SUPPORTING INFORMATION

Evaluation of 3-hydroxypyridin-2-one (2,3-HOPO) based macrocyclic chelator for $^{89}Zr^{4+}$ and its use for immunoPET imaging of HER2 positive model of ovarian carcinoma in mice

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MATERIALS AND METHODS

Ligand Synthesis

Electrospray ionization (ESI) high-resolution mass spectra (HRMS) were obtained by the Mass Spectrometry Facility, College of Chemistry, University of California, Berkeley, CA. Flash chromatography was performed using EM Science Silica Gel 60 (230 - 400 mesh). NMR spectra were obtained using either Bruker AM-300 or AV-600 spectrometers operating at 300 (75) MHz and 600 (150) MHz for ^{1}H (or ^{13}C) respectively. ^{1}H (or ^{13}C) chemical shifts are reported in parts per million (ppm) relative to the solvent resonances, taken as δ 7.26 (δ 77.0) for $CDCl_3$. For the deprotected macrocycles **1** and **2**, the observed NMR spectra were very complicated due to the presence of differing conformers/isomers in solution, and are not reported.[3] Analytical HPLC was performed on an Agilent 1200 instrument (Agilent, Santa Clara, CA) equipped with a diode array detector (λ = 280 or 315 nm, 600 nm reference), a thermostat set at 25 °C, and a Zorbax Eclipse XDB-C18 column (4.6 x 150 mm, 5 µm, Agilent, Santa Clara, CA). The mobile phase of a binary gradient (Method 1: 2-40% B/20 min; solvent A, 0.1% TFA; solvent B, ACN or Method 2: 10-60% B) at a flow rate of 1 mL/min was used for analytical HPLC. All compounds were ≥95% pure. 3-(Benzyloxy)-6-methyl-1-(2-oxo-2-(2-thioxothiazolidin-3-yl)ethyl)-4-(2-thioxothiazolidine-3-carbonyl)pyridin-2(1H)-one **3**, N-[2-[2-(2-methoxyethoxy)ethoxy]ethoxy]-bis(2-aminoethyl)amine **4** and 5-amino-6-[(2-aminoethyl)-[2-[bis(2-aminoethyl)amino]ethyl]amino]hexylcarbamic acid tert-butyl ester **6** were prepared as previously described.[1-3] Unless otherwise noted, all other chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO USA), and solutions were prepared using ultrapure water (18 MΩ-cm resistivity).

Synthesis of di-macrocycle **1** (Scheme S1). Overview: An excess of di-thiazolide **3** was condensed with N-[2-[2-(2-methoxyethoxy)ethoxy]ethoxy] diethylenetriamine **4** to provide the activated di-amide **5**, which was reacted with amine **6** under high dilution conditions to form the regioisomeric di-macrocycles **7** and **8**. Protective groups were removed using a solution of concentrated hydrochloric acid in acetic acid to provide di-macrocycles **1** and **2**. The structures of **1** and **2** were assigned from fragmentation patterns using tandem mass

spectrometry. Lower Rf isomer **8** was isolated in poorer yield; as a result, biological studies were not conducted using derivative **2**.

