Supplementary Information for

A solid phase-assisted approach for the facile synthesis of a highly water-soluble zirconium-89 chelator for radiopharmaceutical development

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Table of Contents

1) General Considerations	2
2) Synthesis and Characterisation of oxoDFO* (3)	3
3) Complexation Reaction of oxoDFO* (3) with Non-radioactive ^{nat} Zr(IV)	9
4) Determination of Lipophilicity (log <i>D</i> 7.4)	.10
5) ¹ H NMR, ¹³ C NMR and Mass Spectral Analysis of Compounds	.11
6) References	22

1) General Considerations

All chemicals were of reagent grade quality or better, obtained from commercial suppliers and used without further purification. Solvents were used as received or distilled and if needed dried over molecular sieves prior to their use. HATU was purchased from abcr GmbH. HPLC solvents were purchased from Sigma-Aldrich. TentaGel S Ram Resin was purchased from Rapp Polymere GmbH. Polypropylene syringes for manual peptide couplings, fitted with polypropylene frits (pore size 25 µm) and a polypropylene plunger, were obtained from MultiSyntech GmbH. Solvents were used as received or distilled and if needed dried over molecular sieves prior to their use. Thin layer chromatography (TLC): Merck TLC plates silica gel 60 on aluminium with the indicated solvent system; the spots were visualized by UV light (254 nm). Chromatography: Merck Silica gel 60 (0.063-0.200 mm) with the indicated solvent system. ¹H NMR and ¹³C NMR spectra in indicated deuterated solvents: *Bruker ARX-400* (¹H: 400 MHz, ¹³C: 100.6 MHz) and Bruker ARX-500 (¹H: 500 MHz, ¹³C: 126 MHz) at room temperature; chemical shifts (δ) in ppm (parts per million); coupling constant J in Hz, residual solvent peaks were used as an internal reference; abbreviations for the peak multiplicities: s (singlet), d (doublet), t (triplet), m (multiplet), br (broad). ESI mass spectra were recorded on a Bruker Esquire 6000 spectrometer. Ultraperformance liquid chromatography mass spectrometry (UPLCMS): Waters Acquity UPLC System with an Acquity UPLC PDA Detector and an Acquity UPLC BEH C₁₈ 1.7 µm reverse phase column (2.1 x 50 mm); flow rate: 0.6 mL/min with a linear gradient of A (acetonitrile) and B (millipore H₂O containing 0.1% formic acid): $t = 0 \min, 5\%$ B, $t = 0.5 \min, 5\%$ B, $t = 4 \min, 100\%$ B, $t = 5 \min, 100\%$ B, characteristic fragments in m/z. High performance liquid chromatography (HPLC): Preparative column Macherev-Nagel (21 \times 250 mm) at a flow rate of 16 mL min⁻¹ with a linear gradient of A (acetonitrile; Sigma-Aldrich HPLC-grade) and B (distilled water containing 0.1% TFA).

2) Synthesis and Characterisation of oxoDFO* (3)

2-(2-(2-bromoethoxy)ethyl)isoindoline-1,3-dione (4)



Using a procedure reported by Kadi *et al.*¹, to a stirred solution of potassium phthalimide (2.00 g, 10.8 mmol) in dimethylformamide (DMF) (30 mL), was added bis(2-bromoethyl) ether (5.00 g, 21.6 mmol) under a N₂ atmosphere. After 48 h at 80°C, the solvent was removed using high vacuum pump to give an orange oil that was dissolved in EtOAc (100 mL) a washed with water (2×100 mL). The aqueous layer was then extracted with EtOAc (3×100 mL). The combined organic layers were washed with saturated solution of NaHCO₃ (2×100 mL), brine (2×100 mL), dried with MgSO₄ and concentrated under reduced pressure. The resulting brown oil was purified using silica gel chromatography (hexane/EtOAc: 1/1, $R_f = 0.5$) to give the title compound **4** in a 89% yield (2.87 g) as a yellow oil.

