Supplementary material for

New benzoazacrown compound as effective chelator for Bismuth in radiopharmaceuticals

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Content

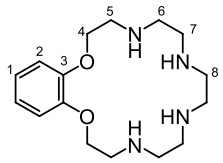
Experimental

All commercially available reagents were used without further purification. The progress of reactions was followed with TLC using aluminum oxide. Bisamide azacrown compound **1** (4,5,6,7,8,9,10,11,12,13-decahydro-1,16,4,7,10,13-benzodioxatetraazacyclooctadecine-3,14(2H,15H)-dione) was synthesized following the literature procedures [1]. ^{1}H and ^{13}C NMR spectra were recorded at 25 °C on Bruker Avance 500 spectrometer. Chemical shifts are reported in parts per million relative to internal standards for ^{1}H and ^{13}C (the given deuterated solvent). Coupling constants *J* are given in Hertz. Spectral assignments were based in part on two-dimensional NMR experiments (^{1}H COSY, HSQC, and HMBC). Melting points were determined on a «Mel-temp II». Elemental analyses were carried out on a Carlo Erba 1108 elemental analyzer. Electrospray ionization mass spectrometry (ESI-MS) analyses were performed using a Finnigan LCQ Advantage mass spectrometer equipped with an octopole ion-trap mass-analyzer.

Measurement of radioactivity was performed by gamma-spectrometer ORTEC DSPec50 (16013585) with coaxial HPGe-detector GEM-C5060P4-B (56-TP23840B) and GR3818 Canberra Ind.

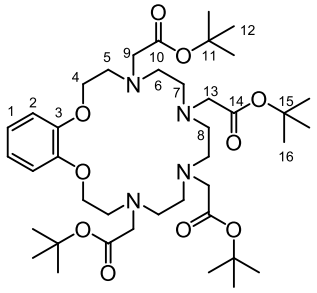
Ligand Synthesis

2,3,4,5,6,7,8,9,10,11,12,13,14,15-tetradecahydro-1,16,4,7,10,13-benzodioxatetraazacyclooctadecine (2)



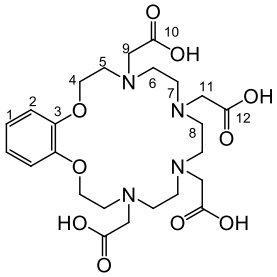
Bisamide azacrown compound **1** (250 mg, 0.7 mmol) was dissolved in 1M solution of BH_3·THF (7 mL) at 0°C and stirred under inert atmosphere overnight. Excess BH_3·THF was destroyed by adding of water (10 mL). To the resulting solution 1M HCl (10 mL) was added and the reaction mixture was refluxed for 5 h. The solution was washed with $CHCl_3$, and then the pH was adjusted to 10 by adding NaOH. The product was extracted with $CHCl_3$. The solvent was evaporated under vacuum to give **2** as yellow solid (192 mg, yield 89%). M.p. 95-97 ^oC. ^1H NMR ($CDCl_3$): ^1H NMR ($CDCl_3$): 2.80 (s, 4H, H(8)), 2.83 (br.s, 8H, H(6,7)), 3.05 (t, 4H, H(5), *J*=4.6), 4.14 (t, 4H, H(4), *J*=4.6), 6.90 (s, 4H, H(1, 2)). ^{13}C NMR ($CDCl_3$): 48.60 (C-8), 48.64 (C-7), 48.66 (C-6), 49.00 (C-5), 68.16 (C-4), 112.74 (C-2), 121.03 (C-1), 148.44 (C-3). Elemental analysis, found (%): C, 61.27; H, 9.01; N, 17.17. $C_{16}H_{28}N_4O_2$; calculated (%): C, 62.31; H, 9.15; N, 18.17. ESI-MS, calculated, m/z: 308.4; found: 309.6 $[MH]^+$.

