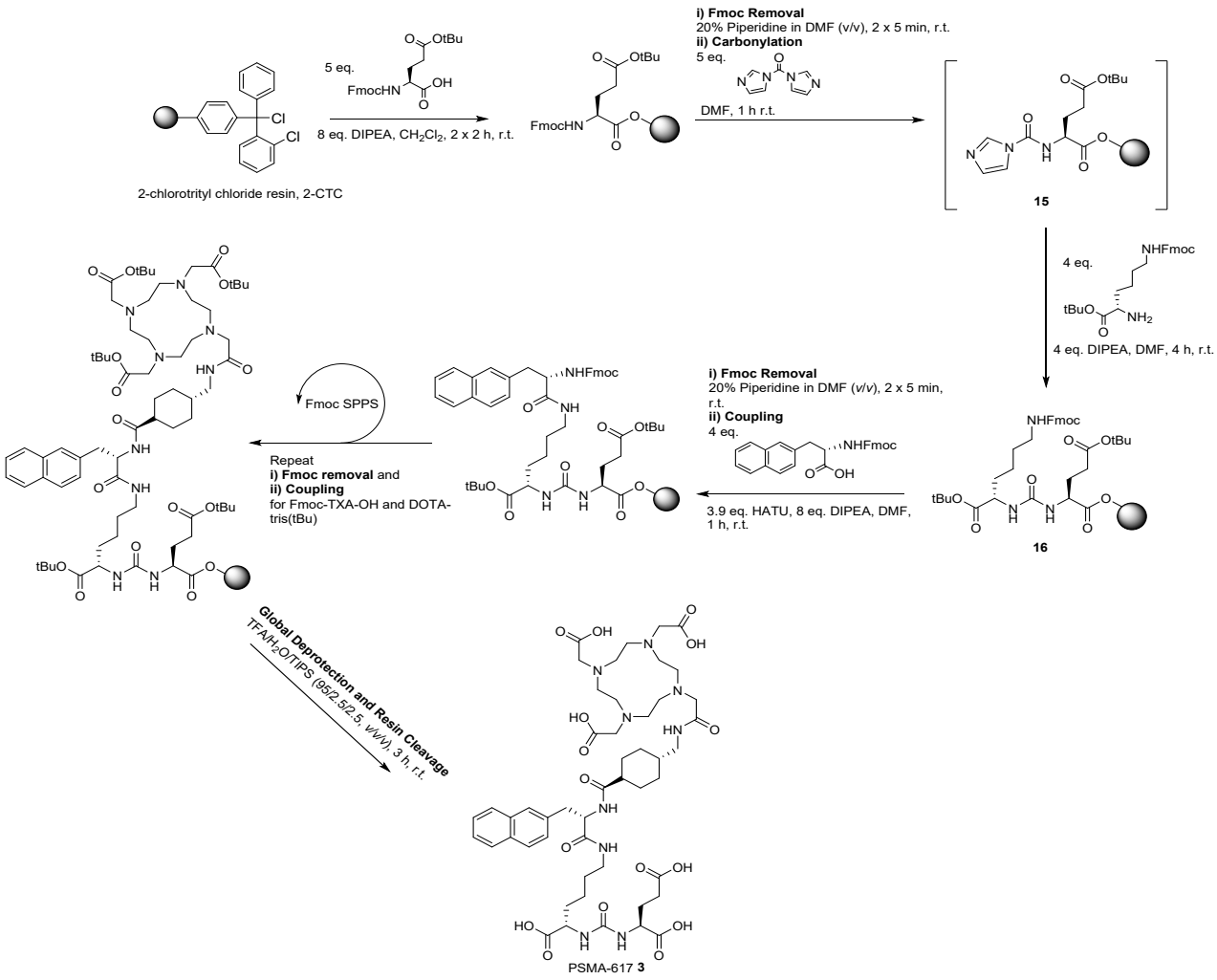
The glutamate-immobilised resin was preswollen in DMF (5 mL, 30 min) and the Fmoc was removed by treating the resin with 20% piperidine in DMF (*v/v*, 5 mL, 2 x 5 min, r.t.). CDI (5 eq., 0.5 mmol, 81 mg) was dissolved in DMF (5 mL) and added to the resin. The mixture was gently agitated at r.t. for 1 h, then the resin was drained and washed with DMF (3 x 5 mL). To the resin, H-L-Lys(Fmoc)-OtBu•HCl (4 eq., 0.4 mmol, 184.4 mg) and DIPEA (4 eq., 0.4 mmol, 50 mg, 68 µL) in DMF (5 mL) was added. The mixture was gently agitated at r.t. for 4 h, then the resin was drained and washed with DMF (3 x 5 mL).

Fmoc was removed from the KuE-immobilised resin by treatment with 20% piperidine in DMF (*v/v*, 5 mL, 2 x 5 min, r.t.). To the resin, a solution of Fmoc-L-2-Nal-OH (4 eq., 0.4 mmol, 175 mg), HATU (3.9 eq., 0.39 mmol, 148.3 mg), and DIPEA (8 eq., 0.8 mmol, 103.4 mg, 140 µL) in DMF (5 mL) was added and gently agitated at r.t. for 1 h. The resin was filtered then washed with DMF (3 x 5 mL). These Fmoc removal and coupling steps were repeated for Fmoc-transexamic acid (4 eq., 0.4 mmol, 151.8 mg) and DOTA-tris(tBu) ester (4 eq., 0.4 mmol, 229.1 mg).

Some resin beads were retrieved and treated with a solution of TFA/triisopropylsilane/water (95:2.5:2.5, *v/v/v*, 0.5 mL, 24 h) to cleave some product from the resin for analysis. After the product was confirmed, the entire resin was treated with a solution of TFA/triisopropylsilane/water (95:2.5:2.5, *v/v/v*, 5 mL, 24 h), simultaneously cleaving PSMA-617 from the resin and removing all protecting groups. Following cleavage, the sample was filtered and lyophilised affording the crude product.

A sample was purified by semi-preparative RP HPLC and lyophilised, affording an amorphous white solid in 31% purified yield (9.2 mg pure from 29.4 mg crude).

2.4 Synthesis of PSMA-617 on 2-Chlorotrityl Chloride Resin



Scheme S6 Synthesis of PSMA-617 **3** on 2-CTC resin.

To 2-CTC resin (1 eq., 0.1 mmol, 313.5 mg, 0.319 mmol/g loading), preswollen in CH_2Cl_2 (5 mL, 5 min), a solution of Fmoc-Glu(OtBu)-OH (4 eq., 0.4 mmol, 170.2 mg) and DIPEA (8 eq., 0.8 mmol, 103.4 mg, 77 µL) in CH_2Cl_2 (5 mL) was added. The mixture was gently agitated at r.t. for 2 h, then filtered and washed with CH_2Cl_2 (3 x 3 mL). This procedure was repeated with freshly prepared reagents.

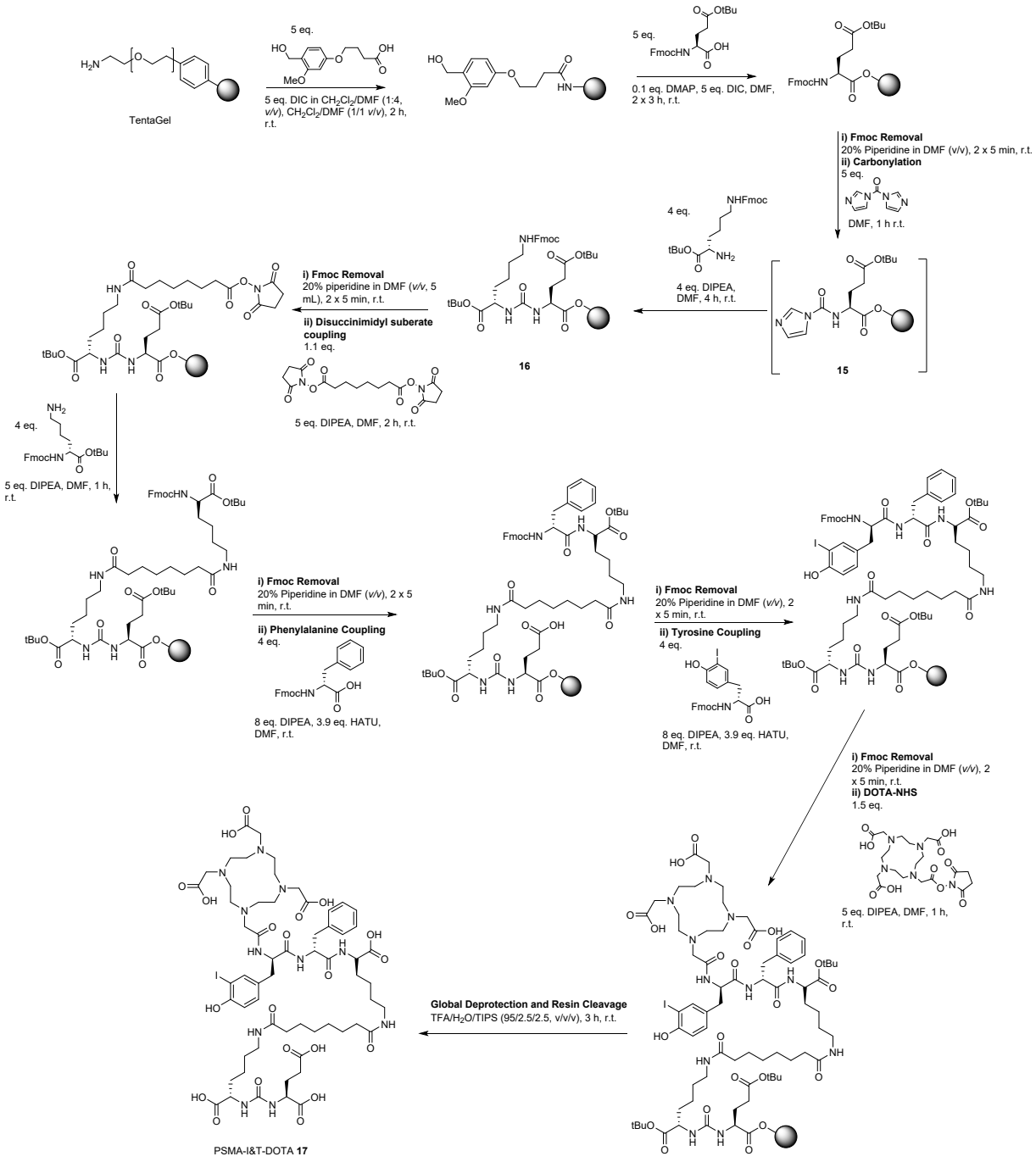
To quench any unreacted resin handles, a capping solution of MeOH/DIPEA/CH_2Cl_2 (15/5/80, *v/v/v.* 5 mL) was added to the resin. The mixture was gently agitated at r.t. for 20 min, then filtered and washed with CH_2Cl_2 (3 x 3 mL).