¹H NMR (400 MHz, CDCl₃): δ (ppm) 3.40 (t, *J* = 6.2 Hz, 2H), 3.77 (t, *J* = 5.7 Hz, 2H), 3.78 (t, *J* = 6.2 Hz, 2H), 3.91 (t, *J* = 5.7 Hz, 2H), 7.69-7.74 (m, 2H), 7.82-7.89 (m, 2H). ¹³C NMR (126 MHz, CDCl₃): δ (ppm) 30.35, 37.35, 67.97, 70.62, 123.43, 132.23, 134.11, 168.42.

tert-butyl (benzyloxy)(2-(2-(1,3-dioxoisoindolin-2-yl)ethoxy)ethyl)carbamate (5)



To a stirred solution of *tert*-butyl *N*-(benzyloxy)-carbamate (2.33 g, 10.4 mmol) in DMF (18 mL), were added NaI (57 mg, 0.4 mmol) and NaH (318 mg, 13.3 mmol) under a N₂ atmosphere. After 15 min at 85 °C, compound **4** (2.83 g, 9.5 mmol) was added to the media and the reaction mixture was stirred at the same temperature for 24 h. After cooling down, the solution was carefully quenched by the addition of water (100 mL). The aqueous layer was then extracted with Et₂O (3 × 100 mL). The combined organic layers were washed with 1% aqueous Na₂S₂O₃ (100 mL), brine (100 mL), dried with MgSO₄ and evaporated under reduced pressure. The resulting orange oil was purified using silica gel chromatography (hexane/EtOAc: 7/3, R_f = 0.3) to provide the title compound **5** in 79% yield (3.31 g) as a colourless oil.

¹H NMR (400 MHz, CDCl₃): δ (ppm) 1.45 (s, 9H), 3.56 (t, *J* = 4.9 Hz, 2H), 3.61 (t, *J* = 5.2 Hz, 2H), 3.69 (t, *J* = 5.8 Hz, 2H), 3.87 (t, *J* = 5.8 Hz, 2H), 4.77 (s, 2H), 7.30-7.36 (m, 5H), 7.66-7.69 (m, 2H), 7.79-7.81 (m, 2H). ¹³C NMR (126 MHz, CDCl₃): δ (ppm) 28.33, 37.41, 49.68, 67.19, 67.62, 77.05, 81.43, 123.30, 128.44, 128.48, 129.49, 132.19, 133.98, 135.70, 156.69, 168.27. HRMS (ESI+): calcd for C₂₄H₂₈N₂O₆Na⁺ 463.18396, found 463.18325.

tert-butyl(2-(2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)ethoxy)ethyl)(benzyloxy) carbamate (6)



To a stirred solution of **5** (4.08 g, 9.2 mmol) in EtOH (50 mL) was added hydrazine (1.8 mL, 55.4 mmol). After 90 mins at 90 °C, the white precipitate was filtered through a glass sintered

funnel and washed several times with Et₂O (4 × 50 mL). Evaporation under reduced pressure afforded a yellow oil. The crude of the formed free amine was directly used for the next step. The crude material obtained in the aforementioned step was dissolved in a mixture of 1,4-dioxane/15% aqueous Na₂CO₃ solution (1/1) (50 mL) followed by the addition of Fmoc-Cl (2.87 g, 11.1 mmol). After 3 h at room temperature, the reaction mixture was diluted with Et₂O (50 mL), washed with water (2 × 100 mL) and the aqueous layer was extracted with Et₂O (2 × 50 mL). The combined organic layers were washed with 10% aqueous solution of HCl (3 × 50 mL), brine (3 × 50 mL), dried with MgSO₄ and concentrated *in vacuo*. The resulting yellow oil was purified using silica gel chromatography (hexane/EtOAc: 7/3, R_f = 0.1) to give the title compound **6** in 99% yield over two steps (4.92 g) as a colourless oil.

¹H NMR (400 MHz, CDCl₃): δ (ppm) 1.51 (s, 9H), 3.35-3.39 (m, 2H), 3.53 (t, J = 4.8 Hz, 2H), 3.58-3.63 (m, 4H), 4.20 (t, J = 7.1 Hz, 1H), 4.37 (d, J = 7.1 Hz, 2H), 4.84 (s, 2H), 5.41 (s, br, 1H), 7.27-7.41 (m, 9H), 7.62 (d, J = 7.4 Hz, 2H), 7.76 (d, J = 7.5 Hz, 2H). ¹³C NMR (126 MHz, CDCl₃): δ (ppm) 28.33, 40.86, 47.18, 49.14, 66.75, 66.93, 69.97, 77.00, 81.76, 120.05, 125.31, 127.13, 127.74, 128.57, 128.69, 129.57, 135.59, 141.30, 144.04, 156.68, 157.49. HRMS (ESI+): calcd for C₃₁H₃₆N₂O₆Na⁺ 555.24656, found 555.24622.