4,7,10,13-tetra(*tert*-butyl)-2,3,4,5,6,7,8,9,10,11,12,13,14,15-tetradecahydro-1,16,4,7,10,13-benzodioxatetraazacyclooctadecine--4,7,10,13-yl-tetraacetate (3)



To the mixture of macrocycle **2** (150 mg, 0.49 mmol) and K_2CO_3 (537 mg, 3.89 mmol) in MeCN (10 ml) *tert*-butyl bromoacetate (284 µl, 1.95 mmol) was added dropwise. The mixture was refluxed for 18 hours. Solvent was evaporated in vacuum. The residue was dissolved in $CHCl_3$ and washed with H_2O. After evaporation the organic layer, a crude product was purified by column chromatography (alumina neutral, benzene/ethanol). The product **3** was obtained as yellow oil (156 mg, 42% yield). ^1H NMR ($CDCl_3$): 1.43 (s, 18H, H (16)), 1.46 (s, 18H, H(12)), 2.76 (s, 4H, H(8)), 2.80 (t, 4H, H(7), *J*=6.4), 2.92 (t, 4H, H(6), *J*=6.4), 3.16 (t, 4H, H(5), *J*=5.5), 3.31 (s, 4H, H (13)), 3.42 (s, 4H, H(9)), 4.10 (t, 4H, H(4), *J*=5.5), 6.88 (s, 4H, H(1, 2)). ^{13}C NMR ($CDCl_3$): 28.17 (C-12, C-16), 52.00 (C-8), 52.38 (C-7), 52.48 (C-6), 53.56 (C-5), 55.95 (C-13), 56.43 (C-9), 68.12 (C-4), 80.55 (C-15), 80.72 (C-11), 114.20 (C-2), 121.19 (C-1), 149.07 (C-3), 171.03 (C-14), 171.10 (C-10). Elemental analysis, found (%): C, 62.35; H, 8.59; N, 7.74. $C_{40}H_{68}N_4O_{10}$; calculated (%): C, 62.80; H, 8.96; N, 7.32. ESI-MS, calculated, m/z: 765.0; found: 765.9 $[MH]^+$.

2,3,4,5,6,7,8,9,10,11,12,13,14,15-tetradecahydro-1,16,4,7,10,13-benzodioxatetraazacyclooctadecine--4,7,10,13-yl-tetraacetic acid (H_4BATA)



Water (10 mL) was added to **3** (110 mg, 0.14 mmol) and refluxed for 20 h. The solution was washed with $CHCl_3$ and the aqueous layer was separated and evaporated under vacuum. The product **4** was obtained as a beige solid (77 mg, yield 99%). ^1H NMR (D_2O): 3.25 (s, 4H, H(8)), 3.45 (br.s, 4H, H(7)), 3.62 (s, 4H, H(11)), 3.70 (br.s, 4H, H(6)), 3.78 (br.s, 4H, H(5)), 3.89 (s, 4H, H(9)), 4.36 (br.s, 4H, H(4), *J*=5.49), 7.03 (br.s, 4H, H(1, 2)). ^{13}C NMR (D_2O): 50.63 (C-7), 52.46 (C-6,8), 54.48 (C-9), 54.71 (C-5), 55.00 (C-11), 62.44 (C-4), 113.90 (C-2), 122.50 (C-1), 146.86 (C-3), 170.26 (C-10), 172.92 (C-12). Elemental analysis, found (%): C, 50.49; H, 6.50; N, 9.49. $C_{24}H_{36}N_4O_{10}$·1,5H_2O; calculated (%): C, 50.79; H, 6.93; N, 9.87. ESI-MS, calculated, m/z: 540.6; found: 541.8 $[MH]^+$, 563.9 $[MNa]^+$.