The remainder of the synthesis is the exact same as described in section **2.3**, from when the glutamate becomes immobilised.

Some resin beads were retrieved and treated with a solution of TFA/triisopropylsilane/water (95:2.5:2.5, *v/v/v*, 0.5 mL, 24 h) to cleave some product from the resin for analysis. After the product was confirmed, the entire resin was treated with a solution of TFA/triisopropylsilane/water (95:2.5:2.5, *v/v/v*, 5 mL, 24 h), simultaneously cleaving PSMA-617 from the resin and removing all protecting groups. Following cleavage, the sample was filtered and lyophilised affording the crude product.

A sample was purified by semi-preparative RP HPLC and lyophilised, affording an amorphous white solid in 9.8% purified yield (1.9 mg pure from 19.2 mg crude).

2.5 Synthesis of PSMA-I&T Backbone with DOTA Chelator on TentaGel®



Scheme S7 Synthesis of PSMA-I&T-DOTA on TentaGel®.

To TentaGel® (1 eq., 0.1 mmol, 385 mg, 0.26 mmol/g), preswollen in CH_2Cl_2/DMF (1:1, *v/v*, 5 mL, 30 min), HMPB linker (5 eq., 0.5 mmol, 120 mg) and DIC (5 eq., 0.5 mmol, 63 mg, 77 µL) dissolved in CH_2Cl_2/DMF (1:4, *v/v*, 5 mL) was added. The mixture was gently agitated at r.t. for 2 h. Reaction completion was monitored with a Kaiser test and, if positive, the procedure

was repeated with freshly prepared reagents. After completion, the resin was drained and washed with DMF (3 x 5 mL).

To the HMPB-loaded resin, preswollen in DMF (5 mL, 30 min), a solution of Fmoc-L-Glu(OtBu)-OH (5 eq., 0.5 mmol, 213 mg), DMAP (0.1 eq., 0.01 mmol, 1 mg), and DIC (5 eq., 0.5 mmol, 63 mg, 77 µL) in DMF (5 mL) was added. The reaction was gently agitated at r.t. for 3 h. Then the resin was filtered and washed with DMF (3 x 5 mL) and the procedure was repeated with freshly prepared reagents and gently agitated for another 3 h. After this, the resin was filtered then washed with DMF (3 x 5 mL).

The glutamate-immobilised resin was preswollen in DMF (5 mL, 30 min) and the Fmoc was removed by treating the resin with 20% piperidine in DMF (*v/v*, 5 mL, 2 x 5 min, r.t.). CDI (5 eq., 0.5 mmol, 81 mg) was dissolved in DMF (5 mL) and added to the resin. The mixture was gently agitated at r.t. for 1 h, then the resin was drained and washed with DMF (3 x 5 mL). To the resin, H-L-Lys(Fmoc)-OtBu•HCl (4 eq., 0.4 mmol, 184.4 mg) and DIPEA (4 eq., 0.4 mmol, 50 mg, 68 µL) in DMF (5 mL) was added. The mixture was gently agitated at r.t. for 4 h, then the resin was drained and washed with DMF (3 x 5 mL).

Fmoc was removed from the KuE-immobilised resin by treatment with 20% piperidine in DMF (*v/v*, 5 mL, 2 x 5 min, r.t.). A solution of disuccinimidyl suberate (1.1 eq., 0.11 mmol, 40.5 mg) and DIPEA (5 eq., 0.5 mmol, 64.6 mg, 87 µL) in DMF (5 mL) was added to the resin. The mixture was gently agitated at r.t. for 2 h, then drained and rinsed with DMF (2 x 3 mL) then CH_2Cl_2 (1 x 3 mL).

To the resin, preswollen in DMF (5 mL, 30 min), a solution of Fmoc-D-Lys-OtBu (4 eq., 0.4 mmol, 187.4 mg) and DIPEA (5 eq., 0.5 mmol, 64.6 mg, 87 µL) in DMF (1 mL) was added. The mixture was gently agitated at r.t. for 1 h, then drained and rinsed with DMF (3 x 3 mL).

The Fmoc was then removed by treating the resin with 20% piperidine in DMF (*v/v*, 5 mL, 2 x 5 min, r.t.), then the resin was washed with DMF (3 x 5 mL). To the resin, a solution of Fmoc-D-Phe-OH (4 eq., 0.4 mmol, 155 mg), HATU (3.9 eq., 0.39 mmol, 148.3 mg), and DIPEA (8 eq., 0.8 mmol, 103.4 mg, 140 µL) in DMF (5 mL) was added and gently agitated at r.t. for 1 h.

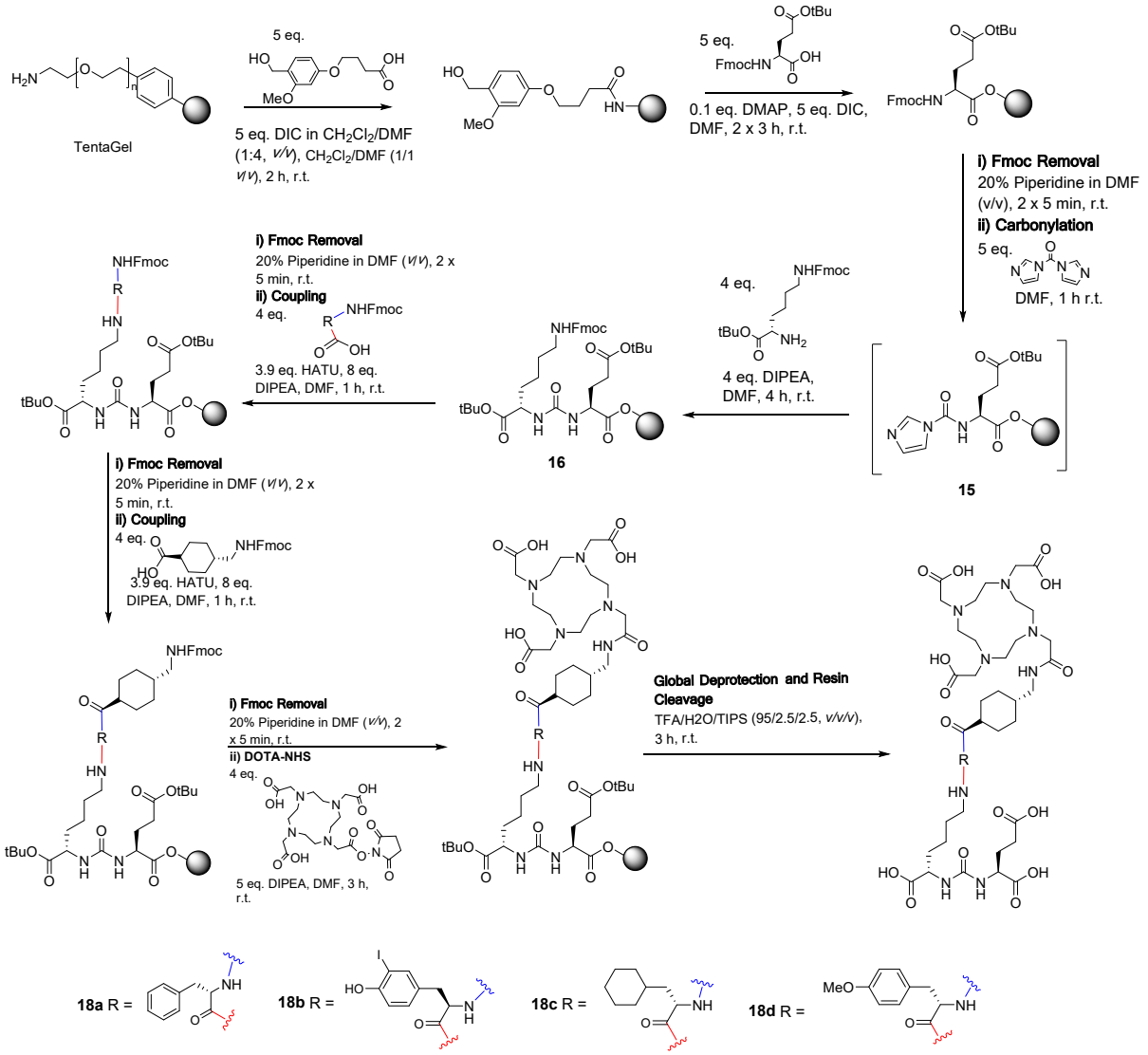
The resin was filtered then washed with DMF (3 x 5 mL). These Fmoc removal and coupling steps were repeated for Fmoc-L-Tyr(3-I)-OH (4 eq., 0.4 mmol, 211.7 mg).

To remove the Fmoc, the resin was treated with 20% piperidine in DMF (*v/v*, 5 mL, 2 x 5 min, r.t.), then washed with DMF (3 x 5 mL). To the resin, a solution of DOTA-NHS ester (1.5 eq., 0.15 mmol, 75.2 mg) and DIPEA (5 eq., 0.5 mmol, 64.6 mg, 87 µL) in DMF (5 mL) was added and the resin gently agitated at r.t. for 1 h. Reaction completion was monitored by Kaiser test and, if positive, the coupling was repeated with freshly prepared reagents.

Some resin beads were retrieved and treated with a solution of TFA/triisopropylsilane/water (95:2.5:2.5, *v/v/v*, 0.5 mL, 1 h) to cleave some product from the resin for analysis. After the product was confirmed, the entire resin was treated with a solution of TFA/triisopropylsilane/water (95:2.5:2.5, *v/v/v*, 5 mL, 1 h), simultaneously cleaving PSMA-I&T-DOTA from the resin and removing all protecting groups. Following cleavage, the sample was filtered and lyophilised affording the crude product.

A sample was purified by semi-preparative RP HPLC and lyophilised, affording an amorphous white solid in 6.4% purified yield (0.8 mg pure from 12.5 mg crude).

2.6 Synthesis of PSMA-617 Analogues



Scheme S8 Synthesis of PSMA-617 analogues **18a-18d**, whereby **18a** uses phenylalanine, **18b** uses tyrosine(3-I), **18c** uses cyclohexyl alanine, and **18d** uses 4-methoxyphenylalanine in place of PSMA-617's native napthylalanine.

To TentaGel® (1 eq., 0.1 mmol, 385 mg, 0.26 mmol/g), preswollen in CH_2Cl_2/DMF (1:1, *v/v*, 5 mL, 30 min), HMPB linker (5 eq., 0.5 mmol, 120 mg) and DIC (5 eq., 0.5 mmol, 63 mg, 77 µL) dissolved in CH_2Cl_2/DMF (1:4, *v/v*, 5 mL) was added. The mixture was gently agitated at r.t. for 2 h. Reaction completion was monitored with a Kaiser test and, if positive, the procedure was repeated with freshly prepared reagents. After completion, the resin was drained and washed with DMF (3 x 5 mL).