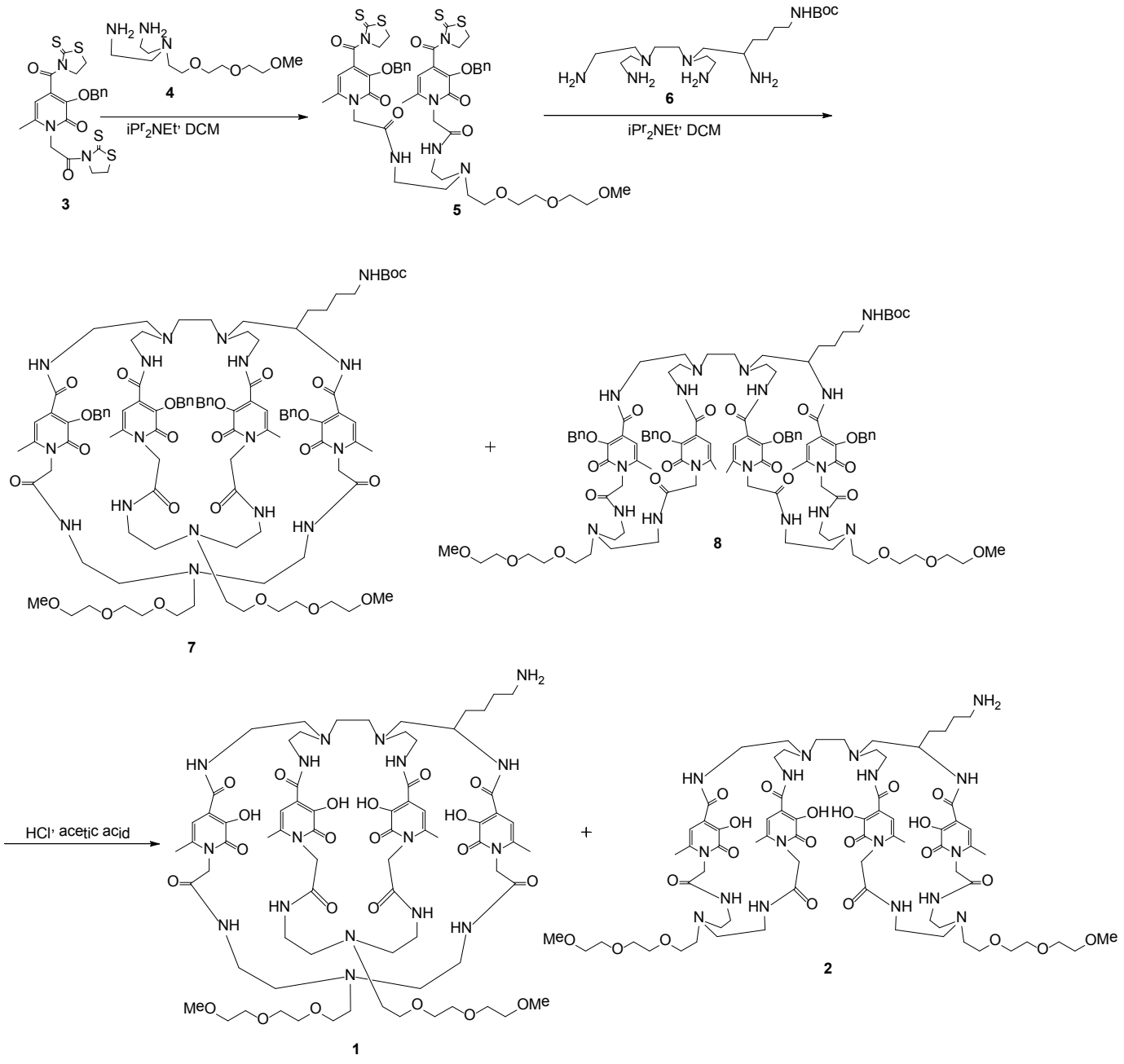
N,N″-bis[3-benzyloxy-1-carbamidomethyl-6-methyl-2-oxo-1,2-dihydropyridine-4-carbonyl(2-mercaptothiazolide)]-N′-[2-[2-(2-methoxyethoxy)ethoxy]ethoxy]-bis(2-aminoethyl)amine **5**. N-[2-[2-(2-methoxyethoxy)ethoxy]ethoxy]-bis(2-aminoethyl)amine **4** (382 mg, 1.54 mmol) was dissolved in dichloromethane (33 mL) and diisopropylethylamine (0.8 mL) and added using a syringe pump (NE1000) to a solution of 3-benzyloxy-1-carbonyl(2-mercaptothiazolide)methyl-6-methyl-2-oxo-1,2-dihydropyridine-4-carbonyl(2-mercaptothiazolide) **3** (1.99 g, 38.3 mmol) in dichloromethane (50 mL) over a period of 23 hrs at a rate of 1.50 mL/hr. After a further 24 hr, solvent was removed under reduced pressure, and the crude product was purified by silica gel chromatography using 0.1% triethylamine, 2 – 3.5% methanol in dichloromethane as eluents. Fractions containing product were combined, solvent was removed under reduced pressure, and the residue dried in vacuo to provide compound **5** (997 mg, 60.7%). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.45 – 7.30 (m, 10H, PhH), 6.21 (s, 2H, ArH), 5.19 (s, 4H, $PhCH_2O$), 4.76 (4H, s, $CH_2C{=}O$), 4.29 (t, 4H, NCH_2CH_2S), 3.61 – 3.51 (m, 10H, CH_2O), 3.35 (s, 3H, OCH_3), 3.30 (m, 4H, $CH_2NC{=}O$), 2.89 (t, 4H, NCH_2CH_2S), 2.66 (m, 6H, CH_2N), 2.36 (s, 6H, CH_3). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 200.7, 167.0, 165.9, 159.6, 141.8, 141.3, 137.7, 133.2, 128.4, 128.2, 128.1, 104.1, 73.8, 71.8, 70.6, 70.4, 70.2, 58.9, 55.1, 54.4, 52.9, 48.2, 38.0, 29.1, 20.5. FTMS pESI: calculated for $C_{49}H_{60}N_7O_{11}S_4$ $[MH]^+$, 1050.3228, found, 1050.3223.

Benzyl and tert-butyloxycarbonyl-protected di-macrocycles **7** and **8**. A solution of N,N″-bis[3-benzyloxy-1-carbamidomethyl-6-methyl-2-oxo-1,2-dihydropyridine-4-carbonyl(2-mercaptothiazolide)]-N′-[2-[2-(2-methoxyethoxy)ethoxy]ethoxy]-bis(2-aminoethyl)amine **5** (924 mg, 880 μmol) in dichloromethane (49.5 mL) and triethylamine (0.5 mL) and a solution of 5-amino-6-[(2-aminoethyl)-[2-[bis(2-aminoethyl)amino]ethyl]amino]hexylcarbamic acid tert-butyl ester **6** (213 mg, 528 μmol) in dichloromethane, isopropyl alcohol (ca. 5%), and diisopropylethylamine (ca. 3%) (50 mL) were added dropwise to dichloromethane (2 L) over a period of four days using two syringe pumps at a rate of 0.5 mL/hr. After an additional two days of reaction, solvent was removed under reduced pressure, and the crude products were purified by silica gel chromatography using 0.1% triethylamine, 5 – 7.5% methanol in dichloromethane as eluents. The silica gel column was prepared so as to have a short section (ca. 1.25”) of aluminum oxide (basic,