Thermodynamic stability studies

The setup for potentiometric titrations has been described before [2]. The titrant was a carbonate-free NaOH (≈0.1 M) solution. The exact concentration of NaOH solution was obtained by application of the Gran's method upon titration of a previously standardized amounts of HCl with and determining the equivalent point by the Gran's method using the program GLEE [4] which gives the standard electrode potential, E_0, and the slope, s. The ionic product of water pKw = 13.78 at 25.0°C in 0.10 ± 0.01 M KNO_3 [5] was kept constant. A stock solution of **H_4BATA** was prepared at *ca*. 0.01 M. An analytical solution of $Bi(NO_3)_3$ was prepared at 0.025 M in 0.7 M aqueous HCl to avoid metal hydrolysis. Potentiometric titrations were run with *ca*. 0.016 mmol of ligand in a total volume of 16.00 mL at 25.0 ± 0.5 ºC. Data were collected in the pH range 2.5‑11.0. Each titration consisted of 80–100 equilibrium points and a minimum of two replicates were performed. The protonation constants of the **H_4BATA** and the stability constants of the complexes were calculated from the electromotive force titration data with the Hyperquad program [4].

The overall equilibrium (formation) constants β_{HhL} and β_{MmHhLl} are defined by $\beta_{HhL}=[H_hL_l]/[H]^h[L]^l$ and $\beta_{MmHhLl}=[M_mH_hL_l]/[M]^m[H]^h[L]^l$, 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.

Typical protocol for ligand radiolabeling with ^{207}Bi

All reagents and solvents were purchased from commercially available sources and used as received. The initial concentration of ^{207}Bi was determined by radioactivity counting. The average ^{207}Bi concentration was 0.2 nM. ^{207}Bi was equilibrated with 1, 5, 10, 77, 100, 460, 520, 770 μM of the **H_4BATA**. Solution was buffered at pH 6.1 or 8.0 with 0.01 M MES solution.

The mixtures were incubated 1 hour at 80°C or at room temperature and analyzed both by TLC. The TLC plates (cellulose on Al support) were developed in a mixture of 0.9% NaCl / 10mM NaOH. Plates were cut in half and radioactivity on each part was measured by gamma-spectrometry. The activity was quantified by the 570 keV gamma emission of ^{207}Bi. Autoradiography was performed with a Perkin Elmer Cyclone Plus Storage Phosphor System and associated software. Percentages of ^{207}Bi incorporation were deduced from the ratio of the radioactivity intensities at R_f = 0.9±0.1 (which corresponds to **H_4BATA**·$^{207}Bi^{3+}$) and R_f ~ 0 (which corresponds to the remaining ^{207}Bi salts). They are average values of at least two experiments (error estimated: ± 6%).

In order to study labeling efficiency 30 μL containing 300 Bq [^{207}Bi]$BiCl_3$ in 0.1 M HCl were equilibrated in 0.01M MES/ 0.01M PBS/ 0.1M NaOAc or 0.05M Na_2CO_3 buffers at room temperature or 80°C with 1, 5, 10, 77, 100, 500, 770 μM of the **H_4BATA** in a plastic Eppendorf tube with the total volume 300 μL.

In vitro stability study

The solution of radiolabelled complex **H_4BATA**·$^{207}Bi^{3+}$ with *ca*.0.5 mM was buffered at pH 6.1 with 0.01 M MES solution at R_T (radiochemical purity: 97%). Stability in isotonic solution, in high excess of biological cation Ca^{2+} ($5\cdot10^{-3}$ M), Mg^{2+} ($5\cdot10^{-3}$ M), Zn^{2+} (10^{-4} M) together and Cu^{2+} (10^{-4} M) separately. After 0, 15, 30, 60, 120 minutes and 1 day an aliquot of each sample was taken for TLC analysis. Fetal bovine serum (triple 0.1um sterile filtered) was purchased in HyClone (South Logan, Utah), all storage measures are followed. The ratio of complex **H_4BATA**·$^{207}Bi^{3+}$ and serum volumes was established 1:100. After 0, 15, 30, 60, 120 minutes, 1 day, 2 days an aliquot of each sample was taken and after protein precipitation by ethanol radioactivity of each supernatant and aliquot of initial sample with the same volume and geometry was measured by gamma-spectrometry and percentage of the complex was

determined. Also aliquots of supernatants were analyzed by TLC on cellulose plates with Al support.

