Investigation of the complexation of ^{nat}Zr(IV) and ^{89}Zr(IV) by hydroxypyridinones for the development of chelators for PET imaging applications

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Supplementary information

1. Ligands synthesis

General. All reagents and solvents were obtained commercially and used without further purification unless otherwise noted. 1-Benzyloxy-6-carboxy-2-(1*H*)-pyridinone (**1**),[1] 3-(Benzyloxy)-1 (2'-carboxyethyl)-2-(1*H*)-pyridinone (**5**),[2] 1-(2'-Carboxyethyl)-2-methyl-3-(benzyloxy)-4(1*H*)-pyridinone **(8)**[3] and 1-hydroxy-2(1*H*)-pyridinone-2-carboxylic acid propylamide (**L^{1}'**H)[4] were prepared as previously reported. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Advance 300 MHz instrument, and chemical shifts are reported in ppm on the δ scale relative to TMS. Electrospray ionization-mass spectra (ESI-MS) were acquired using an Agilent LC⁄MS system equipped with a multimode ion or using an Advion CMS spectrometer. Elemental analyses were performed by Galbraith Lab. Inc. (Knoxville, TN) using combustion analysis methods for C, H, and N.

1,2-HOPOBn succinimidyl ester (2). To HOPOBn acid (**1**) (2.022 g, 8,25 mmol) in dry DMF (60 mL) was added *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDCI, 3.165 g, 16.5 mmol) followed by *N*-Hydroxysuccinimide (1.97 g, 16.5 mmol). The solution was stirred overnight at room temperature and solvent was evaporated in vacuo. The crude compound was purified on a silica gel column using CH_2Cl_2/AcOEt (9:1) as eluent, affording a colorless oil (1.75 g, 62%). ^{1}H NMR ($CDCl_3$, 300 MHz, ppm): δ 2.91 (s, 4H), 5.38 (s, 2H), 6.96 (m, 2H), 7.38 (m, 5H), 7.54 (m, 2H). ESI-MS: m/z = 343.1 $[M+H]^+$, 365.1 $[M+Na]^+$.

1,2-HOPOBn-CONHMe (3). To compound **2** (1.025 g, 2.95 mmol) dissolved in dry THF (50 mL) was added a 2 M solution of methylamine in THF (7.50 mL, 14.8 mmol). The solution was stirred for 3 h at room temperature. After evaporation of volatiles in vacuo, the crude mixture was purified on a silica gel column using CH_2Cl_2/MeOH (9:1) as eluent, affording a white solid (655 mg, 86%). ^{1}H NMR ($CDCl_3$, 300 MHz, ppm): δ 2.87 (d, 3H), 5.28 (s, 2H), 6.53 (m, 1H), 6.72 (m, 1H), 6.84 (s, 1H, broad), 7.28 (m, 1H), 7.33 (m, 5H). ESI-MS: m/z = 259.1 $[M+H]^+$, 281.1 $[M+Na]^+$.

1,2-HOPO-NHMe (L^{1}H). Compound **3** (503 mg, 1.95 mmol) was dissolved in a 1:1 mixture of AcOH/HCl_{conc} (10 mL) and stirred at room temperature for 4 days. Volatiles were evaporated in vacuo and the oily residue was triturated in Et_2O until it turns into a solid. It was filtered and dried in a oven at 100 °C overnight, affording a beige solid (298 mg, 91%). %). ^{1}H NMR ($CDCl_3$, 300 MHz, ppm): δ 3.06 (d, 3H), 7.06 (m, 1H), 7.49 (t, 1H, *J* = 8.1 Hz), 7.67 (m, 2H), 7.89 (s, 1H, broad), 9.58 (s, 1H, broad). %). ^{13}C NMR ($CDCl_3$, 75 MHz, ppm): δ 25.9, 103.7, 119.3, 137.2, 142.2, 157.5, 160.7). ESI-MS: m/z = 169.1

$[M+H]^+$, 167.1 $[M-H]^-$. Elemental analyses: Calculated for $C_7H_8N_2O_3$: C, 50.00; H, 4.80; N, 16.66%. Found: C, 49.56; H, 4.52; N, 16.58.

3,2-HOPOBn succinimidyl ester (6). To compound **5** (1.347 g, 4.93 mmol) in dry DMF (40 mL), were added successively EDCI (1.93 g, 10.1 mmol) and *N*-hydroxysuccinimide (1.16 g, 10.1 mmol). After stirring the solution overnight at room temperature, a white precipitate has formed. DMF was evaporated in vacuo and the crude residue was washed with AcOEt and filtered. The white solid was dissolved in $CHCl_3$, washed with a 1N HCl solution, dried over $MgSO_4$ and evaporated, affording a white solid (1.653 g, 90 %). ^{1}H NMR (DMSO-d_6, 300 MHz, ppm) δ 2.81 (s, 4H), 3.16 (t, 2H, *J* = 6.9 Hz), 4.20 (t, 2H, *J* = 6.9 Hz), 5.01 (s, 2H), 6.13 (m, 1H), 6.92 (m, 1H), 7.30 (m, 1H), 7.37 (m, 5H). ESI-MS: m/z =371.1 $[M+H]^+$, 393.1 $[M+Na]^+$, 741.2 $[2M+H]^+$, 763.2 $[2M+Na]^+$.