10-(benzyloxy)-1-(9*H*-fluoren-9-yl)-3,11-dioxo-2,7-dioxa-4,10-diazatetradecan-14-oic acid (7)



TFA (11 mL) was added to a stirred solution of compound **6** (4.92 g, 9.2 mmol) in CH₂Cl₂ (11 mL). After 2.5 h at room temperature, the volatile compounds were removed using nitrogen flow to provide a pink oil that was purified using silica gel chromatography (hexane/ EtOAc: 5/5, $R_f = 0.2$) as a yellow oil.

To a stirred solution of the previous crude oil (4.34 g, 10.0 mmol) in DMF (30 mL) under a N₂ atmosphere was added succinic anhydride (1.00 g, 10.0 mmol) followed by DIPEA (10 mL). After 1 h at room temperature, the media was evaporated using high vacuum to give an orange oil. The crude product was purified using silica gel column (CH₂Cl₂/MeOH: 9/1) to give the title compound **7** in 88% yield (4.71 g) as an orange oil.

¹H NMR (400 MHz, CDCl₃): δ (ppm) 2.62-2.67 (m, 2H), 2.70-2.74 (m, 2H), 3.32-3.36 (m, 2H), 3.51 (t, J = 3.5 Hz, 2H), 3.63-3.64 (m, 2H), 3.81 (m, 2H), 4.17-4.24 (m, 1H), 4.34-4.41 (m, 2H), 4.86 (s, 2H), 5.49 (s, br, 1H), 7.27-7.31 (m, 2H), 7.36-7.40 (m, 7H), 7.55-7.60 (m, 2H), 7.73-7.75 (d, J = 7.5 Hz, 2H). ¹³C NMR (126 MHz, CDCl₃): δ (ppm) 27.45, 28.64, 41.01, 46.28, 47.32, 66.78, 66.95, 69.79, 77.36, 120.03, 125.25, 127.15, 127.75, 128.85, 129.13, 129.36, 134.58, 141.39, 144.12, 156.71, 174.56, 176.35. HRMS(ESI+): calcd for C₃₀H₃₃N₂O₇+ 533.22823, found 533.22839.

Benzyl protected OxoDFO* (10)



TentaGel S RAM Resin (0.55 g, 0.14 mmol) was deprotected using a solution of 20% piperidine in DMF (8 mL) a first time for 2 minutes and a second time for 10 minutes. After each deprotection, the resin was washed with DMF (5×8 mL), CH₂Cl₂ (5×8 mL) and DMF

 $(5 \times 8 \text{ mL})$. To a solution of HATU (205 mg, 0.54 mmol) in 3.5/3.5/93 (v/v/v) DIPEA (26 mg/mL, 0.20 mmol)/2,6-lutidine (32 mg/mL, 0.30 mmol)/DMF (1 mL) was added monomer 7 (294 mg, 0.55 mmol). The coupling solution was mixed with the resin and the reaction mixture was shaken at room temperature for 2 h. The complete conversion was confirmed by Kaiser Test and repeated if necessary.² This procedure was repeated 3 times to afford tetramer **9** on the resin. After Fmoc deprotection with 20% piperidine in DMF (8 mL), the resin was cleaved using 38/1/1 (v/v/v) TFA/TIPS/H₂O (8 mL). After shaking for 4 h, the recovered solution was evaporated using nitrogen flow and the resulting oil was washed with ice-cold Et₂O (3 × 10 mL), sonicated, centrifugated and evaporated using high vacuum to provide the title compound **10** in quantitative yield (163 mg) as a yellow oil with a purity $\geq 82\%$.

¹H NMR (500 MHz, MeOD, recorded after complete H/D exchange): δ (ppm) 2.45-2.49 (m, 8H), 2.72-2.75 (m, 8H), 3.07 (t, *J* = 3.5 Hz, 2H), 3.48-3.49 (m, 6H), 3.63-3.69 (m, 16H), 3.76-3.86 (m, 8H), 4.91-4.92 (m, 8H), 7.37-7.43 (m, 20H). ¹³C NMR (126 MHz, MeOD): δ (ppm) 28.52, 28.88, 30.56, 31.05, 40.47, 40.66, 45.65, 46.84, 67.83, 70.30, 71.35, 77.51, 129.70, 129.95, 130.67, 136.19, 174.77, 176.28, 177.46. HRMS(ESI+): calcd for C₆₀H₈₄N₉O₁₆Na²⁺ 604.79614, found 604.79591.