Brockmann I) on its bottom. Fractions containing product were combined, solvent was removed under reduced pressure, and the residue dried in vacuo to provide the protected di-macrocycles **7** (202 mg, 22.7%) and **8** (78 mg, 8.7%). The residue containing lower Rf compound **8** was additionally dissolved in dichloromethane (10 mL) and washed with water (2 x 10 mL) to remove triethylammonium salts prior to yield calculation. Compound **7**: ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.45 – 7.29 (m, 20H, PhH), 6.48 – 6.37 (m, 4H, ArH), 5.56 – 4.78 (m, 8H, $PhCH_2O$), 4.77 (8H, br s, $CH_2C=O$), 3.76 – 3.57 (m, 20H, CH_2O), 3.36 (s, 6H, OCH_3), 2.90 (m, 17H, $CH_2NC=O$, $CHNC=O$), 2.65 – 2.33 (m, 24H, CH_2N), 2.18 (m, 12H, CH_3), 1.39 (s, 9H, CH_3), 1.03 – 0.98 (m, 6H, CH_2). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 167.5, 163.5, 160.4, 155.9, 128.9, 128.6, 128.5, 128.4, 128.3, 74.5, 74.4, 71.9, 71.0, 70.5, 58.9, 52.9, 46.2, 40.4, 38.9, 28.4, 23.1, 20.3, 20.1, 20.0, 19.9, 11.6, 8.0. FTMS pESI: calculated for $C_{105}H_{145}N_{17}O_{24}$ $[M+2H]^{2+}$, 1014.0319, found, 1014.0342. Compound **8**: ^{1}H NMR (600 MHz, MeOD): δ = 7.41 – 7.30 (m, 20H, PhH), 6.32 – 6.30 (m, 4H, ArH), 5.26 – 4.97 (m, 8H, $PhCH_2O$), 4.71 (8H, br s, $CH_2C=O$), 3.69 – 3.56 (m, 20H, CH_2O), 3.35 (s, 6H, OCH_3), 3.34 - 2.90 (m, 17H, $CH_2NC=O$, $CHNC=O$), 2.70 – 2.21 (m, 24H, CH_2N), 2.27 (m, 12H, CH_3), 1.41 (s, 9H, CH_3), 1.34 – 1.16 (m, 6H, CH_2). ^{13}C NMR (150 MHz, MeOD): δ = 168.1, 164.0, 160.1, 143.1, 141.4, 141.3, 136.7, 136.6, 128.5, 128.4, 128.3, 128.2, 104.2, 78.6, 74.2, 74.0, 71.5, 70.3, 70.2, 70.1, 70.0, 57.7, 53.2, 52.8, 52.0, 48.4, 37.8, 37.6, 29.4, 27.4, 23.1, 19.3, 19.2. FTMS pESI: calculated for $C_{105}H_{145}N_{17}O_{24}$ $[M+2H]^{2+}$, 1014.0319, found, 1014.0351.

Di-macrocycle **1**. Benzyl and tert-butyloxycarbonyl-protected di-macrocycle **7** (51 mg, 25 μmol) was dissolved in 12N hydrochloric acid (1.0 mL) and glacial acetic acid (1.0 mL). The solution was stirred under inert atmosphere for 23 hr, whereupon HCl was removed with a stream of inert gas. Solvents were removed under reduced pressure and the residue was dried in vacuo. The residue was dissolved in methanol (600 + 300 μL) and transferred to two O-ring microcentrifuge tubes. Ether (ca. 1.5 mL) was added, and the tubes were placed at 4 ^{o}C for 1 hr. The tubes were centrifuged at 12,000 rpm for 3 minutes, decanted, the pellets were washed with ether (ca. 1.5 mL) and allowed to air dry. The pellets were dried in vacuo to provide di-macrocycle **7**, pentahydrochloride salt (39.8 mg, 90%). FTMS pESI: calculated for $C_{72}H_{113}N_{17}O_{22}$ $[M+2H]^{2+}$, 783.9118, found, 783.9140. Analysis (C,H,N): Calc. for $C_{72}H_{111}N_{17}O_{22}.5(HCl).10(H_2O)$, 44.81, 7.11, 12.35; found, 45.05, 7.45, 12.04. Di-macrocycle **2** was formed from compound **8** following a similar procedure (89%). FTMS pESI: calculated for $C_{72}H_{110}FeN_{17}O_{22}$ $[M+Fe]^{2+}$, 810.3681, found, 810.3706. Tandem mass spectrometry (TOF MSMS

ES$^+$) performed on compound **2**, 1566.83 MS1 peak $[M+H]^+$, revealed peaks at mass 761.39 $[M+H]^+$ and 832.45 $[M+H]^+$consistent with fragmentation across the ethylene diamine bridge. Similar fragmentation was not observed upon analysis of compound **1**, where the main fragment observed was 1548.77 $(M-H_2O)^+$.