3,2-HOPOBn-CONHMe (7). To compound **6** (1.224 g, 3.30 mmol) in dry DMF (30 mL) was added a 2 M methylamine solution in THF (4.14 mL, 8.25 mmol). The solution was stirred at room temperature overnight and the volatiles were evaporated in vacuo. The crude material was purified on a silica gel column using CH_2Cl_2/MeOH (95:5) as eluent, affording a colorless oil (812 mg, 86%). ^{1}H NMR ($CDCl_3$, 300 MHz, ppm): δ 2.59 (d, 3H), 2.70 (t, 2H, *J* = 6.3 Hz), 4.25 (t, 2H, 6.3 Hz), 5.02 (s, 2H), 6.04 (t, 1H, *J* = 7.2 Hz), 6.69 (m, 1H), 7.11 (m, 1H), 7.31 (m, 5H). ESI-MS: m/z =287.2 $[M+H]^+$.

3,2-HOPO-NHMe (L^2H). To compound **7** (596 mg, 2.08 mmol) dissolved in MeOH (10 mL) was added 10 % Pd/C (30 mg). The solution was shacked in a Paar apparatus with 30 psi hydrogen pressure for 48 h. Pd/C was then removed by 3 successive centrifugations at 8500 rpm for 15 min. Evaporation of the methanol in vacuo afforded a white solid (395 mg, 97%). ^{1}H NMR (DMSO-d_6, 300 MHz, ppm): δ 2.50-2.54 (m, 5H), 4.09 (m, 2H), 6.06 (m, 1H), 6.67 (m, 1H), 7.05 (m, 1H), 7.86 (s, 1H, broad), 8.97 (s, 1H, broad). ^{13}C NMR (DMSO-d_6, 75 MHz, ppm): δ 25.4, 34.3, 45.6, 105.0, 114.5, 128.4, 146.6, 157.6, 169.8.). ESI-MS: m/z =197.1 $[M+H]^+$, 219.1 $[M+Na]^+$, 195.1 $[M-H]^-$. Elemental analyses: Calculated for $C_9H_{12}N_2O_3$: C, 55.09; H, 6.16; N, 14.28%. Found: C, 54.96; H, 6.09; N, 13.86.

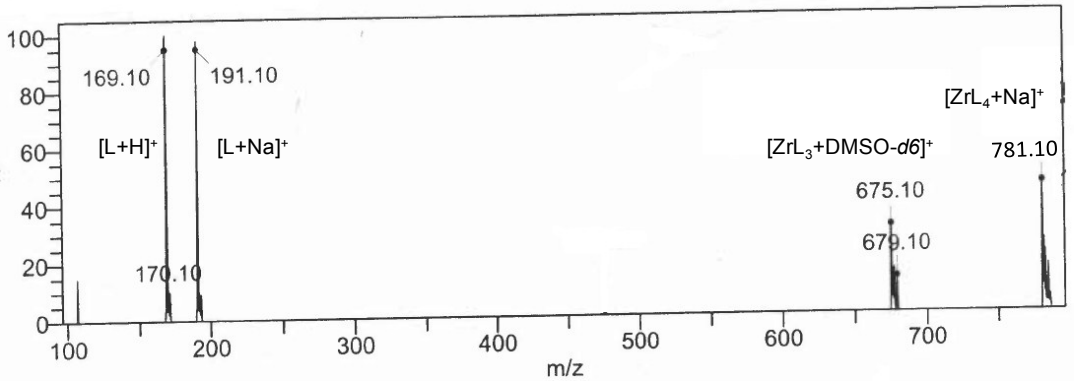
3,4-HOPOBn succinimidyl ester (9). To compound **8** (2.511 g, 8.74 mmol) dissolved in dry DMF (50 mL) was added EDCI (2.513 g, 13.11 mmol) and *N*-hydroxysuccinimide (1.507 g, 13.11 mmol). After one night at room temperature, the solvent was removed in vacuo. The oily residue was recrystallyzed in AcOEt, affording a yellowish solid (1.478g, 44%). %). ^{1}H NMR (DMSO-d_6, 300 MHz, ppm): δ 2.22 (s, 3H), 2.82 (s, 4H), 3.19 (t, 2H, *J* = 6.9 Hz), 4.24 (t, 2H, *J* =6.9 Hz), 5.00 (s, 2H), 6.13 (d, 1H, *J* = 7.8 Hz), 7.29-7.40 (m, 5H), 7.68 (d, 1H, *J* = 7.8 Hz). ESI-MS: m/z =385.1 $[M+H]^+$.

3,4-HOPOBn-NHMe (10). The same procedure as compound **7** was used, affording a white solid (74 %). ^{1}H NMR ($CDCl_3$, 300 MHz, ppm): δ 2.11 (s, 3H), 2.47 (t, 2H, *J* = 6.6 Hz), 2.74 (d, 3H, *J* = 4.8 Hz), 4.07 (t, 2H, *J* = 6.6 Hz), 5.06 (s, 2H), 6.18 (d, 1H, *J* = 7.5 Hz), 7.24 (d, 1H, *J* = 7.5 Hz), 7.33 (m, 5H), 7.81 (m, broad, 1H). ESI-MS: m/z =301.1 $[M+H]^+$, 323.1 $[M+Na]^+$, 601.3 $[2M+H]^+$, 623.3 $[2M+Na]^+$.