To the HMPB-loaded resin, preswollen in DMF (5 mL, 30 min), a solution of Fmoc-L-Glu(OtBu)-OH (5 eq., 0.5 mmol, 213 mg), DMAP (0.1 eq., 0.01 mmol, 1 mg), and DIC (5 eq., 0.5 mmol, 63 mg, 77 µL) in DMF (5 mL) was added. The reaction was gently agitated at r.t. for 3 h. Then the resin was filtered and washed with DMF (3 x 5 mL) and the procedure was repeated with freshly prepared reagents and gently agitated for another 3 h. After this, the resin was filtered then washed with DMF (3 x 5 mL).

The glutamate-immobilised resin was preswollen in DMF (5 mL, 30 min) and the Fmoc was removed by treating the resin with 20% piperidine in DMF (*v/v*, 5 mL, 2 x 5 min, r.t.). CDI (5 eq., 0.5 mmol, 81 mg) was dissolved in DMF (5 mL) and added to the resin. The mixture was gently agitated at r.t. for 1 h, then the resin was drained and washed with DMF (3 x 5 mL). To the resin, H-L-Lys(Fmoc)-OtBu•HCl (4 eq., 0.4 mmol, 184.4 mg) and DIPEA (4 eq., 0.4 mmol, 50 mg, 68 µL) in DMF (5 mL) was added. The mixture was gently agitated at r.t. for 4 h, then the resin was drained and washed with DMF (3 x 5 mL).

Fmoc was removed from the KuE-immobilised resin by treatment with 20% piperidine in DMF (*v/v*, 5 mL, 2 x 5 min, r.t.).

To Synthesise 18a:

To KuE-immobilised resin, a solution of Fmoc-L-Phe-OH (4 eq., 0.4 mmol, 155 mg), HATU (3.9 eq., 0.39 mmol, 148.3 mg), and DIPEA (8 eq., 0.8 mmol, 103.4 mg, 140 µL) in DMF (5 mL) was added and gently agitated at r.t. for 1 h. The resin was filtered then washed with DMF (3 x 5 mL).

To Synthesise 18b:

To KuE-immobilised resin, a solution of Fmoc-L-Tyr(3-I)-OH (4 eq., 0.4 mmol, 211.7 mg), HATU (3.9 eq., 0.39 mmol, 148.3 mg), and DIPEA (8 eq., 0.8 mmol, 103.4 mg, 140 µL) in DMF (5 mL) was added and gently agitated at r.t. for 1 h. The resin was filtered then washed with DMF (3 x 5 mL).

To Synthesis 18c:

To KuE-immobilised resin, a solution of Fmoc-L-Cha-OH (4 eq., 0.4 mmol, 145.4 mg), HATU (3.9 eq., 0.39 mmol, 148.3 mg), and DIPEA (8 eq., 0.8 mmol, 103.4 mg, 140 µL) in DMF (5

mL) was added and gently agitated at r.t. for 1 h. The resin was filtered then washed with DMF (3 x 5 mL).

To Synthesise 18d:

To KuE-immobilised resin, a solution of Fmoc-L-Phe(4-OMe)-OH (4 eq., 0.4 mmol, 167 mg), HATU (3.9 eq., 0.39 mmol, 148.3 mg), and DIPEA (8 eq., 0.8 mmol, 103.4 mg, 140 µL) in DMF (5 mL) was added and gently agitated at r.t. for 1 h. The resin was filtered then washed with DMF (3 x 5 mL).

To All Analogues 18a-d:

These Fmoc removal and coupling steps were repeated for Fmoc-transexamic acid (4 eq., 0.4 mmol, 151.8 mg). Fmoc was removed from the resulting peptide by treatment with 20% piperidine in DMF (*v/v*, 5 mL, 2 x 5 min, r.t.). To the resin, DOTA-NHS ester (4 eq., 0.4 mmol, 200.6 mg) in DMF (5 mL) was added. The resulting mixture was gently agitated at r.t. for 3 h, then drained and washed with DMF (3 x 5 mL) then CH_2Cl_2 (1 x 5 mL).

Some resin beads from each derivative were retrieved and treated with a solution of TFA/triisopropylsilane/water (95:2.5:2.5, *v/v/v*, 0.5 mL, 1 h) to cleave some product from the resin for analysis.

3. Spectra

3.1 ELYRAG

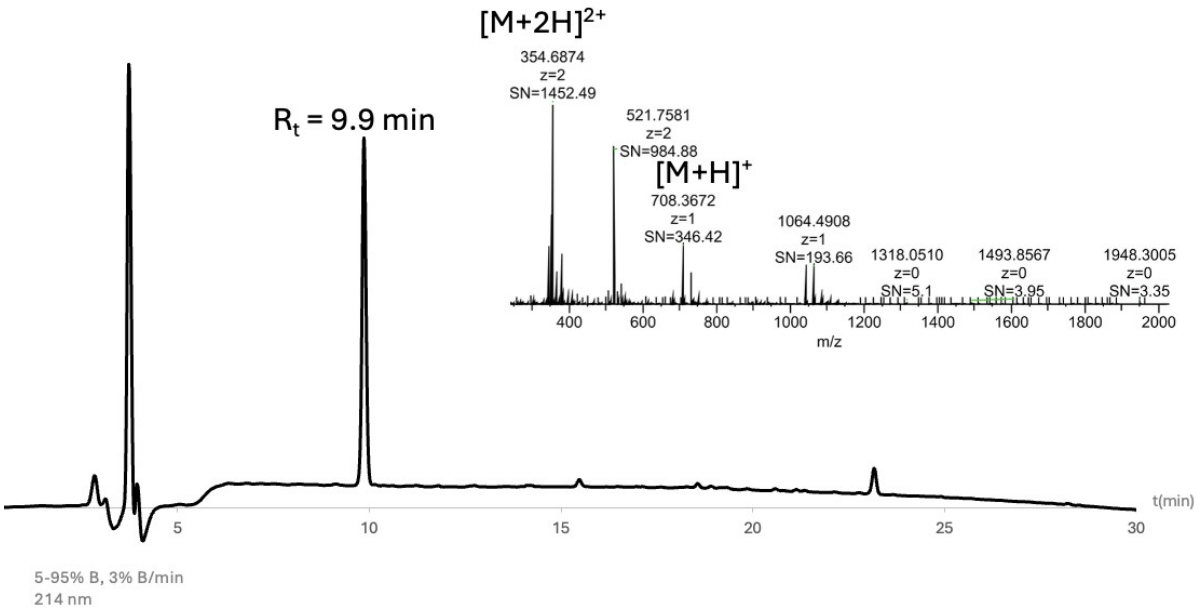


Figure S1 Analytical HPLC showing ELYRAG, Fmoc removed, at R_t = 9.9 min. HRMS (EI): $[M+H]^+$ calculated for 708.3675, observed *m/z* 708.3672.

3.2 Optimisation EXP 1-7

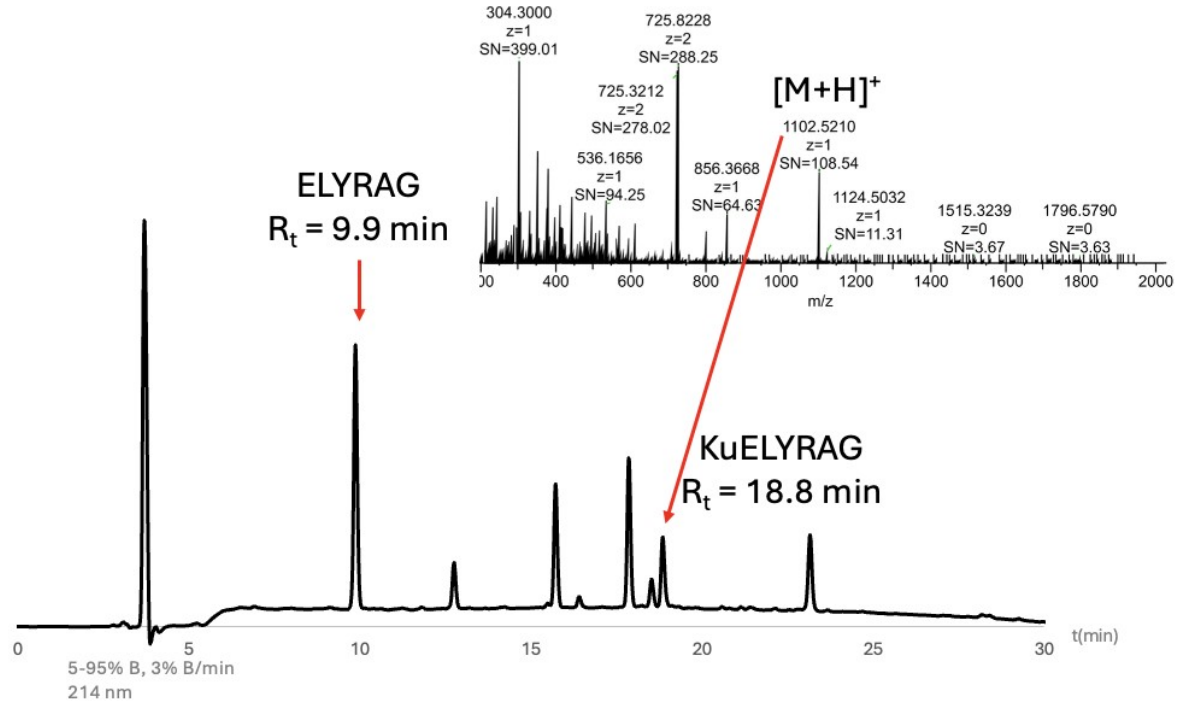


Figure S2 Analytical HPLC following **EXP 1**. ELYRAG at R_t = 9.9 min; KuELYRAG at R_t = 18.8 min. ELYRAG was 21.3% converted to KuELYRAG as judged by peak area at 214 nm. HRMS (EI): $[M+H]^+$ calculated for 1102.5204, observed *m/z* 1102.5210.