Scheme S1. Synthesis of 3,2-HOPO ligands **1** and **2**.

Di-macrocycle **1**, 4-isothiocyanatophenylthiourea derivative (**1**-NCS). Di-macrocycle **1** (31.0 mg, 17.7 μmol) was dissolved in dimethylformamide (886 μL) and triethylamine (49.4 μL). The solution was transferred in 5

aliquots to a microcentrifuge tube containing 1,4-phenyldiisothiocyanate (34.1 mg, 177 μmol) dissolved in dimethylformamide (345 μL) and mixed at 800 rpm under inert atmosphere for 1.5 hours. The resulting solution was distributed to 5 additional microcentrifuge tubes. Ether (ca. 1.8 mL per tube) was added, and the resulting suspension placed at 4 °C for ca. 2 hr. The tubes were centrifuged at 12,000 rpm for 3 minutes, decanted, the pellets were washed with ether (ca. 2 mL) and allowed to air dry. The pellets were dissolved in dimethylformamide (40 μL) and methanol (300 μL), and precipitated and washed with ether as described above. The pellets were dried in vacuo to provide di-macrocycle **1**, 4-isothiocyanatophenylthiourea derivative (**1**-NCS) (29.3 mg, 94%). FTMS pESI: calculated for C80H116N19O22S2 [MH]+, 1758.7984, found, 1758.8022.

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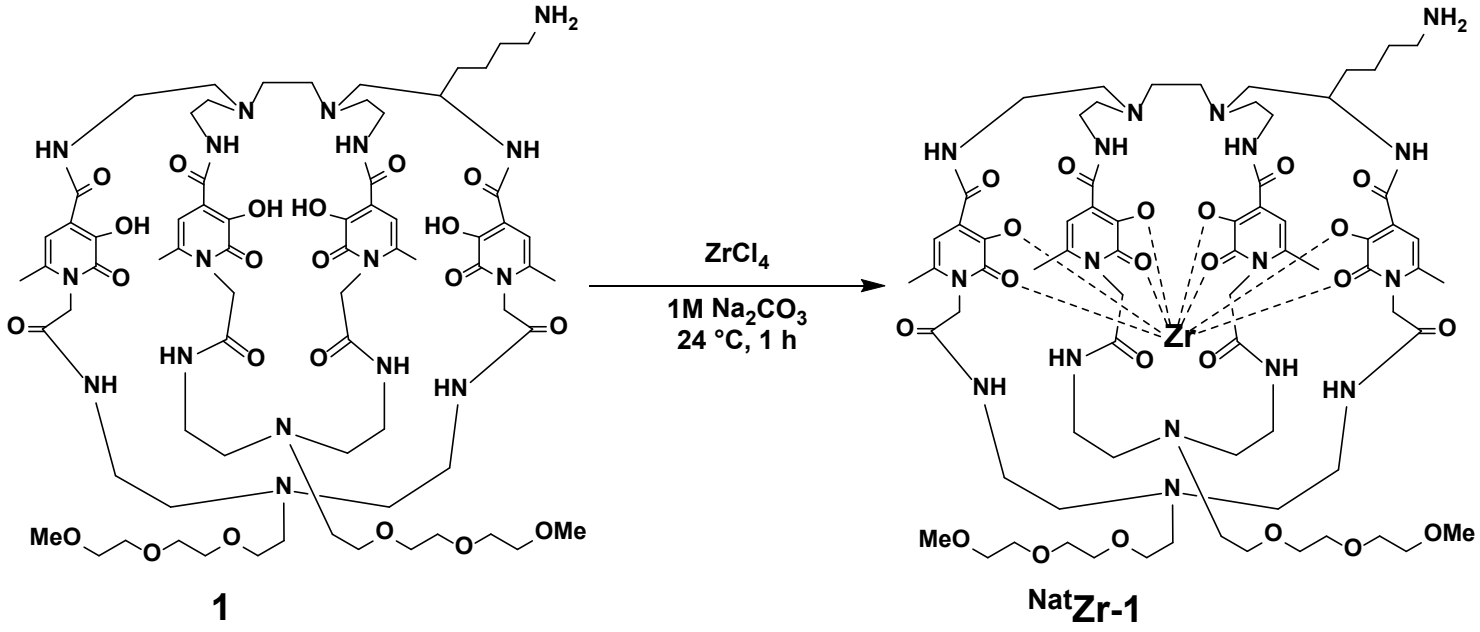


Scheme S2. Synthesis of ^{Nat}Zr-**1**.