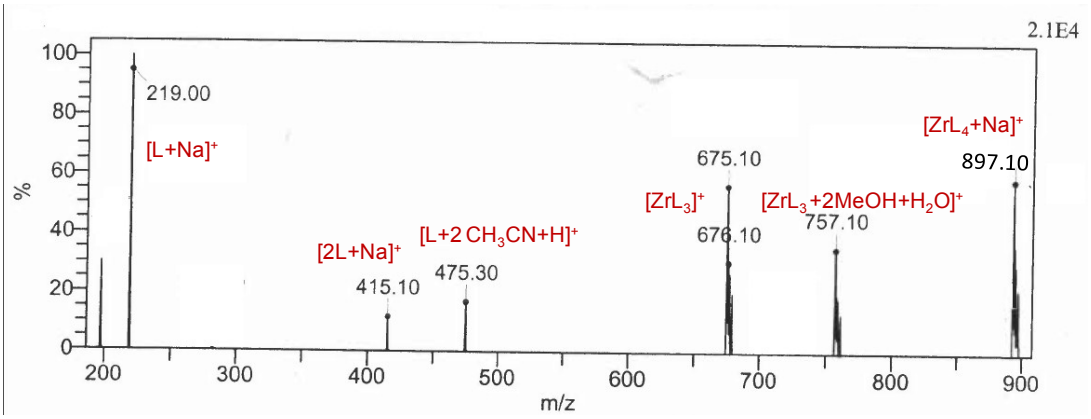
3,4-HOPO-NHMe (L^3H). The same hydrogenation procedure than as L^2H was used, providing a beige solid in 96% yield. ^{1}H NMR (D_2O, 300 MHz, ppm): δ 2.41 (s, 3H), 2.62 (s, 1H), 2.68 (t, 2H, *J* = 6.6 Hz), 4.34 (t, 2H, *J* = 6.6 Hz), 4.79 (s, 2H), 6.47 (d, 1H, *J* = 7.2 Hz), 7.55 (d, 1H, *J* = 7.2 Hz). ^{13}C NMR (D_2O, 75 MHz, ppm): δ 11.3, 25.8, 36.3, 50.6, 112.4, 137.3, 138.4, 144.8, 169.0, 172.5.). ESI-MS: m/z =211.1 $[M+H]^+$, 233.1 $[M+Na]^+$, 421.2 $[2M+H]^+$, 443.1 $[2M+Na]^+$, 209.1 $[M-H]^-$, 245.1/247.7 $[M+Cl]^-$, 269.1 $[M+AcO]^-$. Elemental analyses: Calculated for $C_{10}H_{14}N_2O_3$: C, 57.13; H, 6.71; N, 13.33%. Found: C, 56.73; H, 6.56; N, 12.93.

Mass spectra of ZrL_4 complexes.

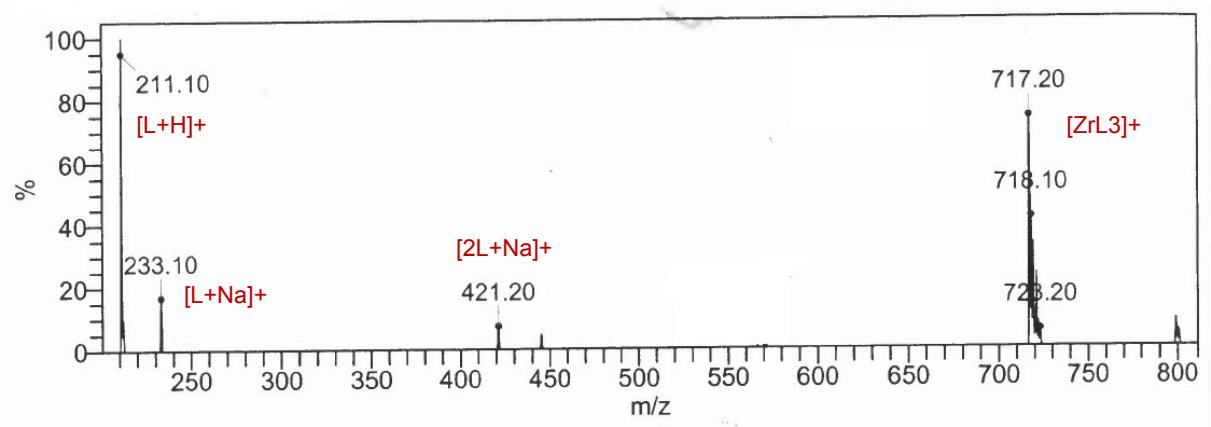
With L = L^1 Isotopic distribution (m/z) calculated for $[ZrL_4+Na]^+$: 781.1 (100.000%), 782.1 (55.793%), 783.1 (48.814%), 784.1 (14.438%), 785.1 (36.673%), 786.1 (11.898%), 787.1 (8.174%), 788.1 (2.266%), 789.1 (0.434%):