OxoDFO* (3)



A mixture of protected oxoDFO* **10** (14.7 mg, 0.01 mmol) and MeOH (7 mL) was sonicated for 10 min in an ultrasonic bath. The resulting solution was transferred to an autoclave, 10% Pd/C (3.6 mg, 0.03 mmol) was added and the mixture was stirred for 4 h under a H₂ atmosphere

(5 bar). The reaction mixture was then filtered through a sintered glass funnel (porosity 4) at ambient pressure and the latter was washed with MeOH (5 × 10 mL). The combined filtrates were evaporated under reduced pressure and the resulting oil was washed with acetonitrile (3 × 5 mL), sonicated, centrifugated and concentrated under reduced pressure to give the title compound in 91% yield (9.3 mg) as a yellow oil with a purity \geq 77%.

¹H NMR (500 MHz, MeOD, recorded after complete H/D exchange): δ (ppm) 2.51-2.53 (m, 8H), 2.79-2.80 (m, 8H), 3.11 (t, *J* = 4.3 Hz, 2H), 3.33-3.35 (m, 6H), 3.51-3.54 (m, 6H), 3.65-3.67 (m, 8H), 3.72-3.74 (m, 2H), 3.77-3.82 (m, 8H). ¹³C NMR (126 MHz, MeOD): δ (ppm) 28.69, 28.92, 30.75, 31.36, 40.47, 40.75, 67.76, 67.89, 70.25, 71.35, 175.19, 175.54, 177.82. HRMS(ESI+): calcd for C₃₂H₆₀N₉O₁₆⁺ 826.41525, found 826.41537.

OxoDFO*-NCS (11)



To a stirred solution of 4-isothiocyanatobenzoic acid (6.5 mg, 0.036 mmol) in DMF (2 mL) under a N₂ atmosphere was added HATU (20.5 mg, 0.054 mmol) followed by DIPEA (12.54 μ L, 0.072 mmol). After 40 min at room temperature, oxoDFO* **3** (30 mg, 0.036 mmol) and DIPEA (12.54 μ L, 0.072 mmol) were added and the reaction mixture was stirred at the same temperature for 12 h. The solvent was then removed under vacuum. The resulting oil was washed with acetone (3 × 10 mL) and taken up in MeOH. The resulting suspension was filtered and washed with MeOH (3 × 10 mL). The combined filtrates were evaporated under reduced pressure to give an orange oil that was purified using preparative HPLC at a flow rate of 16 mL/min with a linear gradient of A (Acetonitrile (Sigma-Aldrich, HPLC grade) and B (distilled

water containing 0.1% TFA): $t = 0 \min A 23\% + B 77\%$, $t = 10 \min A 23\% + B 77\%$, $t = 15 \min A 30\% + B 70\%$, $t = 30\min A 30\% + B 70\%$. After lyophilization, the title compound **8** was obtained as a white powder in 15% yield (5.4 mg) with a purity $\ge 95\%$.

¹H NMR (500 MHz, MeOD, recorded after complete H/D exchange): δ (ppm) 2.45-2.51 (m, 8H), 2.75-2.79 (m, 8H), 3.33 (m, 7H), 3.52-3.55 (m, 8H), 3.65-3.69 (m, 10H), 3.78 (m, 7H), 4.56 (s, br, 2H), 7.37-7.38 (d, *J* = 7.20 Hz, 2H), 7.88-7.89 (d, *J* = 6.80 Hz, 2H). ¹³C NMR (126 MHz, MeOD): δ (ppm) 28.70, 28.93, 30.76, 31.37, 40.46, 40.98, 41.08, 67.78, 70.26, 126.74, 129.14, 130.12, 134.48, 135.56, 168.91, 169.66, 175.18, 177.82. HRMS (ESI-): calcd for C₄₀H₆₁N₁₀O₁₇S⁻ 985.3936, found 985.3954.