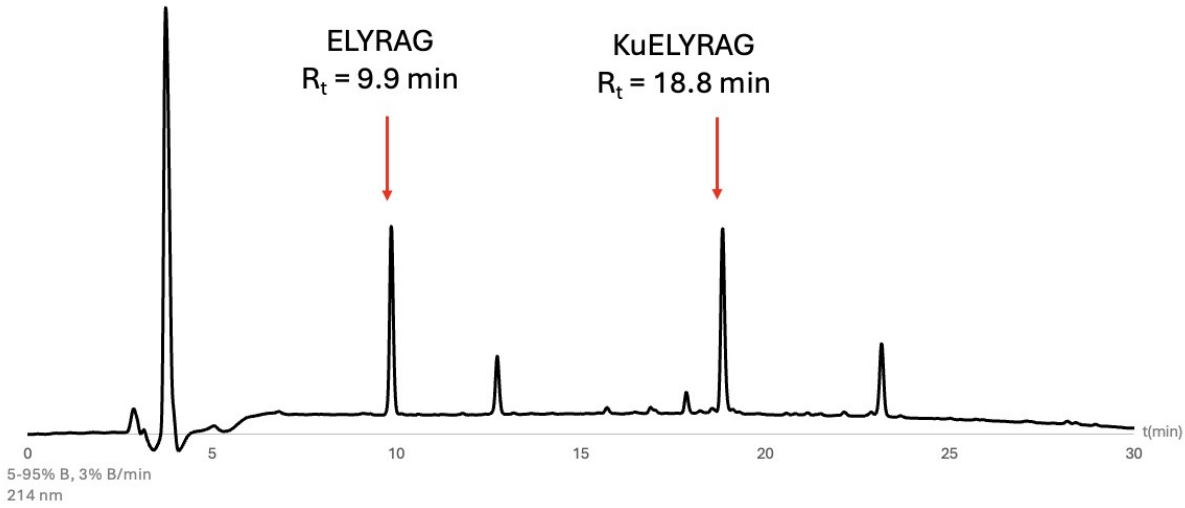


Figure S3 Analytical HPLC following **EXP 2**. ELYRAG at R_t = 9.9 min; KuELYRAG at R_t = 18.8 min, confirmed by HRMS. ELYRAG was 50% converted to KuELYRAG as judged by peak area at 214 nm.

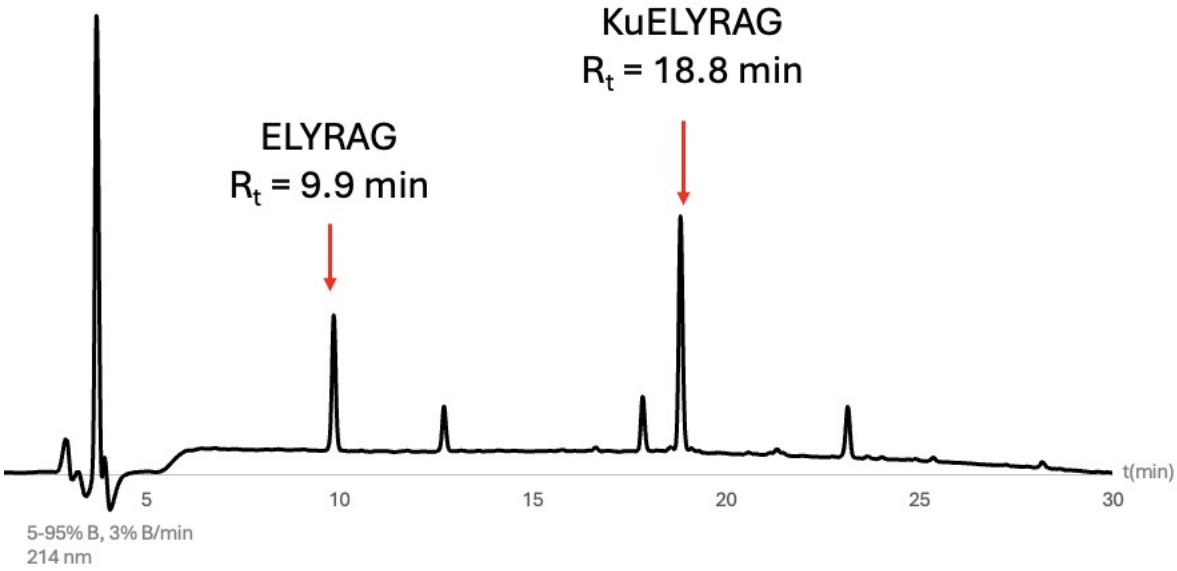


Figure S4 Analytical HPLC following **EXP 3**. ELYRAG at R_t = 9.9 min; KuELYRAG at R_t = 18.8 min. ELYRAG was 63% converted to KuELYRAG as judged by peak area at 214 nm.

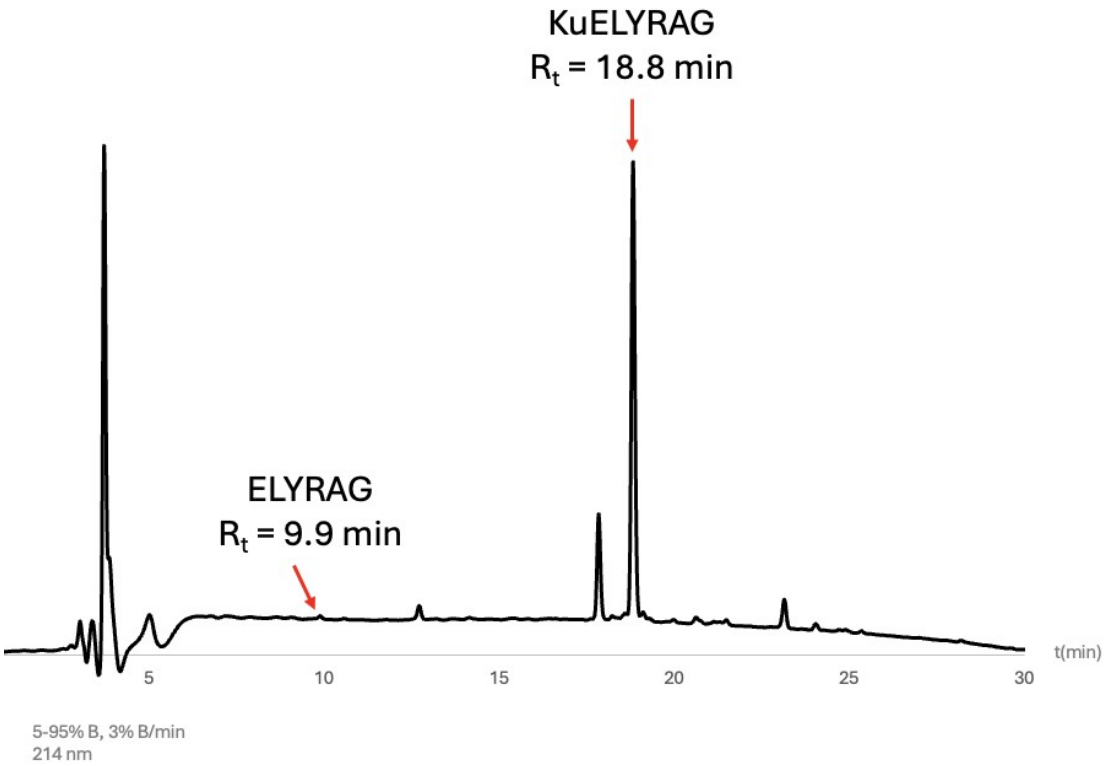


Figure S5 Analytical HPLC following **EXP 4**. ELYRAG at R_t = 9.9 min; KuELYRAG at R_t = 18.8 min. ELYRAG was >99% converted to KuELYRAG as judged by peak area at 214 nm.

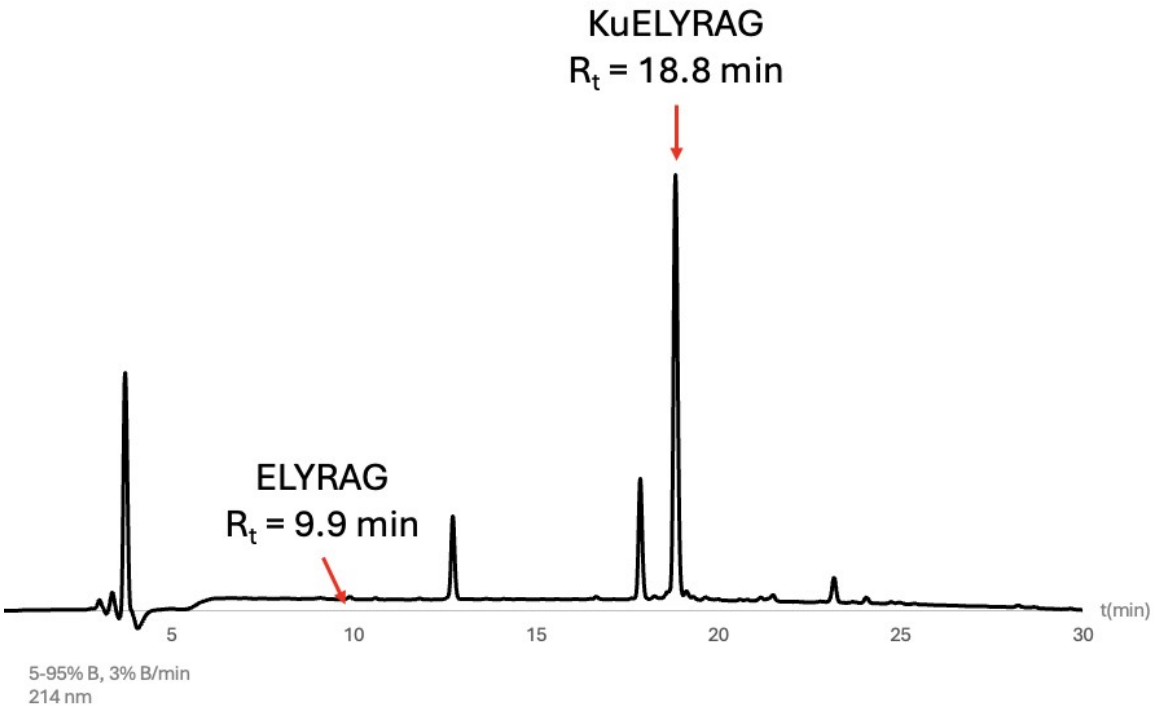


Figure S6 Analytical HPLC following **EXP 5**. ELYRAG at R_t = 9.9 min; KuELYRAG at R_t = 18.8 min. ELYRAG was >99% converted to KuELYRAG as judged by peak area at 214 nm.