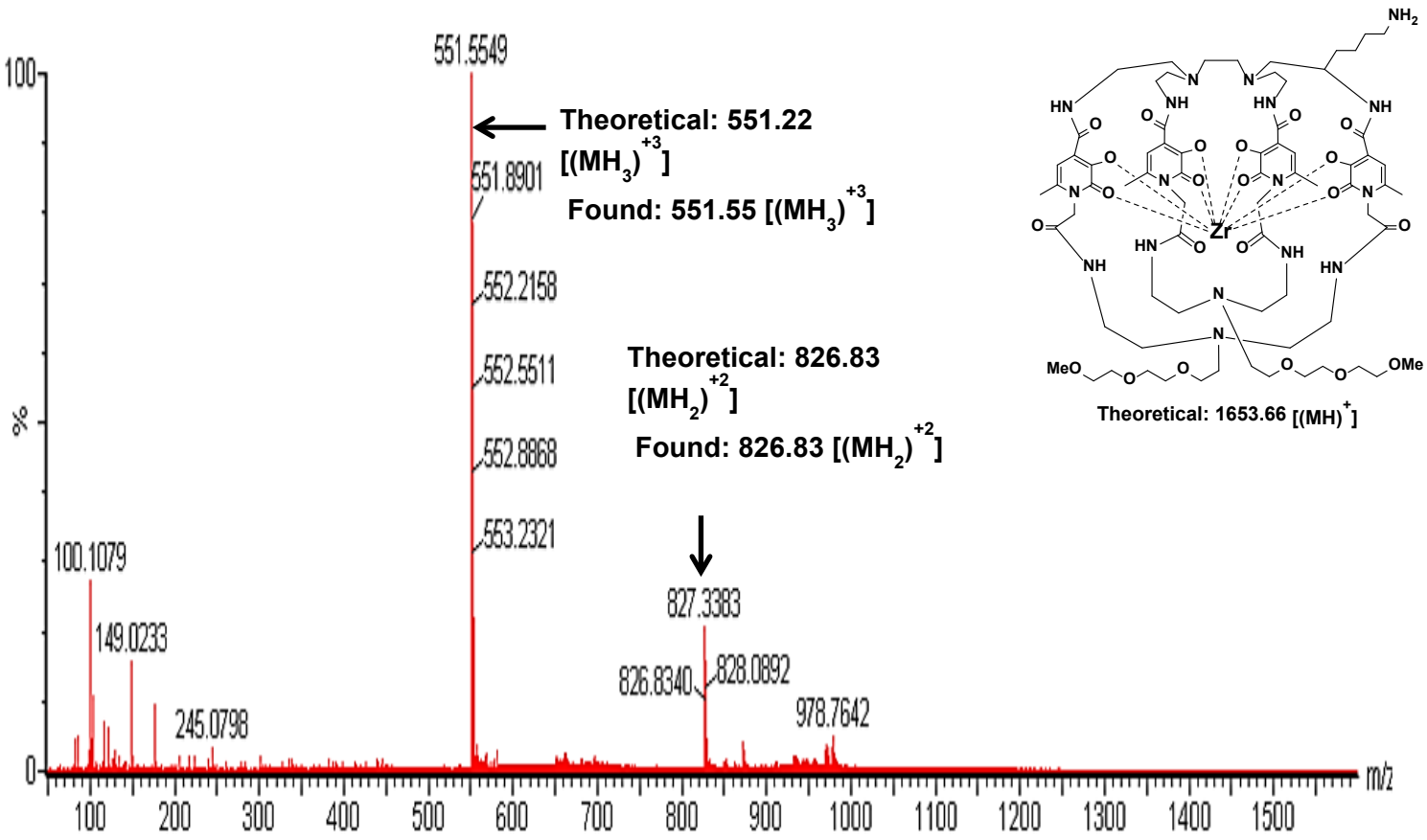
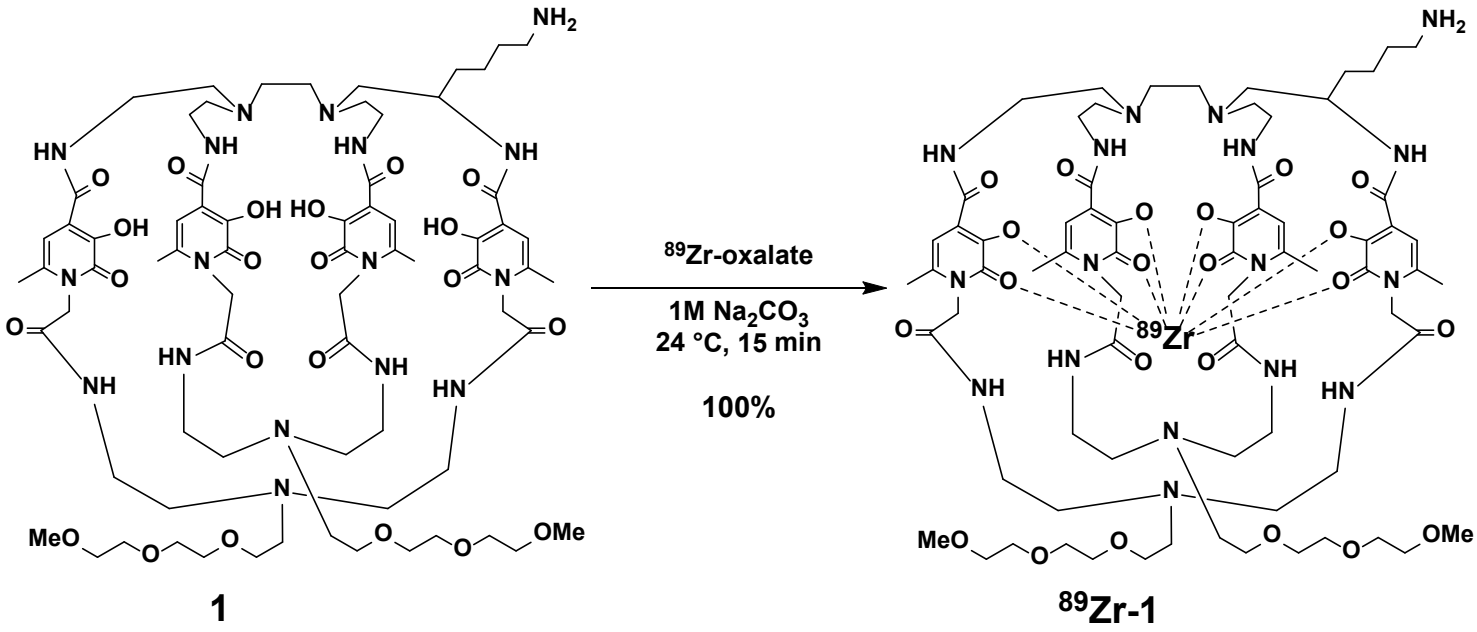