3) Complexation Reaction of oxoDFO* (3) with Non-radioactive ^{nat}Zr(IV)

OxoDFO*-natZr (natZr-3)

HN HN HN H2 NH2 NH2

Using a procedure reported by Patra *et al.*³, a solution of ZrCl₄ (0.5 mg, 2.2 μ mol) in 0.1 M HCl (200 μ L) was added to a stirred solution of ligand oxoDFO*, **3**, (1.6 mg, 1.9 μ mol) in 0.1 M HCl (200 μ L). The pH of the solution was adjusted to 7.5 by slow addition of 0.1 M K₂CO₃ and stirred at RT overnight. The mixture was lyophilized to give a white powder. Formation of

the desired product was confirmed by a single peak in LC trace with HRMS also confirming complex formation: HRMS(ESI+): calcd for $C_{32}H_{55}N_9O_{16}{}^{90}ZrNa^+$ 934.2706, found 934.2706.

4) Determination of Lipophilicity (log D_{7.4})

Lipophilicity was assessed by the shake flask method. To a solution containing 600 μ L *n*-octanol and 600 μ L of PBS (pH 7.4) (obtained from saturated *n*-octanol-PBS solution), 30 μ L of a 1 μ M solution of oxoDFO*, **3**, oxoDFO*-*p*Phen-NCS, **11**, and ^{nat}Zr-**3** in water was added. The resulting solutions were vortexed for 5 min at room temperature, centrifuged (5 min, 1200 rpm), and lipophilicity was calculated by determination of the amount of analyte in each phase by analytical HPLC at a flow rate of 1.0 mL/min with a gradient of 95% buffer B to 90% buffer A over 15 min (buffer B = 0.1% TFA in MilliQ water, buffer A = 100% ACN). Experiments were carried out in triplicates.

5) ¹H NMR, ¹³C NMR and Mass Spectral Analysis of Compounds



Figure S1: ¹H NMR spectrum of *tert*-butyl (benzyloxy)(2-(2-(1,3-dioxoisoindolin-2-yl)ethoxy)ethyl)carbamate (**5**) in CDCl₃



Figure S2: ¹³C NMR spectrum of *tert*-butyl (benzyloxy)(2-(2-(1,3-dioxoisoindolin-2-yl)ethoxy)ethyl)carbamate (**5**) in CDCl₃



Figure S3: HRMS of *tert*-butyl (benzyloxy)(2-(2-(1,3-dioxoisoindolin-2-yl)ethoxy)ethyl)carbamate (5)



Figure S4: ¹H NMR spectrum of *tert*-butyl(2-(2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)ethoxy)ethyl) (benzyloxy)carbamate (**6**) in CDCl₃



Figure S5: ¹³C NMR spectrum of *tert*-butyl(2-(2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)ethoxy)ethyl) (benzyloxy)carbamate (**6**) in CDCl₃



Figure S6: HRMS of *tert*-butyl(2-(2-((((9*H*-fluoren-9yl)methoxy)carbonyl)amino)ethoxy)ethyl) (benzyloxy)carbamate (**6**)



Figure S7: ¹H NMR spectrum of 10-(benzyloxy)-1-(9*H*-fluoren-9-yl)-3,11-dioxo-2,7-dioxa-4,10-diazatetradecan-14-oic acid (7) in CDCl₃



Figure S8: ¹³C NMR spectrum of 10-(benzyloxy)-1-(9*H*-fluoren-9-yl)-3,11-dioxo-2,7-dioxa-4,10-diazatetradecan-14-oic acid (7) in CDCl₃



Figure S9: HRMS spectrum of 10-(benzyloxy)-1-(9*H*-fluoren-9-yl)-3,11-dioxo-2,7-dioxa-4,10-diazatetradecan-14-oic acid (7)



Figure S10: ¹H NMR spectrum of benzyl-protected-oxoDFO* (10) in MeOD



Figure S11: ¹³C NMR spectrum of benzyl-protected-oxoDFO* (10) in MeOD



Figure S12: HRMS spectrum of benzyl-protected-oxoDFO* (10)





Figure S14: ¹H NMR spectrum of oxoDFO* (3) in MeOD



Figure S15: ¹³C NMR spectrum of oxoDFO* (3) in MeOD



Figure S16: HRMS spectrum of oxoDFO* (3)



Figure S17: UV trace and corresponding MS trace of oxoDFO* (3)



Figure S18: ¹H NMR spectrum of oxoDFO*-NCS (11) in MeOD



Figure S19: ¹³C NMR spectrum of oxoDFO*-NCS (11) in MeOD



Figure S20: HRMS spectrum of oxoDFO*-NCS (11)



Figure S21: UV trace and corresponding MS trace of oxoDFO*-NCS (11)



Figure S23: HRMS spectrum of oxoDFO*-^{nat}Zr (^{nat}Zr-**3**)



6) References

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