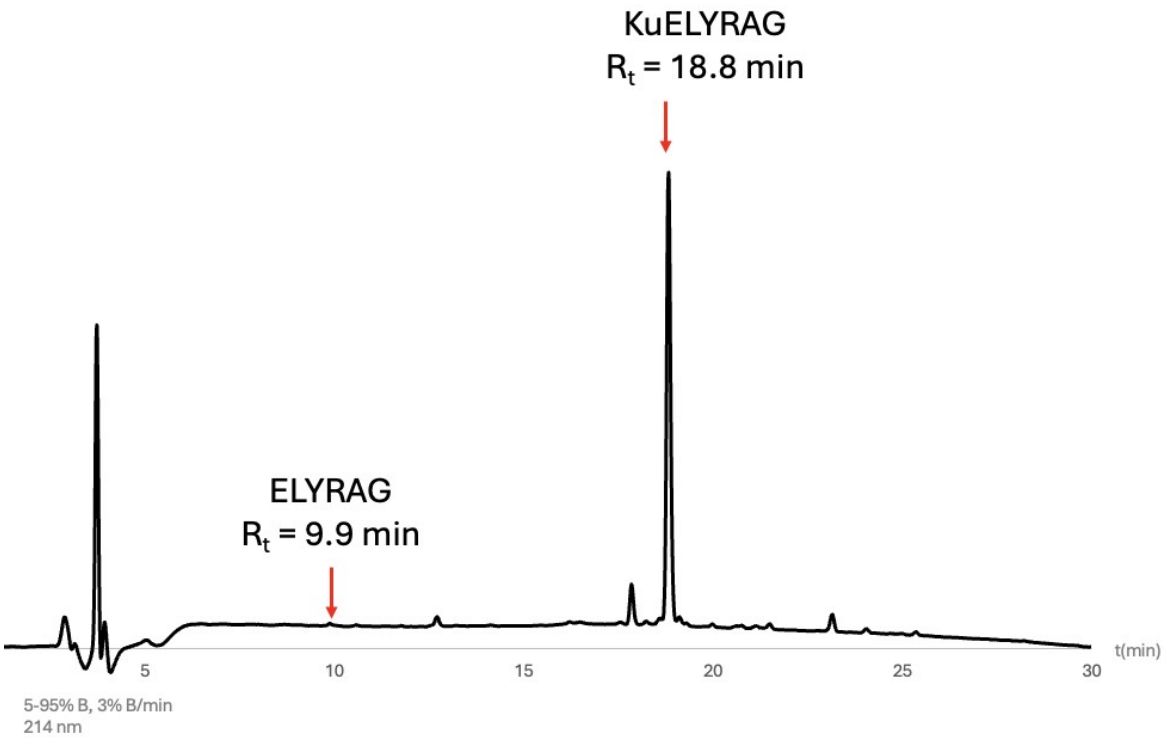


Figure S7 Analytical HPLC following **EXP 6**. ELYRAG at R_t = 9.9 min; KuELYRAG at R_t = 18.8 min. ELYRAG was >99% converted to KuELYRAG as judged by peak area at 214 nm.

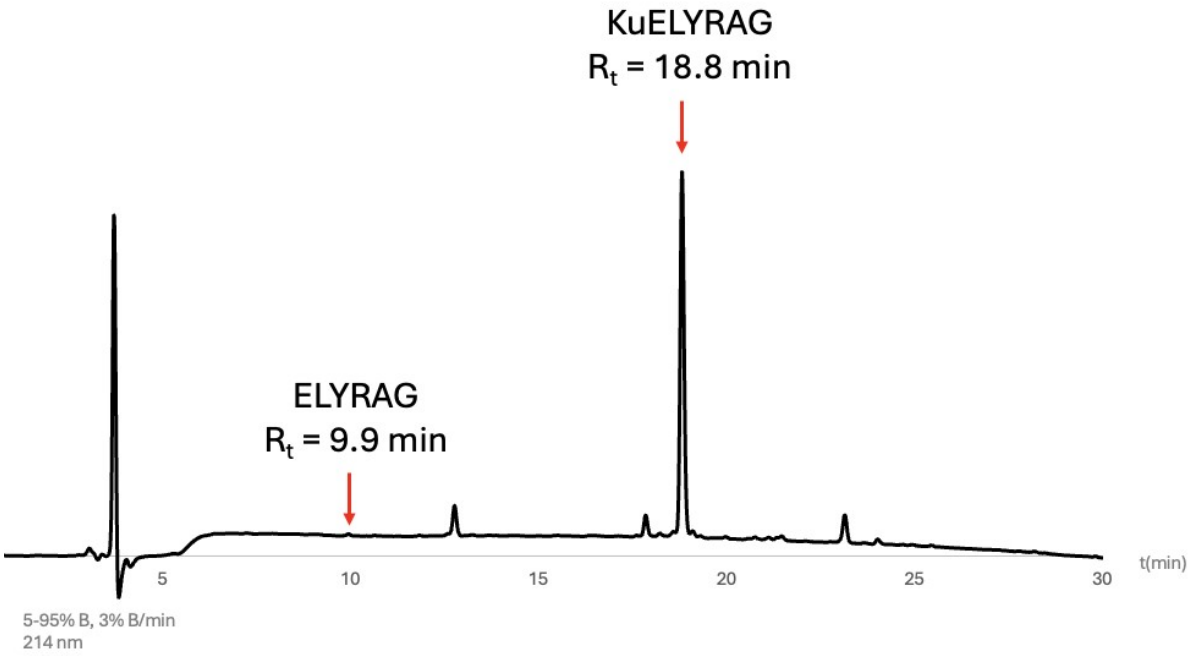


Figure S8 Analytical HPLC following **EXP 7**. ELYRAG at R_t = 9.9 min; KuELYRAG at R_t = 18.8 min. ELYRAG was >99% converted to KuELYRAG as judged by peak area at 214 nm.

3.3 KuE and PSMA-617

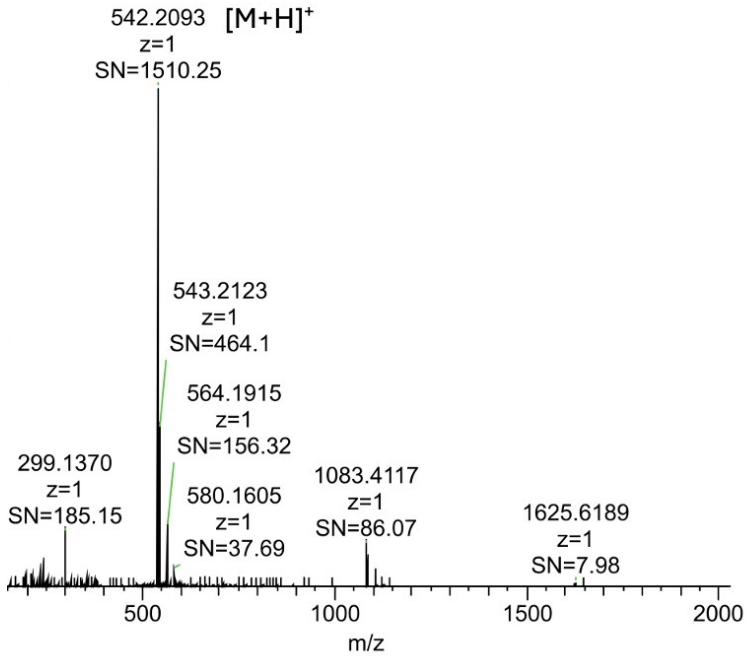


Figure S9 Fmoc-KuE mass spectra. HRMS (EI): $[M+H]^+$ calculated for $C_{27}H_{32}N_3O_9^+$: 542.2133; observed: *m/z* 542.2093.

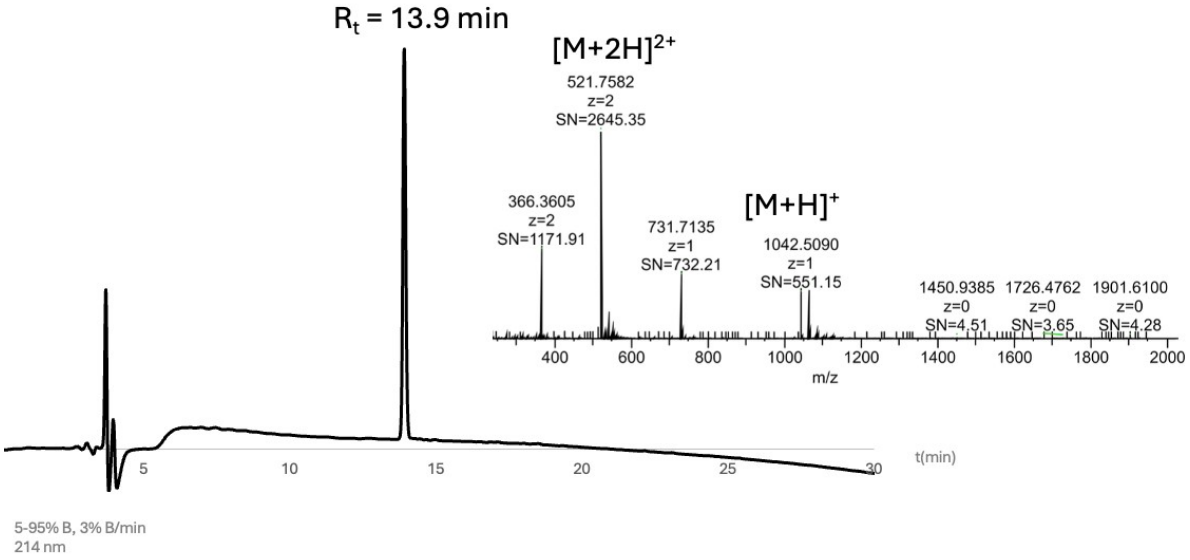


Figure S10 Analytical HPLC of PSMA-617 after purification. HRMS (EI): $[M+H]^+$ calculated for $C_{49}H_{72}N_9O_{16}^+$: 1042.5092, observed: *m/z* 1042.5091.

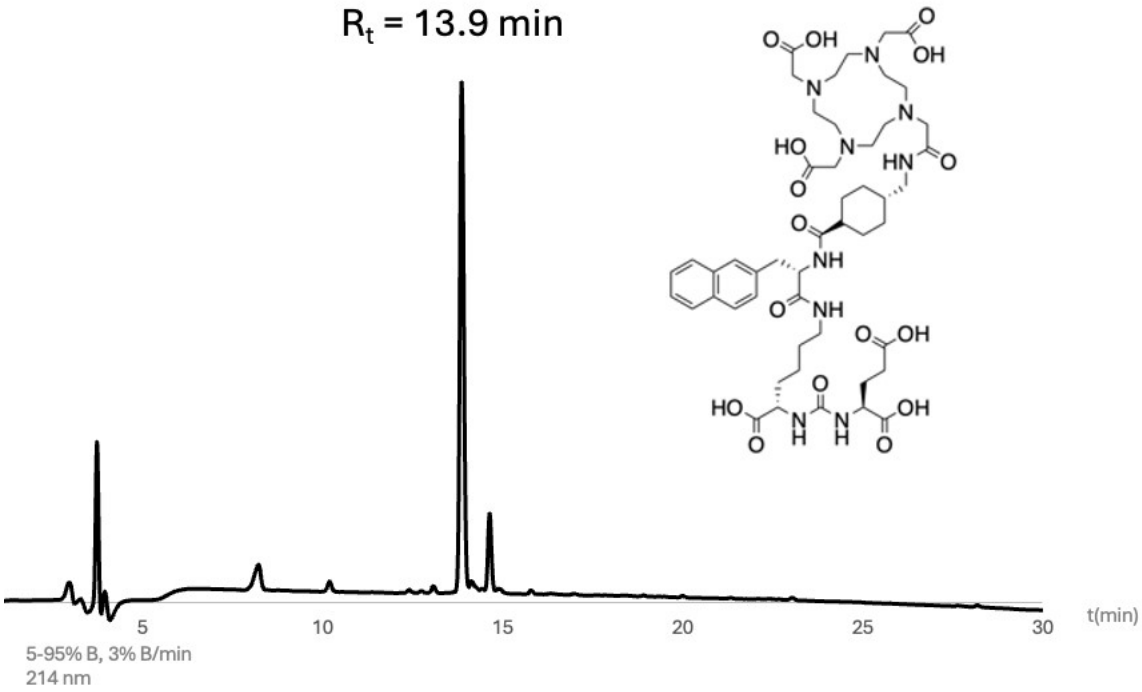


Figure S11 Analytical HPLC of crude PSMA-617 as synthesised on 2-chlorotrityl chloride resin. Crude purity ca. 79% as judged by peak area at 214 nm.