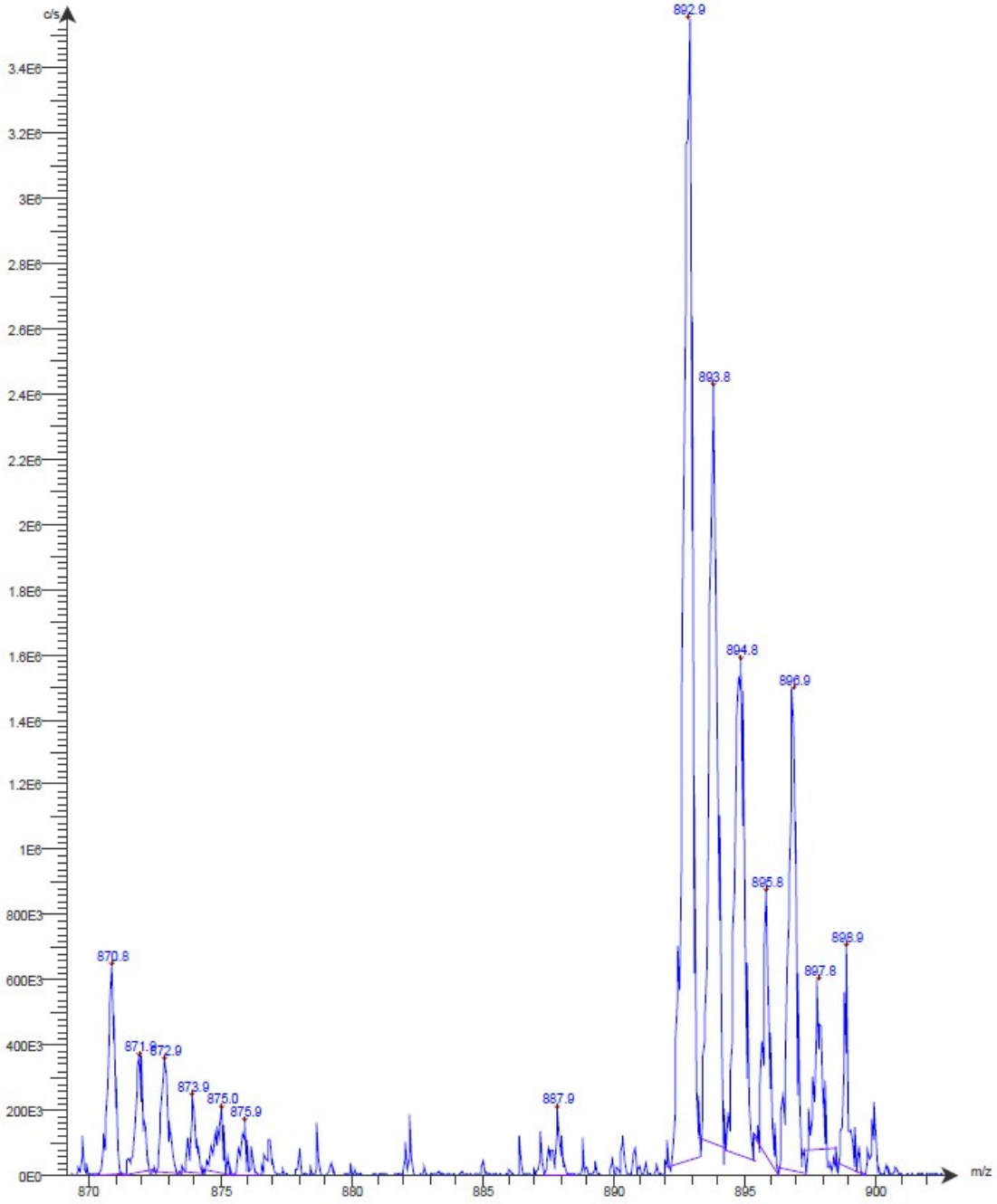
With L = L^2 Isotopic distribution (m/z) calculated for $[ZrL_4+Na]^+$: 893.2 (100.000%), 894.2 (64.630%), 895.2 (54.086%), 896.2 (18.875%), 897.2 (37.833%), 898.2 (15.116%), 899.2 (9.302%), 900.2 (2.984%), 901.2 (0.609%):



With L = L^3 Isotopic distribution (m/z) calculated for $[ZrL_3]^+$: 717.2 (100.000%), 718.2 (57.238%), 719.2 (49.004%), 720.2 (14.780%), 721.2 (36.580%), 722.2 (12.357%), 723.2 (8.127%), 724.2 (2.318%), 725.2 (0.423%)



With L = $L^{1'}$ Isotopic distribution (m/z) calculated for $[ZrL_4+Na]^+$: 893.2 (100.000%), 894.2 (64.630%), 895.2 (54.086%), 896.2 (18.875%), 897.2 (37.833%), 898.2 (15.116%), 899.2 (9.302%), 900.2 (2.984%), 901.2 (0.609%):



2. Crystallographic study

Zr(L^1)$_4$. A clear colourless block-like specimen of Zr(**L^1**)$_4$, approximate dimensions 0.14 mm x 0.16 mm x 0.17 mm, was used for the X-ray crystallographic analysis. The X-ray intensity data were measured on a Bruker SAMRT APEX I system equipped with a graphite monochromator and a MoK_α sealed tube (λ = 0.71073 Å).