In vivo biodistribution study

All *in vivo* experiments were performed in compliance with the ARRIVE guideline and in accordance with EU Directive 2010/63/EU for animal experiments. Solution of 100 μM **H_4BATA** and $^{207}Bi^{3+}$ (2.0-2.5 kBq) was buffered at pH 6.3 with 0.01 M MES at RT and diluted in sterile isotonic solution. Radiochemical purity according to TLC reached 97%. Solution were administered to the normal male BALB/c mice (weight 28–37 g) in 100 μL of solution *via* intraperitoneal injection as it described in [6]. The mice were housed at 12-h light/dark cycle with access to water and food *ad libitum*. The mice (3 per data point) were euthanized at 1 and 6 h by cervical dislocation. Blood was collected right after euthanasia and mixed with a 100 μl heparin solution. The major organs were harvested, washed from blood and peritoneal liquid by 0.9% NaCl solution, wet-weighed and the radioactivity of each one measured by γ-scintillation counter (with HPGe-detector GR3818 Canberra Ind.). The percent injected dose per gram (% ID/g) was determined for each tissue. The values presented are the mean and standard deviation for each tissue.

Computational details

Ligands data were downloaded from Protein Data Bank. Since the ligand protein interaction mentioned in the database may not only occur when the ligand reaches the target organ or system, we took only those ligands that bind at least three different proteins to stress biodistribution factor in the model We divided the whole dataset (1404 molecules) into training and test parts in such a way that 80% of the original data were used for training, and 20% were left to test the model. We trained a neural network with a relatively simple architecture (3 dense layers with 750-512 nodes in each layer) and used 10-fold cross-validation to avoid overfitting to train data. It means that at the same time 9/10 of the training data were used for training and 1/10 for controlling of training process. After convergence, the algorithm took the next 1/10 and repeated the process. Molecules were encoded using a combination of chemical descriptors from RDKit library and Avalon and FCFC6 structure fingerprints [7–9]

Supplementary Figures

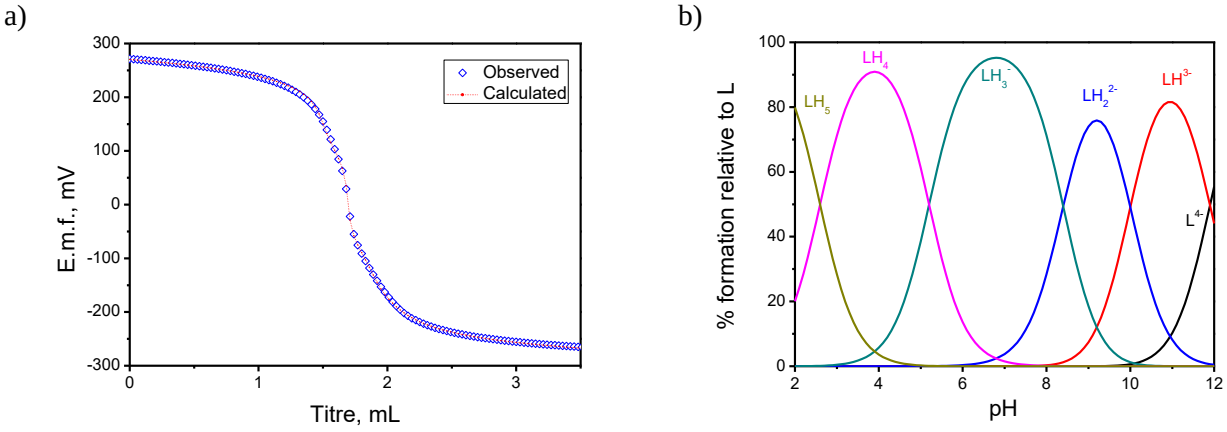


Figure S1. a) Observed and fitted titration curve for **H_4BATA**; b) Species distribution diagram of **H_4BATA**, at C_{H4BATA} = 1 mM.