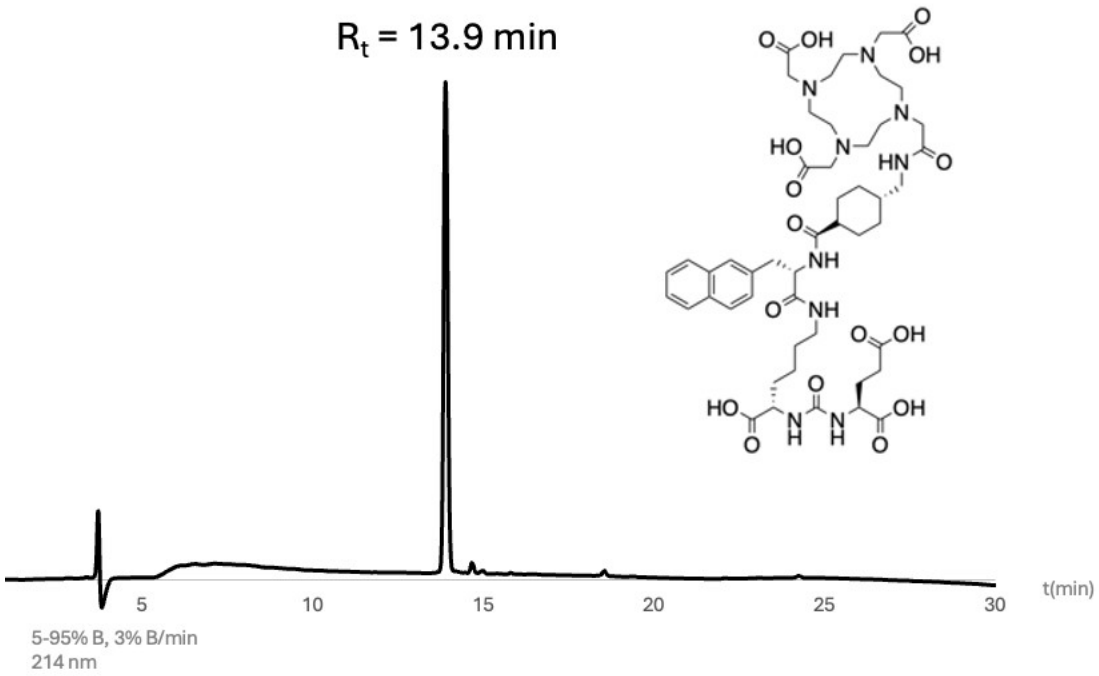


Figure S12 Analytical HPLC of purified PSMA-617 as synthesised on 2-chlorotrityl chloride resin.

3.4 PSMA-I&T-DOTA

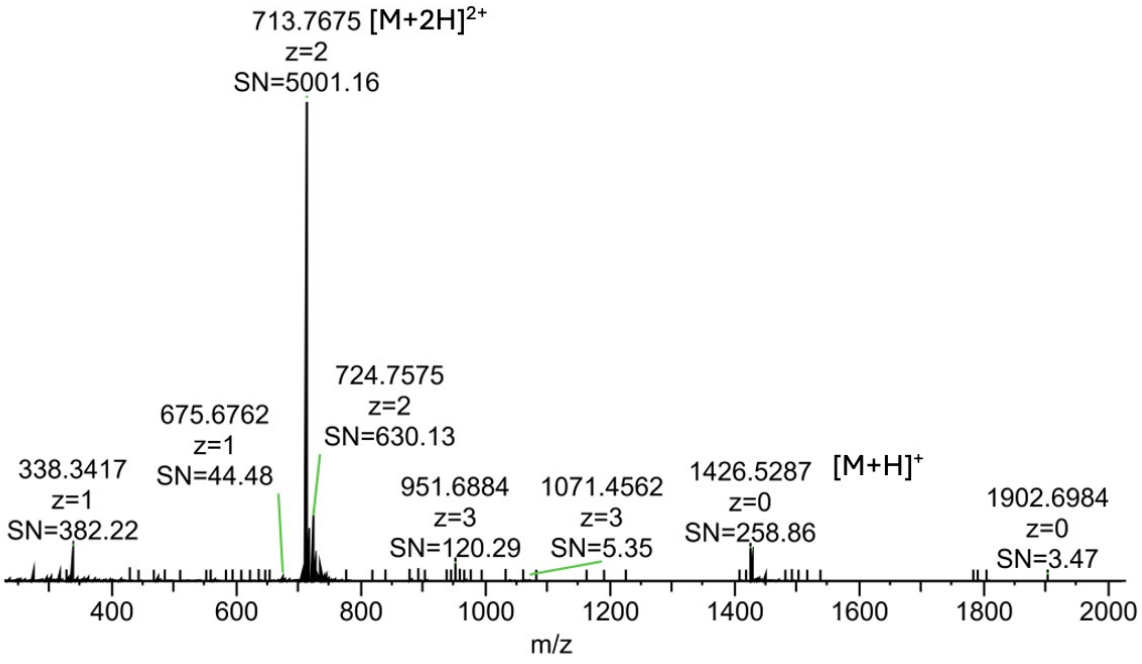


Figure S13 HRMS (EI): $[M+H]^+$ calculated for $C_{60}H_{89}IN_{11}O_{21}^+$: 1426.5274; observed: *m/z* 1426.5284.

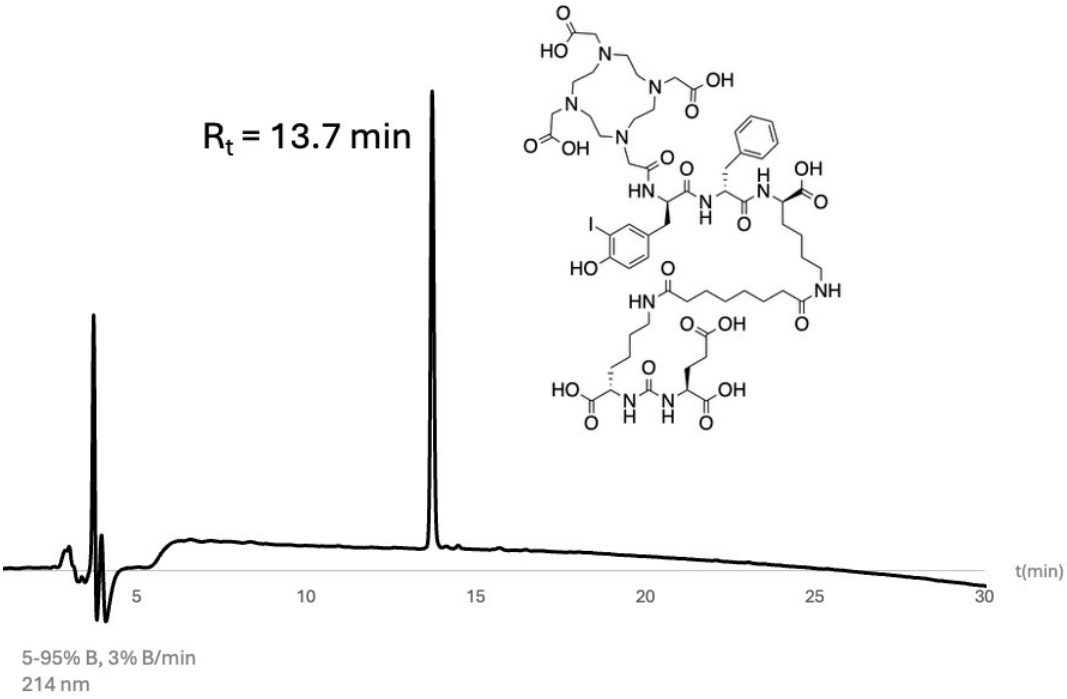


Figure S14 Analytical HPLC of purified PSMA-I&T with DOTA at R_t = 13.7 min.

3.5 PSMA-617 Analogues 18a-d

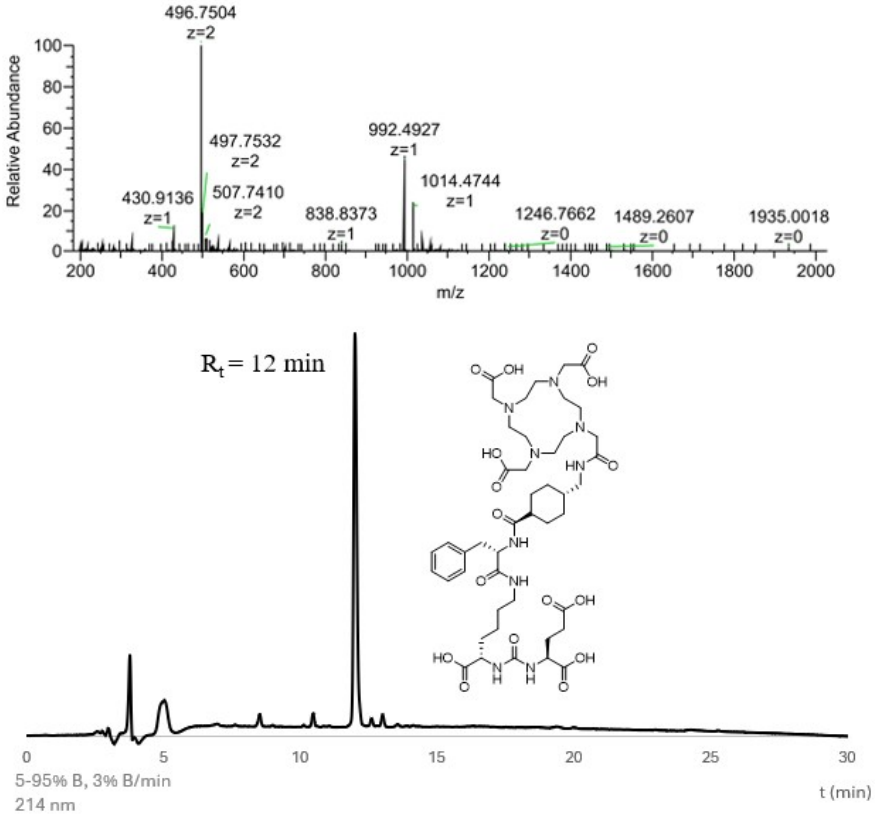


Figure S15 Analytical HPLC of analogue **18a** at R_t = 12 min in *ca.* 92% crude purity. HRMS (EI): $[M+H]^+$ calculated for $C_{45}H_{70}N_9O_{16}^+$: 992.4935; observed: *m/z* 992.4927.