The total exposure time was 2.52 hours. The frames were integrated with the Bruker SAINT software package using a narrow-frame algorithm. The integration of the data using a monoclinic unit cell yielded a total of 13270 reflections to a maximum θ angle of 27.48° (0.77 Å resolution), of which 3652 were independent (average redundancy 3.634, completeness = 99.1%, R_{int} = 2.78%, R_{sig} = 2.79%) and 3061 (83.82%) were greater than 2σ(F^2). The final cell constants of a = 15.6724(9) Å, b = 9.7264(5) Å, c = 21.0842(12) Å, β = 92.6940(10)°, volume = 3210.4(3) Å^3, are based upon the refinement of the XYZ-centroids of 5185 reflections above 20 σ(I) with 4.930° < 2θ < 54.92°. Data were corrected for absorption effects using the multi-scan method (SADABS). The ratio of minimum to maximum apparent transmission was 0.885. The calculated minimum and maximum transmission coefficients (based on crystal size) are 0.9297 and 0.9416.

The structure was solved and refined using the Bruker SHELXTL Software Package, using the space group C 1 2/c 1, with Z = 4 for the formula unit, $C_{28}H_{36}N_8O_{16}Zr$. The final anisotropic full-matrix least-squares refinement on F^2 with 242 variables converged at R1 = 3.64%, for the observed data and wR2 = 9.46% for all data. The goodness-of-fit was 1.057. The largest peak in the final difference electron density synthesis was 0.800 e^-/Å^3 and the largest hole was -0.640 e^-/Å^3 with an RMS deviation of 0.087 e^-/Å^3. On the basis of the final model, the calculated density was 1.721 g/cm^3 and F(000), 1712 e^-.

Zr(L^2)$_4$. A clear colourless plate-like specimen of Zr(**L^2**)$_4$, approximate dimensions 0.05 mm x 0.07 mm x 0.08 mm, was used for the X-ray crystallographic analysis. The X-ray intensity data were measured on a Bruker Kappa APEX II Duo CCD system equipped with a Mo K_α ImuS micro-focus source (λ = 0.71073 Å).

The total exposure time was 15.96 hours. The frames were integrated with the Bruker SAINT software package using a narrow-frame algorithm. The integration of the data using a triclinic unit cell yielded a total of 38302 reflections to a maximum θ angle of 26.30° (0.80 Å resolution), of which 8678 were independent (average redundancy 4.414, completeness = 99.5%, Rint = 6.02%, Rsig = 6.35%) and 6508 (74.99%) were greater than 2σ(F2). The final cell constants of a = 8.7979(11) Å, b = 14.9292(18) Å, c = 17.7180(19) Å, α = 106.292(6)°, β = 93.813(6)°, γ = 103.366(6)°, volume = 2151.6(4) Å3, are based upon the refinement of the XYZ-centroids of 9945 reflections above 20 σ(I) with 4.806° < 2θ < 56.44°. Data were corrected for absorption effects using the multi-scan method (SADABS). The ratio of minimum to maximum apparent transmission was 0.909. The calculated minimum and maximum transmission coefficients (based on crystal size) are 0.9755 and 0.9817.

The structure was solved and refined using the Bruker SHELXTL Software Package, using the space group P -1, with Z = 2 for the formula unit, C36H44N8O12Zr. The final anisotropic full-matrix least-squares refinement on F2 with 593 variables converged at R1 = 4.35%, for the observed data and

wR2 = 10.37% for all data. The goodness-of-fit was 1.022. The largest peak in the final difference electron density synthesis was 0.528 e-/Å3 and the largest hole was -0.570 e-/Å3 with an RMS deviation of 0.080 e-/Å3. On the basis of the final model, the calculated density was 1.485 g/cm3 and F(000), 1004 e-.

Table S1. Crystallographic summary for $Zr(L^1)_4 \cdot 4H_2O$ and $Zr(L^2)_4 \cdot 5H_2O$.

Compound	$Zr(\mathbf{L^1})_4 \cdot 4H_2O$	$Zr(\mathbf{L^2})_4 \cdot 5H_2O$
Crystal habit	clear colorless block	clear colorless plate
Empirical formula	$C_{28}H_{36}N_8O_{16}Zr$	$C_{36}H_{44}N_8O_{12}Zr$
MW	831.87	872.00
Crystal system	monoclinic	triclinic
Space group	C2/c	P-1
T,K	100(2)	100(2)
a, Å	15.6724(9)	8.7979(11)
b, Å	9.7264(5)	14.9292(18)
C, Å	21.0842(12)	17.7180(19)
α, deg	90.00	106.292(6)
β, deg	92.6940(10)	93.813(6)
γ, deg	90.00	103.366(6)
Cell volume, Å^3	3210.4(3)	2151.6(4)
Z	4	2
ρ_{calc}, g/cm^3	1.321	1.485
λ, Å	0.71073	0.71073
abs coef, mm^{-1}	0.435	0.337