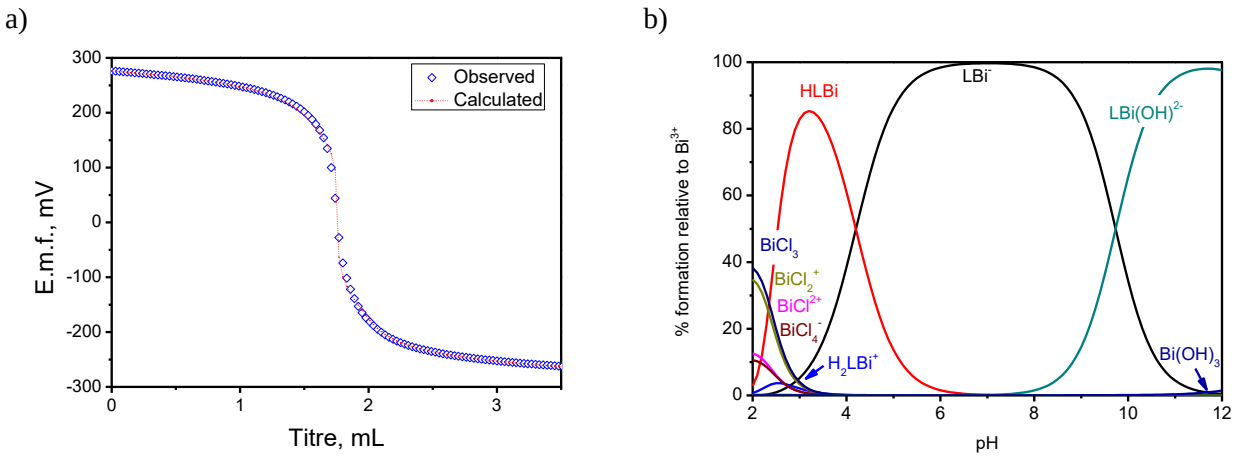


Figure S2. a) Observed and fitted titration curve for **H_4BATA** with Bi^{3+}; b) Species distribution diagram of bismuth(III) in presence of **H_4BATA**, at $C_{Bi}^{3+} = C_{H4BATA}$ = 1 mM.