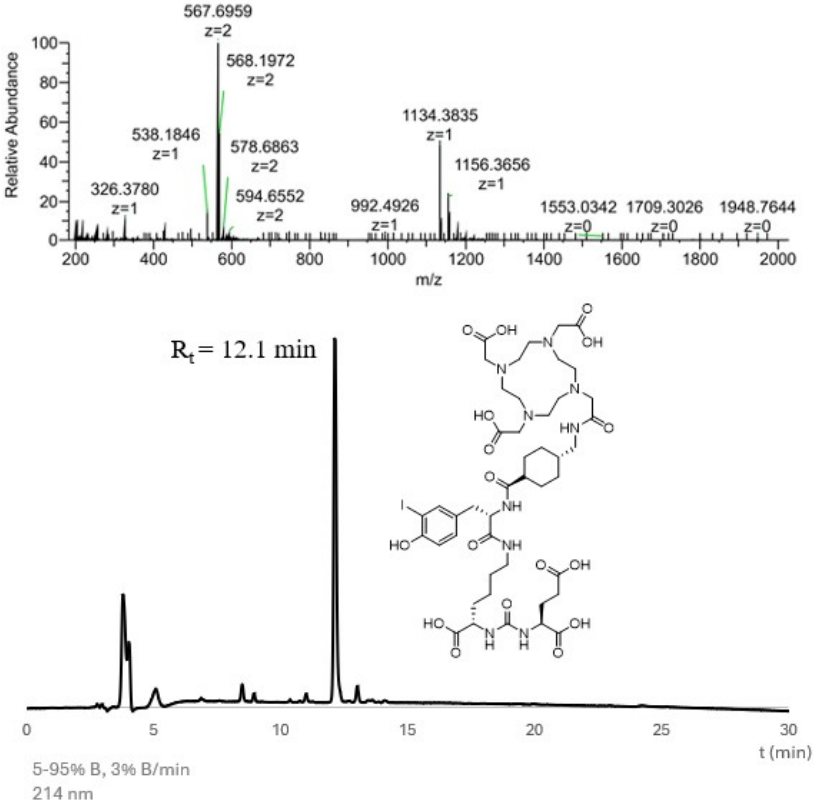


Figure S16 Analytical HPLC of analogue **18b** at R_t = 12.1 min in *ca.* 89% crude purity. HRMS (EI): $[M+H]^+$ calculated for $C_{45}H_{69}IN_9O_{17}^+$: 1134.3851; observed: *m/z* 1134.3835.

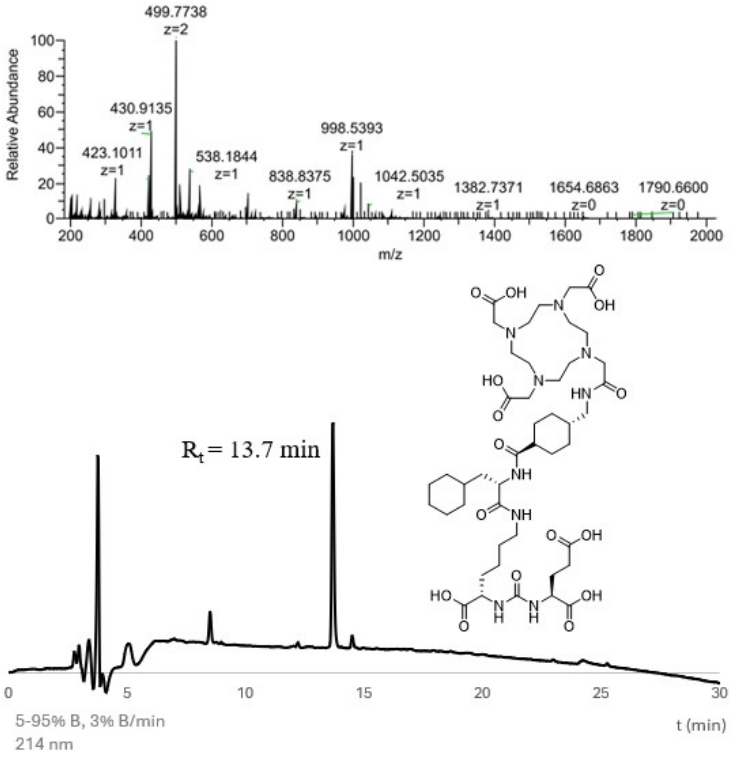


Figure S17 Analytical HPLC of analogue **18c** at R_t = 13.7 min in *ca.* 83% crude purity. HRMS (EI): $[M+H]^+$ calculated for $C_{45}H_{76}N_9O_{16}^+$: 998.5405; observed: *m/z* 998.5393.

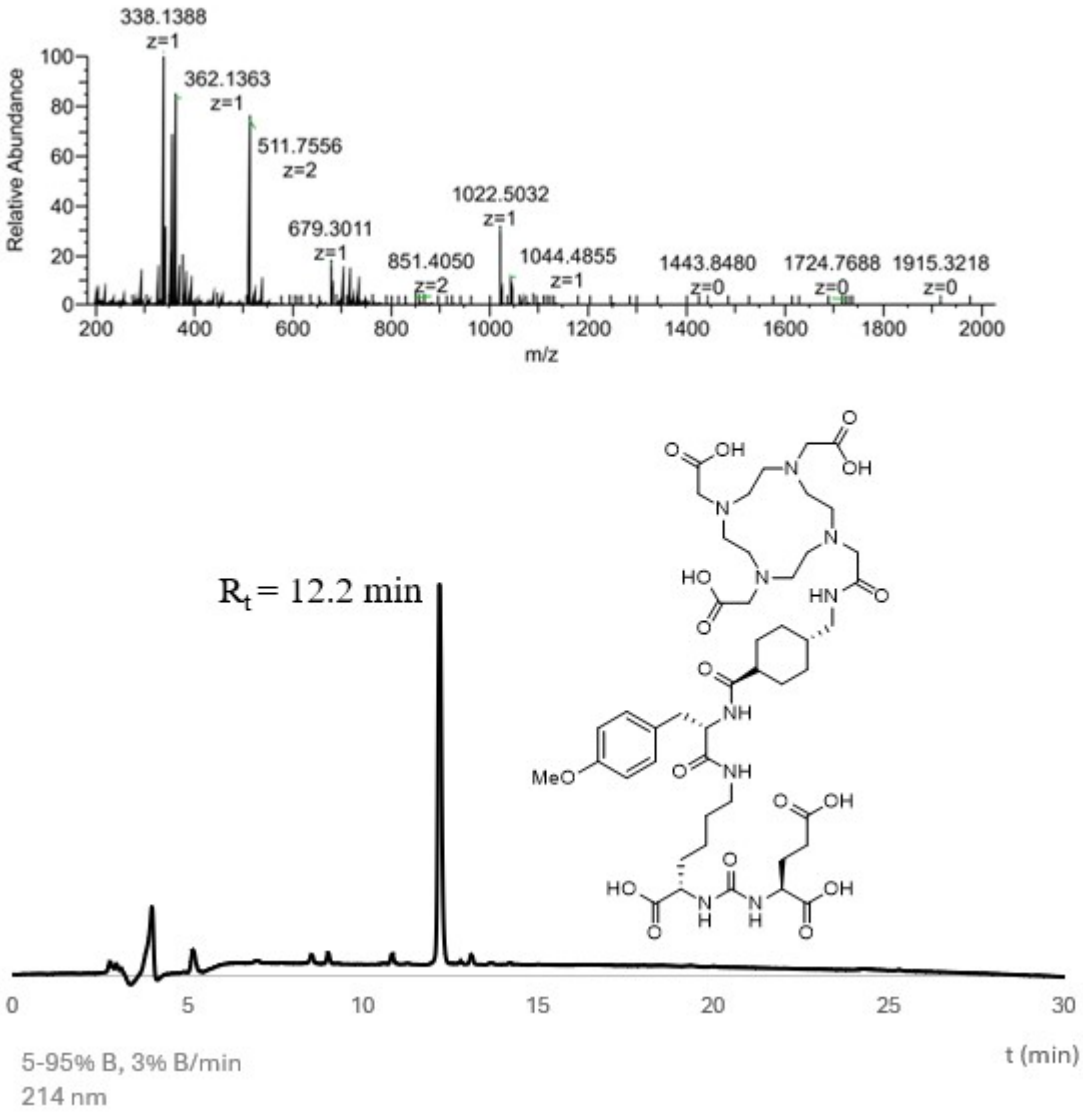
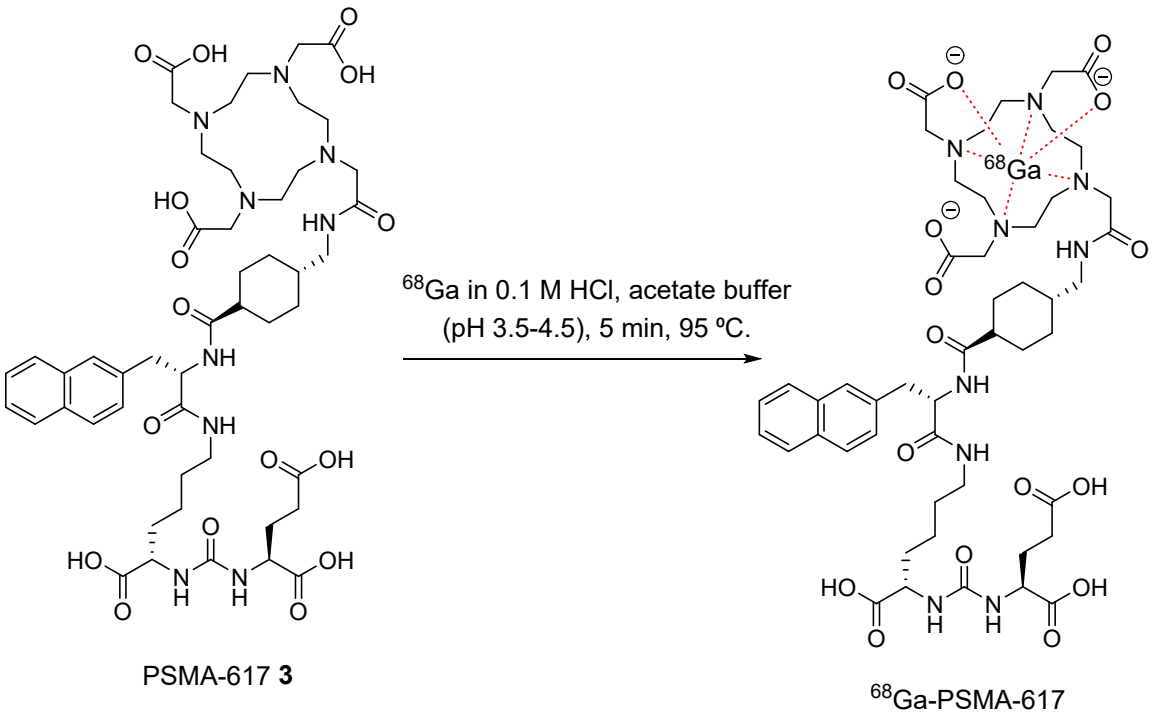