3. Thermodynamic stability studies.

Protonation and complexation titrations were performed in a 50 mL glass-jacketed titration cell (Metrohm 727 TiStand), thermostatized at 25.0 ± 0.1 °C with a Lauda ecoline 003 circulating water bath and sealed from the atmosphere under nitrogen gas. The pH-potentiometric titrations were carried out by using a Metrohm 702 SM Titrino connected to a Metrohm 6.0234.100 combined glass electrode. A Metrohm Dosimat Plus autoburette (5 mL capacity) was used. The titrations were automatically controlled by software upon selection of suitable parameters. The titrant was a carbonate-free KOH solution at *ca.* 0.1 M prepared from a commercial ampoule of analytical grade, and the exact concentration was obtained by application of the Gran's method[5] upon titration of a standard HNO_3 solution. Stock solutions of $\mathbf{L^1}$', $\mathbf{L^2}$ and $\mathbf{L^3}$ were prepared at *ca.* 2×10^{-3} M as well as an analytical solution of $ZrCl_4$ at 0.05 M. Potentiometric titrations were run with *ca.* 0.05 mmol of ligand in a total volume of 30.00 mL at 25.0 $\pm$ 0.1 °C and with the ionic strength kept at 0.20 $\pm$ 0.01 M using KNO_3 as background electrolyte for $\mathbf{L^2}$ and $\mathbf{L^3}$ and at 0.10 $\pm$ 0.01 M using KCl as background electrolyte for $\mathbf{L^1}$'. The $[H^+]$ of the solutions was determined by measurement of the electromotive force of the cell, $E = E^0 + Q \log[H^+] + E_j$. The term pH is defined as $-\log [H^+]$, and a value of $K_w = [H^+][OH^-] = 10^{-13.72}$ was taken from the literature for our experimental conditions.[6] The terms E^0 and Q were determined by titrating a solution of known hydrogen-ion concentration at the same ionic strength. The liquid-junction potential, E_j, was found to be negligible under the experimental conditions used. The potentiometric data were refined with the Hyperquad software.[7]

Spectrophotometric competition titrations were measured on a Jasco V-650 spectrometer. 75 mL of a stock solution containing 0.020 mmol of **L$^{1'}$**, 0.25 mmol of HCl, 0.005 mmol of Zr^{4+} was prepared. The solution was distributed in 30 vials containing each 2.5 mL of this solution. The volume was adjusted to 3 mL by adding KCl as background electrolyte to keep the ionic strength at 0.10 ± 0.01 M, H_2O and KOH to have points distributed in an estimated pH range of 2.5–11.5. The points were incubated at 25.0 °C until reaching equilibrium. UV absorbance spectra were measured after quick transfer of each sample solution from its vial to a semi-micro UV cuvette. The spectroscopic data were refined with the HypSpec software.[8]

The overall equilibrium (formation) constants β_{HhL} and β_{MmHhLl} are defined by $\beta_{MmHhLl} = [M_mH_hL_l]/[M]^m[H]^h[L]^l$ and $\beta_{MH-1L} = \beta_{ML(OH)} \times K_w$, while stepwise equilibrium constants are given by $K_{MmHhLl} = [M_mH_hL_l]/[M_mH_{h-1}L_l][H]$ and correspond to the difference in log units between overall constants of sequentially protonated (or hydroxide) species. Species distribution diagrams were plotted from the calculated constants with the HYSS program.[9]

4. Radiochemistry

General. Zr-89 was used as a [^{89}Zr]-zirconium(IV) oxalate complex in 1M oxalic acid solution either produced and purified at the National Institutes of Health (Bethesda, MD, USA) using the previously reported procedure,[10] or obtained commercially from Perkin-Elmer (Boston, MA, USA).

Formation of $^{89}ZrL_4$ complexes. All solutions described below were prepared with de-ionized (DI) water purified through a Chelex column prior to use. Stock solutions of ^{89}Zr at the desired pH were prepared as follow: to 490 µL DI water was added ^{89}Zr (≈ 10 MBq corresponding to 10-20 µL depending on initial volumic activity) the pH was adjusted by addition of small aliquots of a 0.1 M Na_2CO_3 or 0.1 M HCl. To 45 µL of stock solution (≈ 1 MBq of ^{89}Zr in a typical experiment) were added 20 nmol (5 µL) of ligand dissolved in DMSO. These solutions were incubated at 20°C for 30 min and an aliquot was analyzed by ITLC-SG using a 10 mM EDTA (ethylenediaminetetraacetic acid) solution adjusted to pH = 6 in DI water as eluant, and analyzed with a Cyclone Plus Storage Phosphor System (Perkin-Elmer), with integration of spot intensity performed using OptiQuant software (version 5.0). The percentage of the activity bound to the ligand after TLC was calculated by converting the TLC scan into a chromatogram and integrating the peak corresponding to the spot at the bottom of the TLC. A selection of representative TLCs is displayed in Figure S1.

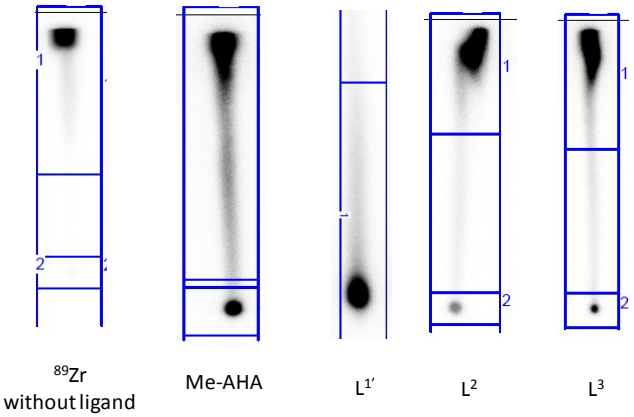


Figure S1. TLC analyses of free ^{89}Zr or ^{89}Zr incubated with ligands for 30 min at pH = 7 on ITLC-SG with EDTA (10 mM, pH = 6) as eluant.