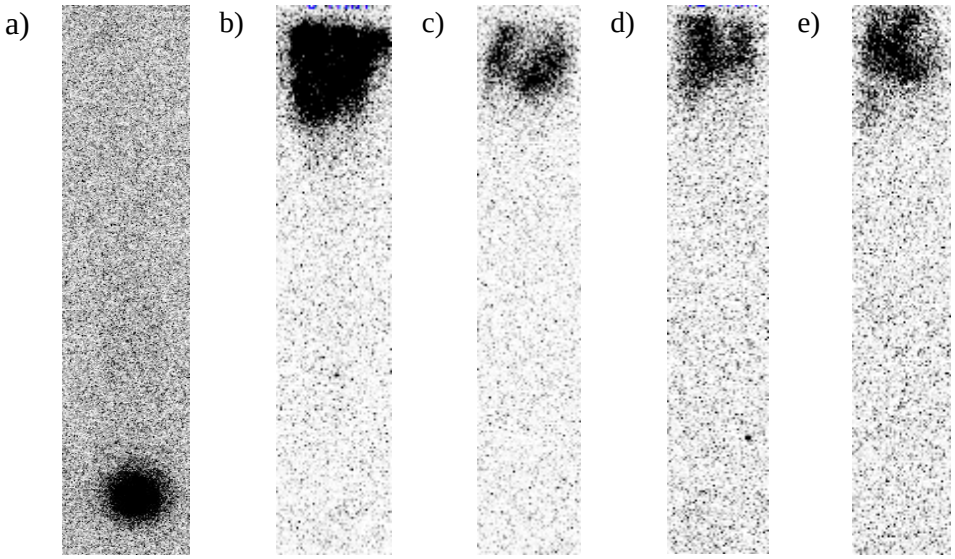


Figure S3. Representative TLC plates, visualized with autoradiographic system:
a) free ^{207}Bi: R_f = 0; b) initial **[**^{207}Bi**]BATA**-Bi; c) **[**^{207}Bi**]BATA**-Bi after 1 day keeping in saline; d) **[**^{207}Bi**]BATA**-Bi after 1 day keeping in solution with Ca^{2+} (5 mM), Mg^{2+} (5 mM) and Zn^{2+} (0.1 mM); e) **H_4BATA**·$^{207}Bi^{3+}$ after 1 day keeping in solution with Cu^{2+} (0.1 mM).

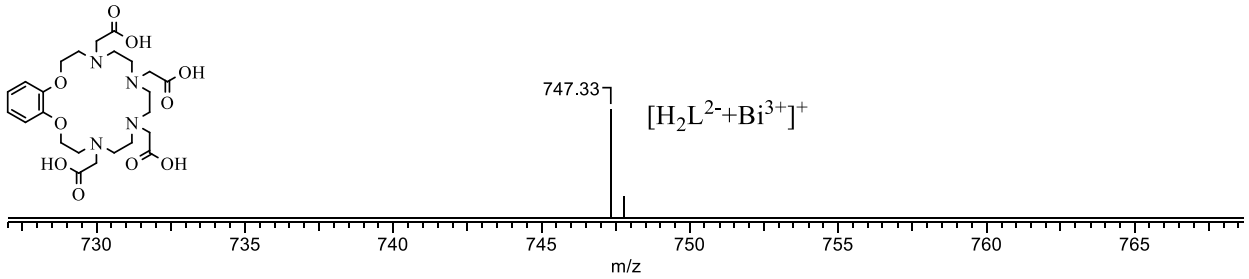


Figure S4. Mass-spectrum of Bismuth complex with **H_4BATA**.

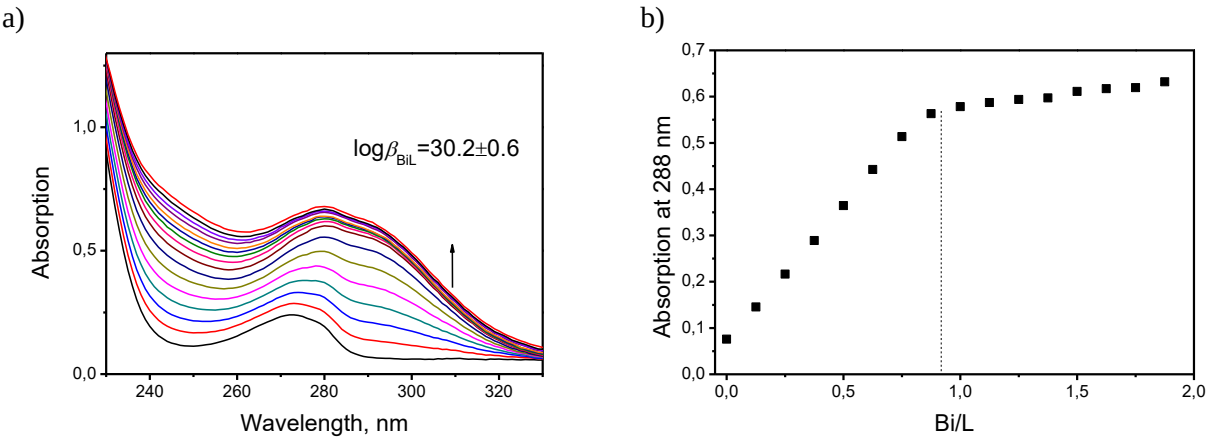


Figure S5. UV spectrophotometric titration of **H_4BATA** with Bi^{3+}: a) recorded spectra; b) absorption at 288 nm related to complex as a function of Bi/L ratio.

References

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