Figure S1. ESI-MS of ^{Nat}Zr-**1**.



Scheme S3. Radiochemical Synthesis of ^{89}Zr-**1**.

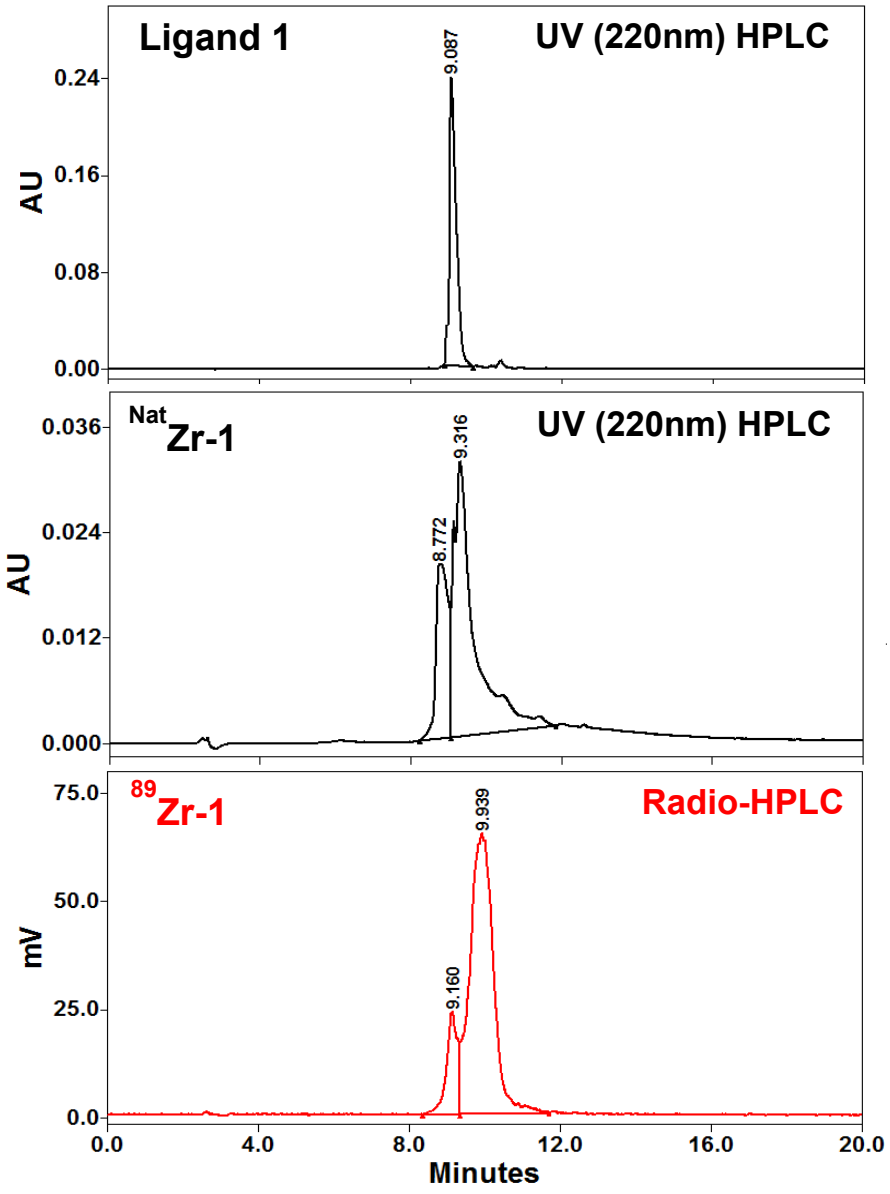


Figure S2. HPLC quality control analysis of ^{89}Zr-**1.**

Table S1. *In vitro* stability: 50mM DTPA challenge (% intact complex)

Day	**^{89}Zr-DFO**	**^{89}Zr-1**
1	55	100
2	54	97
3	53	95
4	47	93
5	44	88
6	43	81
7	41	78

Table S2. *In vitro* stability: Human serum challenge (% intact complex)