Figure S18 Analytical HPLC of analogue **18d** at R_t = 12.2 min in *ca.* 91% crude purity. HRMS (EI): $[M+H]^+$ calculated for $C_{46}H_{71}N_9O_{17}^+$: 1022.5041; observed: *m/z* 1022.5032.

4. Radiolabelling Procedures

4.1 Synthesis of Radiolabelled PSMA-617



Scheme S9 Synthesis of ^{68}Ga-PSMA-617.

Radiolabelling of PSMA-617 with gallium-68 (^{68}Ga) was performed using a ^{68}Ge/^{68}Ga generator. The generator was eluted with 0.1 M HCl, and the eluate was collected in a metal-free vial to minimise interference from trace metal contaminants. For pre-concentration, the ^{68}Ga eluate was passed through a strong cation exchange (SCX) cartridge. The cartridge was rinsed with 5 mL of water to remove residual ions, after which ^{68}Ga was eluted with 900 µL of 5 M NaCl in 0.1 M HCl, affording a purified and concentrated ^{68}Ga fraction suitable for efficient chelation. The radiolabelling reaction was performed in a sterile reaction vial containing 50 µg of PSMA-617 precursor in acetate buffer (pH 3.5-4.5). The purified ^{68}Ga solution (1-2 mL) was added to the reaction mixture, which was then heated at 95 ºC for 5 min to promote rapid complexation of ^{68}Ga with the DOTA chelator of PSMA-617. Under these conditions, the radiochemical yield typically exceeded 98%, as determined by radio-TLC and radio-HPLC. When purification was required, the reaction mixture was passed through a C18 light cartridge, washed with water to remove hydrophilic impurities, and subsequently eluted with 50% ethanol in saline, affording purified ^{68}Ga-PSMA-617. The entire radiolabelling

process was performed using an automated MultiSyn™ synthesis module (iPHASE Technologies).

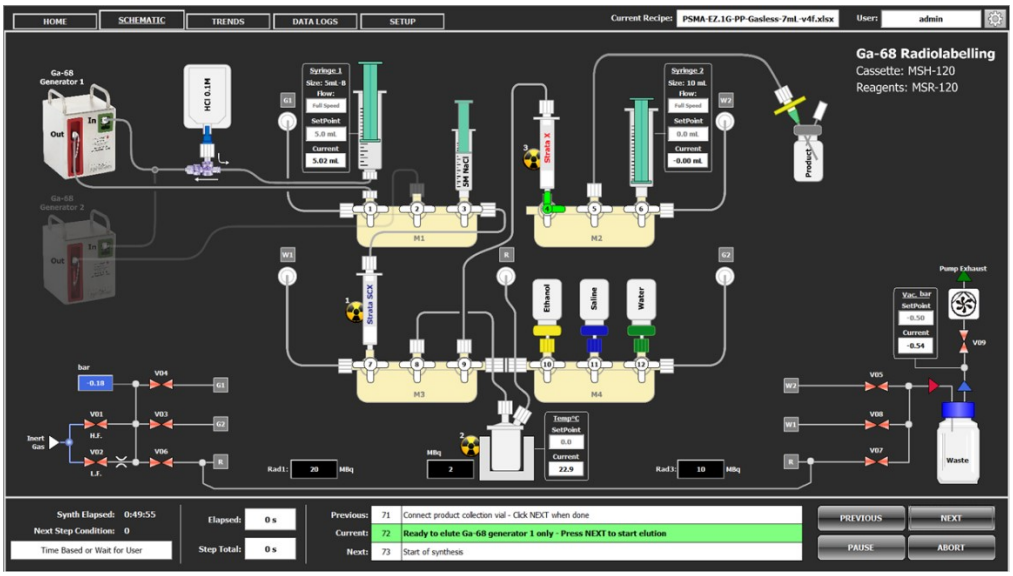


Figure S19 Schematic overview of iPHASE MultiSyn cassette and reagent set-up for automated radiosynthesis of ^{68}Ga-PSMA-617.

Figure S20 iPHASE MultiSyn set-up for automated radiosynthesis of ^{68}Ga-PSMA-617.

4.2 Quality Control

Quality control was performed using a series of analytical procedures to ensure radiochemical integrity, safety, and compliance with European Pharmacopoeia standards. Radiochemical purity was initially evaluated by radio–thin layer chromatography (radio-TLC) using iTLC-SG plates developed in 1 M ammonium acetate/methanol (1:1) as the mobile phase. Under these conditions, free ^{68}Ga remained at the origin (Rf ≈ 0.0–0.1), whereas ^{68}Ga-PSMA-617 migrated with the solvent front (Rf ≈ 0.8–1.0). Further confirmation was obtained by radio-HPLC analysis using a C18 column (250 × 4.6 mm) with a gradient of 5–95% acetonitrile containing 0.1% TFA over 20 min. Under these conditions, ^{68}Ga-PSMA-617 eluted at approximately 8–10 min. The final formulated product was required to meet the following specifications: pH 4.0–8.0, radiochemical purity >95%, and radionuclidic purity with <0.001% ^{68}Ge breakthrough. Typical batch data demonstrated consistently high radiochemical purity across five production runs, with TLC values ranging from 98.9–99.5% and HPLC values from 98.5–99.1%. At the end of synthesis, additional quality control assessments included visual inspection to confirm a clear, particle-free solution and radionuclidic identity verification through half-life measurement (68 min ± 10%).

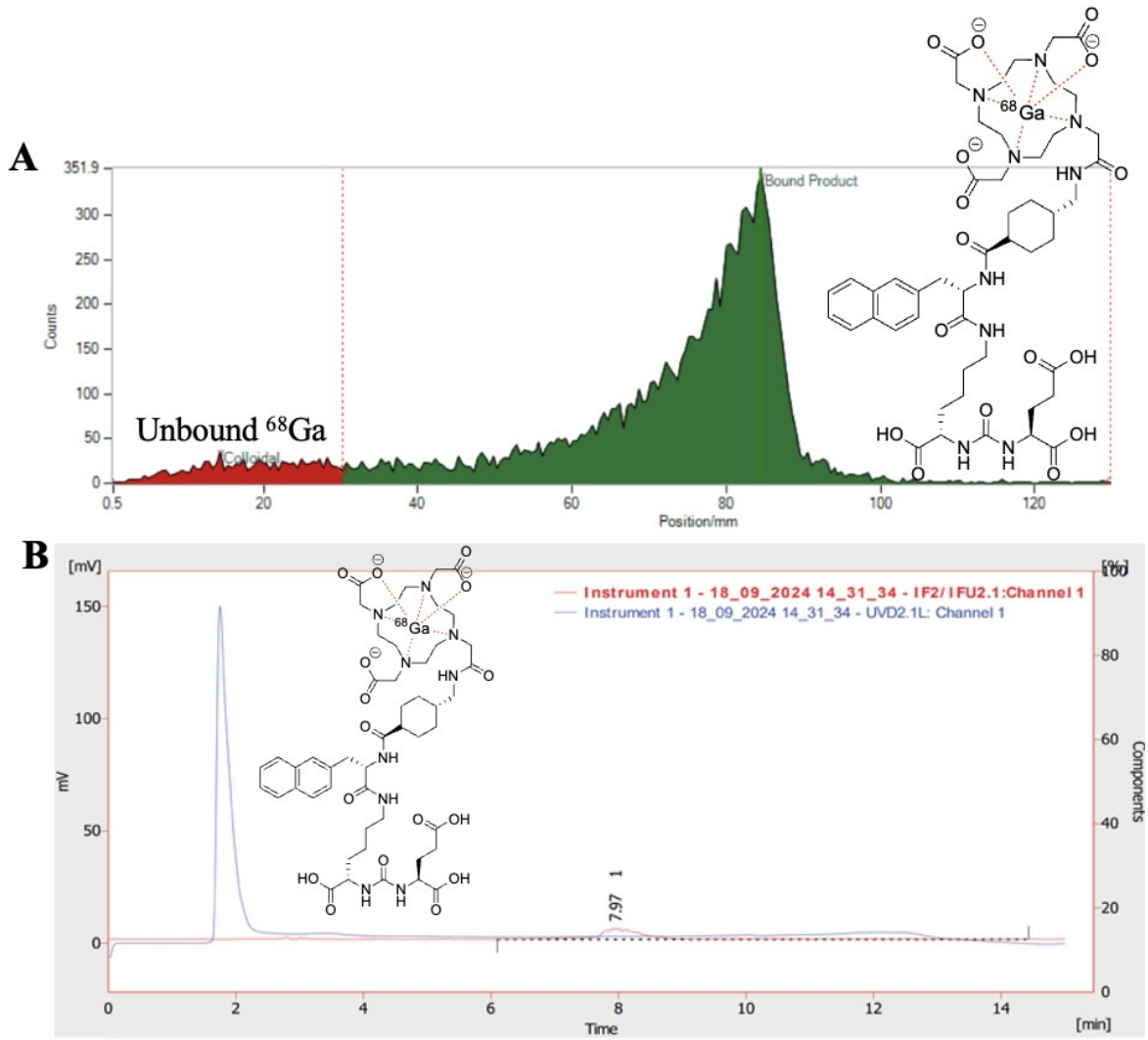


Figure S21 A) Radio-TLC and B) radio-HPLC chromatograms of ^{68}Ga-PSMA-617.