Kinetic inertness study in EDTA and Fe^{3+} solutions. 20 µL of the solution of ^{89}Zr complex prepared as described above at pH 8 were added to 80 µL of EDTA solution (10 mM, pH = 6) or $FeCl_3$ solution (prepared from an aqueous 1 mM $FeCl_3$ solution dissolved to 0.8 mM in sodium phosphate buffer 0.1 M pH 7.2 and resulting in a pH of 6.5). Degradation of the complex was then monitored using the TLC system described above.

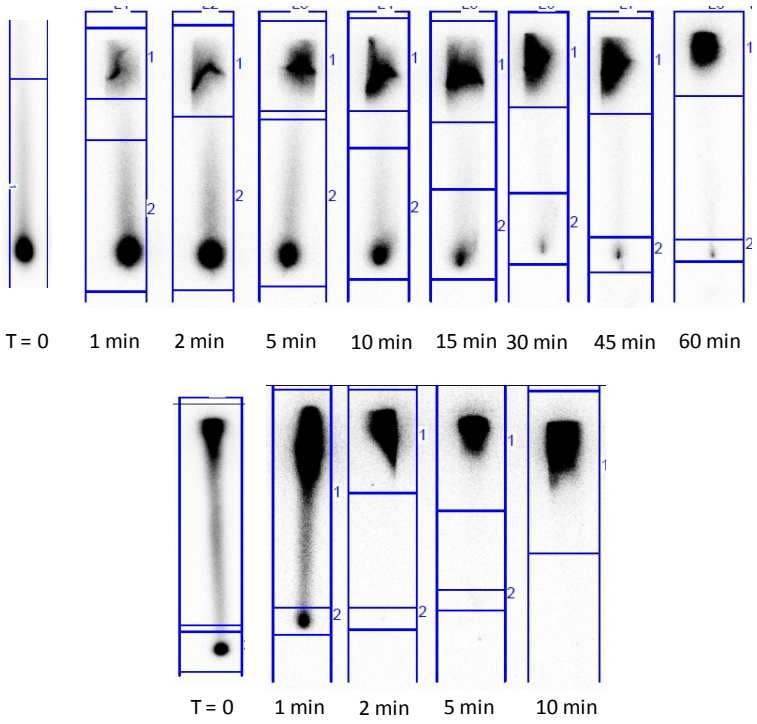


Figure S2. TLC analyses of ^{89}Zr**L$^{1'}$** (top) and ^{89}ZrMe-AHA (bottom) incubated in EDTA (10 mM, pH = 6) from 0 to 60 min, using EDTA (10 mM, pH = 6) as eluent.

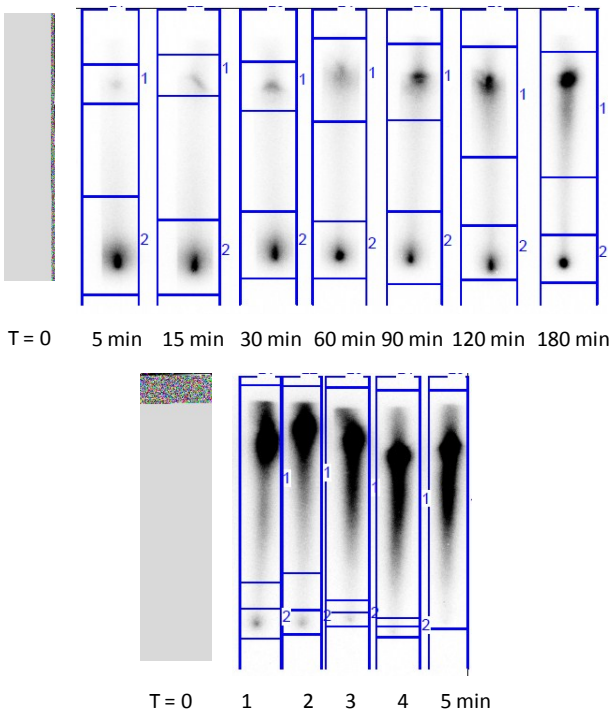


Figure S3. TLC analyses of ^{89}Zr**L**$^{1'}$ (top) and ^{89}ZrMe-AHA (bottom) incubated in $FeCl_3$ (0.8 mM, pH = 6.5) from 0 to 180 min, using EDTA (10 mM, pH = 6) as eluent.