Day	**^{89}Zr-DFO**	**^{89}Zr-1**
1	100	94
2	100	92
3	100	91
4	100	89
5	100	88
6	100	87
7	100	86

Table S3. **Biodistribution (%ID/g) of ^{89}Zr-1 in selected organs at 2, 4, 24, 48, and 72 h p.i.**

Tissue/Organ	%ID/g (^{89}Zr-1)				
	2 h	4 h	24 h	48 h	72 h
Blood	0.045 ± 0.019	0.017 ± 0.008	0.004 ± 0.001	0.001 ± 0.001	0.003 ± 0.002
Heart	0.049 ± 0.010	0.044 ± 0.016	0.027 ± 0.006	0.022 ± 0.011	0.022 ± 0.007
Lung	0.174 ± 0.046	0.114 ± 0.076	0.080 ± 0.020	0.055 ± 0.013	0.045 ± 0.009
Liver	0.640 ± 0.078	0.701 ± 0.099	0.650 ± 0.080	0.496 ± 0.074	0.432 ± 0.031
SMI + contents	0.211 ± 0.188	0.107 ± 0.055	0.026 ± 0.005	0.021 ± 0.005	0.015 ± 0.001
LGI + contents	0.837 ± 0.485	0.563 ± 0.178	0.043 ± 0.015	0.030 ± 0.003	0.025 ± 0.004
Kidney	34.185 ± 2.393	38.504 ± 7.070	29.191 ± 6.989	18.512 ± 1.350	14.733 ± 2.204
Spleen	0.120 ± 0.031	0.120 ± 0.028	0.137 ± 0.117	0.083 ± 0.020	0.074 ± 0.016
Pancreas	0.033 ± 0.005	0.028 ± 0.006	0.021 ± 0.014	0.022 ± 0.009	0.016 ± 0.006
Stomach	0.197 ± 0.226	0.073 ± 0.028	0.024 ± 0.011	0.018 ± 0.007	0.013 ± 0.003
Muscle	0.030 ± 0.017	0.038 ± 0.025	0.007 ± 0.016	0.011 ± 0.018	0.007 ± 0.005
Fat	0.025 ± 0.014	0.018 ± 0.008	0.013 ± 0.006	0.011 ± 0.006	0.012 ± 0.005
Bone	0.170 ± 0.042	0.204 ± 0.088	0.272 ± 0.066	0.249 ± 0.063	0.283 ± 0.083

Table S4. Biodistribution (%ID/g) of ^{89}Zr-DFO in selected organs at 2, 4, 24, 48, and 72 h p.i.

Tissue/Organ	2 h	4 h	24 h	48 h	72 h
Blood	0.009 ± 0.003	0.005 ± 0.001	0.001 ± 0.001	0.001 ± 0.001	0.000 ± 0.001
Heart	0.020 ± 0.003	0.019 ± 0.003	0.014 ± 0.002	0.010 ± 0.002	0.009 ± 0.004
Lung	0.060 ± 0.009	0.038 ± 0.006	0.024 ± 0.006	0.019 ± 0.005	0.017 ± 0.004
Liver	0.234 ± 0.023	0.163 ± 0.051	0.081 ± 0.012	0.070 ± 0.007	0.066 ± 0.009
Small intestine	0.357 ± 0.175	0.130 ± 0.080	0.013 ± 0.002	0.008 ± 0.001	0.006 ± 0.001
Large intestine	0.877 ± 0.435	1.020 ± 0.207	0.024 ± 0.004	0.009 ± 0.002	0.008 ± 0.001
Kidney	2.051 ± 0.238	1.848 ± 0.382	1.340 ± 0.137	0.957 ± 0.216	0.689 ± 0.098
Spleen	0.037 ± 0.005	0.036 ± 0.004	0.036 ± 0.007	0.030 ± 0.008	0.027 ± 0.007
Pancreas	0.015 ± 0.005	0.013 ± 0.002	0.012 ± 0.002	0.009 ± 0.003	0.007 ± 0.002
Stomach	0.140 ± 0.124	0.055 ± 0.038	0.014 ± 0.005	0.005 ± 0.003	0.005 ± 0.002
Muscle	0.011 ± 0.001	0.008 ± 0.003	0.006 ± 0.002	0.004 ± 0.001	0.004 ± 0.002
Fat	0.013 ± 0.003	0.009 ± 0.002	0.007 ± 0.002	0.005 ± 0.008	0.008 ± 0.004
Bone	0.051 ± 0.017	0.058 ± 0.008	0.082 ± 0.016	0.092 ± 0.011	0.078 ± 0.014

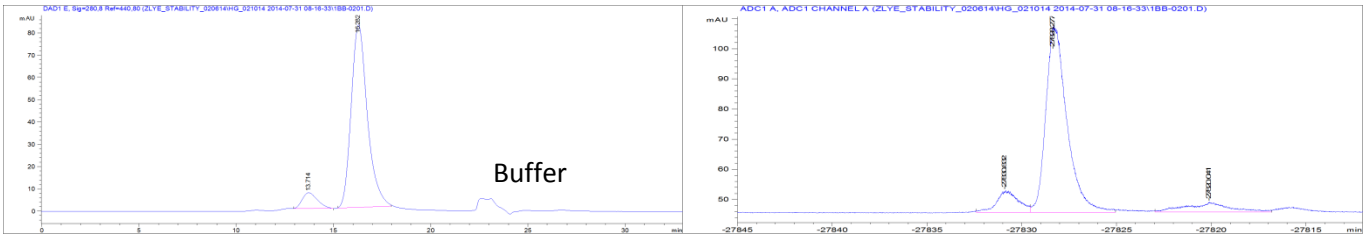
This data was published previously in Pandya DN, Pailloux S, Tatum D, Magda D, Wadas TJ. Di-macrocyclic terephthalamide ligands as chelators for the PET radionuclide zirconium-89. Chem Commun (Camb). 2015; 51: 2301-3.