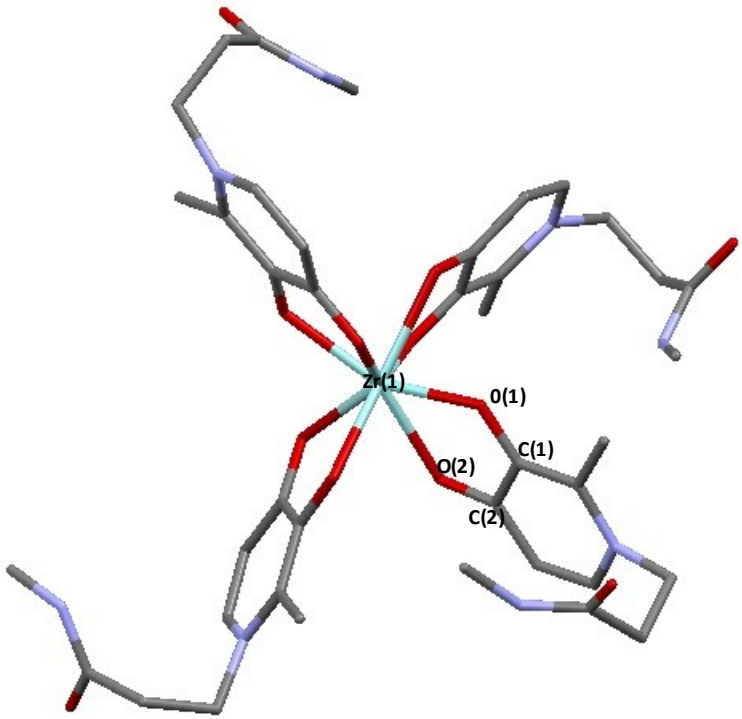


Figure S4. Calculated structure of Zr(**L**^{3})$_4$ in the reaction field of water at 298.15 K. Selected bond lengths (Å) and angles (°): Zr1-O1, 2.187; Zr1-O2, 2.335; O1-Zr1-O1, 69.98; C1-O1-Zr1, 120.29; C2-O2-Zr1, 115.75. Hydrogen atoms omitted for clarity.

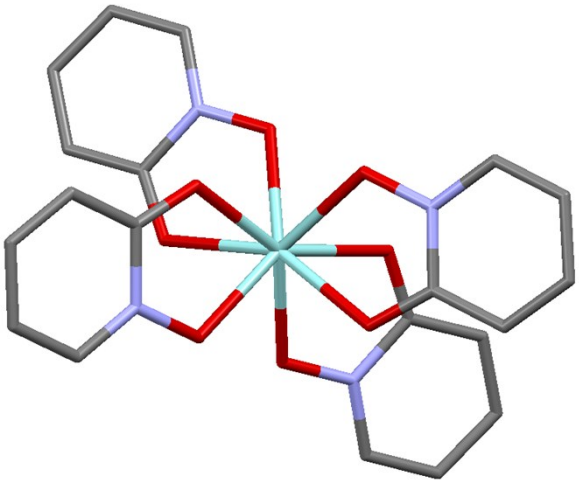


Figure S5. Calculated structure of Zr(**A**)$_4$ in the reaction field of water at 298.15 K. Hydrogen atoms omitted for clarity.

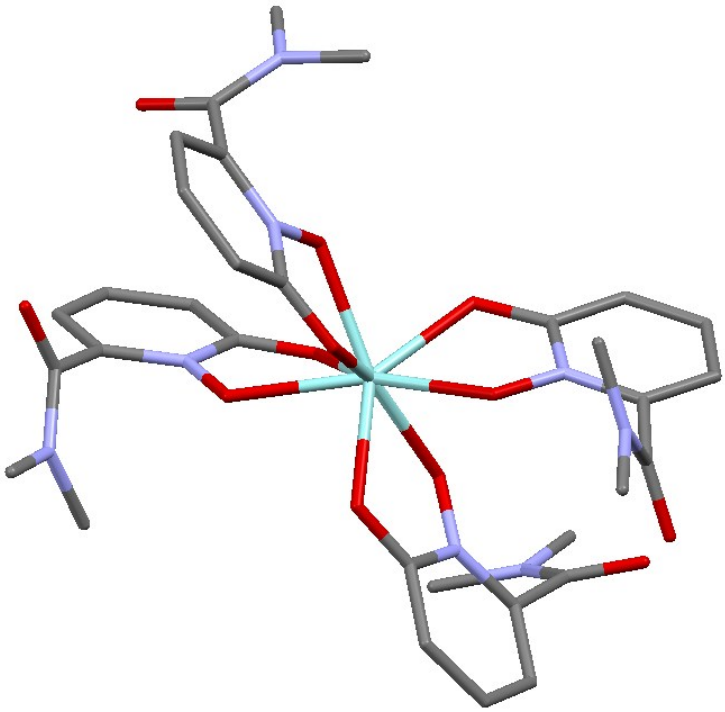


Figure S6. Calculated structure of Zr(**B**)$_4$ in the reaction field of water at 298.15 K. Hydrogen atoms omitted for clarity.

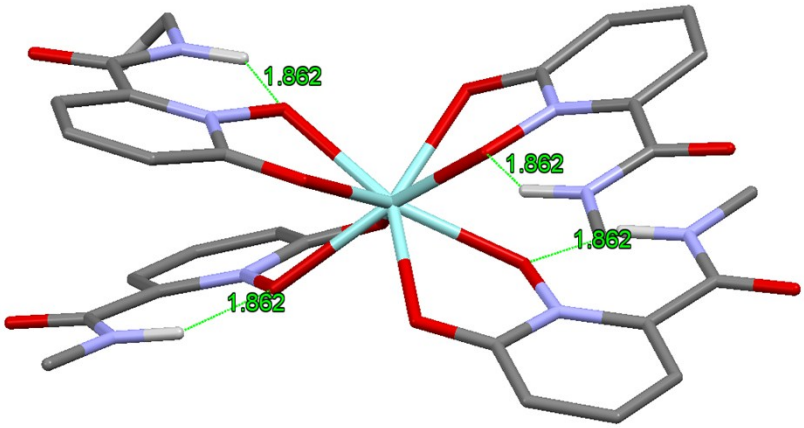


Figure S7. Calculated structure of $Zr(\mathbf{L^1})_4$ in the reaction field of water at 298.15 K. Hydrogen atoms omitted for clarity except hydrogen atoms involved in H-bonds.

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