Table S5. Analytical data for ^{89}Zr-labeled mAbs

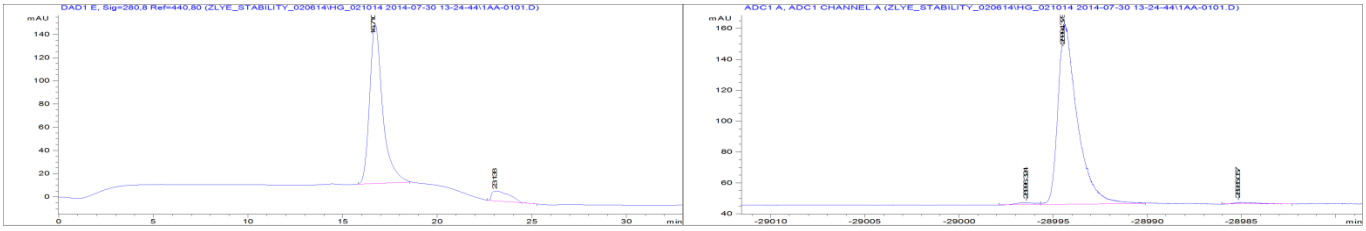
Compound	Loading (per mAb)	HER2 K_D (pM)	Chelation yield (%)	Purity UV 280 nm (%)	Purity radioactivity (%)	Specific Activity (MBq/mg)
1-trastuzumab	1.7	496	60	93	81	30
DFO-trastuzumab	1.3	481	83	99	98	44
1-gD	1.7	-	69	96	64	33
DFO-gD	1.4	-	79	99	96	44

Figure S3. SEC HPLC traces for ^{89}Zr-labeled mAbs, UV 280 nm (left) and PMT (right)

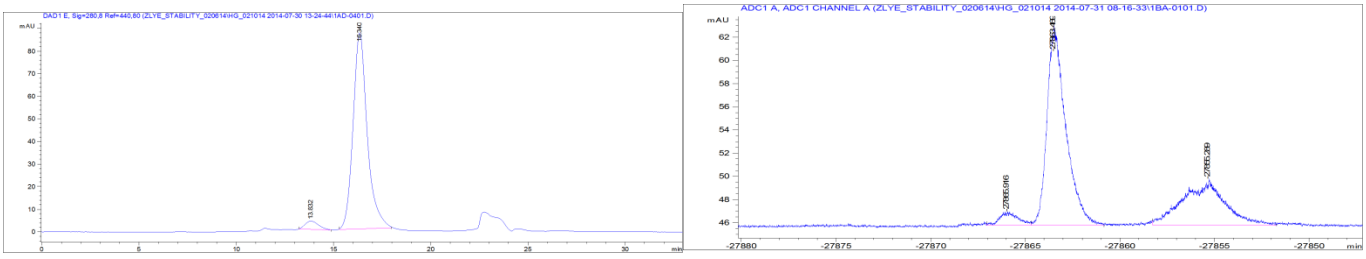
^{89}Zr-**1**-trastuzumab



^{89}Zr-DFO-trastuzumab



^{89}Zr-**1**-gD



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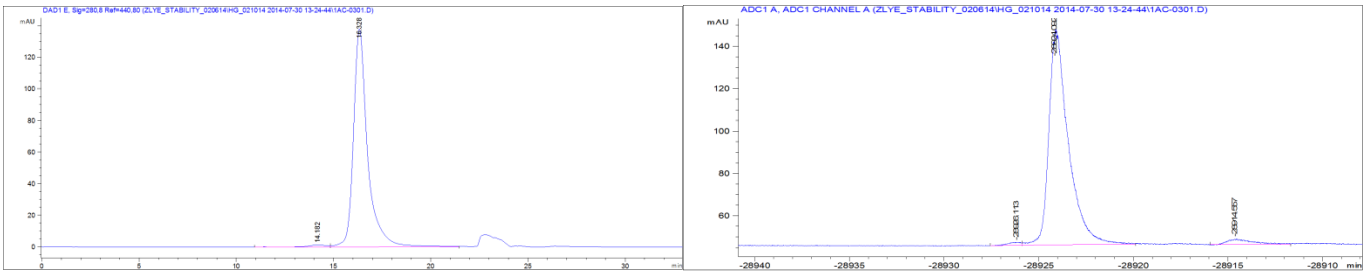

Figure S4 Serum stability of ^{89}Zr-**1**-trastuzumab