Radiolabelled Cyclic Bisarylmercury: High Chemical and in vivo Stability for Theranostics

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1. General Information

All solvent ratios are expressed as v/v. Unless otherwise stated the vials used were 1.5 ml Eppendorf LoBind hinge-top tubes. Routine activity measurements were performed using an Isomed 2000 from MED (Nuklear-Medizintechnik Dresden GmbH, Dresden, Germany) calibrated for $^{197}$Hg by $\gamma$-ray spectroscopy measurements after decaying $^{197m}$Hg. A RITA (Radio Isotope Thin layer Analyzer) thin-layer chromatography radioactivity detector from the company Raytest Isotopenmessgeräte GmbH was used with the RITA Control software (version 1.24) and data was processed using the RITA TLC Analysis software (version 1.97.000). The HPLC specifications were as follows: KNAUER Smartline Pump 1000; KNAUER Smartline Manager 5000; KNAUER Smartline UV Detector 2600; analytical column: Phenomenex Jupiter 4u Proteo 90A (4 µm; 90 Å; 4.60 x 250 mm); semi-prep column: Agilent ZORBAX StableBond 300 C18 (5 µm; 300 Å; 9.4 x 250 mm); Agilent Technologies, Inc. EZChrom Elite Client/Server software, UV wavelength 254 nm. The radio-HPLC specifications were: JASCO Deutschland GmbH ChromNav 2 software; JASCO PU-4180 Analytical Pump;
JASCO MD-4010 PDA Detector; Elysia-raytest GmbH GABI\textsuperscript{a} radioactivity-HPLC-flow-monitor γ detector (CH2); KROMASIL Eternity 5-PhenyHexyl column (5 µm; 100 Å; 10 x 150 mm), UV wavelength: 220 nm (CH2). NMR spectra were measured using either a 9.4 T or 14.1 T Agilent Technologies, Inc. spectrometer. The following NMR-active nuclides were measured at the respective frequencies: \textsuperscript{1}H 400 MHz or 600 MHz, \textsuperscript{13}C 101 MHz or 151 MHz, and \textsuperscript{199}Hg 107 MHz. Chemical shifts were calibrated using residual solvent signals as reported by Fulmer \textit{et al.}\textsuperscript{1} Measurements were performed at 25°C. All spectra data were analyzed using the MestReNova software. Elemental analysis was measured using a Euro EA Elemental Analyzer (CHNS) Model: EuroVector. High-resolution mass spectra were obtained on a Q-TOF MS using electrospray ionization: Agilent 1260 Infinity II HPLC (Santa Clara, CA, USA; pump G7111B, autosampler G7129A, column oven G7116N, UV detector G7717C, eluent MeCN/water acidified with 0.1% formic acid, bypass mode) coupled to UHD Accurate Mass Q-TOF LC MS G6538A.

Materials: All solvents were purchased from Fisher Scientific, with the exceptions of n-hexane and the deuterated solvents, which, as with all other chemicals unless otherwise stated, were purchased from Sigma-Aldrich. Deionized water was formed using a Millipore Direct-Q 3 system.

2. Experimental

\textbf{Scheme S1.} Cyclic bisarylmercury bispidine synthesis.

\begin{center}
\begin{tikzpicture}
\node [draw, circle, thick, fill=white, minimum size=1cm] (A) at (0,0) {\textbf{1}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (B) at (2,0) {\textbf{2}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (C) at (4,0) {\textbf{3}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (D) at (6,0) {\textbf{3*}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (E) at (0,-2) {\textbf{3}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (F) at (2,-2) {\textbf{3a}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (G) at (4,-2) {\textbf{3b}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (H) at (6,-2) {\textbf{3*}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (I) at (0,2) {\textbf{1}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (J) at (2,2) {\textbf{2}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (K) at (4,2) {\textbf{1H}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (L) at (6,2) {\textbf{2/3a: R = \textsuperscript{6}Bu}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (M) at (6,0) {\textbf{2/3b: R = H}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (N) at (6,-2) {\textbf{197[HgCl\textsubscript{3}]}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (O) at (0,-4) {\textbf{1}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (P) at (2,-4) {\textbf{2}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (Q) at (4,-4) {\textbf{3}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (R) at (6,-4) {\textbf{3*}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (S) at (0,0) {\textbf{1}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (T) at (0,-2) {\textbf{1}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (U) at (0,-4) {\textbf{1}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (V) at (0,2) {\textbf{1}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (W) at (0,0) {\textbf{1}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (X) at (0,-2) {\textbf{1}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (Y) at (0,-4) {\textbf{1}};
\node [draw, circle, thick, fill=white, minimum size=1cm] (Z) at (0,2) {\textbf{1}};
\end{tikzpicture}
\end{center}

\textbf{Syntheses}

\textit{Preparation of 3,7-bis(2-bromobenzyl)-1,5-diphenyl-3,7-diazabicyclo[3.3.1]nonan-9-one (1) C\textsubscript{33}H\textsubscript{30}Br\textsubscript{2}N\textsubscript{2}O} A 250 ml round-bottomed flask, with magnetic flea, was charged with 1,3-diphenylpropan-2-one (8.03 g, 38.2 mmol), followed by THF (100 ml) and stirred until a clear, pale yellow solution formed. To this was added 2-bromobenzylamine (14.20 g, 76.3 mmol, Alfa Aesar), a 37 wt% aqueous solution of formaldehyde (11.4 ml, 152.6 mmol) and a catalytic amount of ethanoic acid (a few drops). The reaction mixture was refluxed at 65°C overnight for 19 h forming a dark yellow solution. TLC confirmed the complete conversion of the ketone starting material (R\textsubscript{f} ≈ 0.8, 1:1 EtOAc:hexane, KMnO\textsubscript{4} stain). The ethanoic acid was neutralized by adding saturated NaHCO\textsubscript{3}(aq) until the reaction mixture was slightly alkaline. The THF was removed by evaporation, the reaction mixture dissolved in DCM (50 ml) and washed with water (3×20 ml). The aqueous layers were combined and extracted with DCM (5 ml). The organic layers were combined, washed with brine (10 ml), dried with anhydrous Na\textsubscript{2}SO\textsubscript{4} and filtered. The DCM was evaporated, leaving a brown solid, which was recrystallized by dissolving in boiling EtOH and slowly cooling to room temperature, affording 1 as white crystals (17.95 g, S2
28.5 mmol, 75%). TLC Rf ≈ 0.8 (1:1 EtOAc:hexane, KMnO4 stain). >99% HPLC purity.

Anal. EA: calculated C, 62.87; H, 4.80; N, 4.44, found C, 62.49; H, 5.17; N, 4.38.

ESI-MS: calculated [C31H38Br2N2O]+ 631.0783, found 631.0781.

1H NMR (400 MHz, CDCl3): δ 7.64 (dd, J = 8.0, 1.2 Hz, 2H, NCH2ArH(3)), 7.53 (dd, J = 7.6, 1.7 Hz, 2H, NCH2ArH(6)), 7.34 (td, J = 7.5, 1.3 Hz, 2H, NCH2ArH(5)), 7.31-7.24 (m, 4H, Ph-m) 7.24–7.16 (m, 8H, Ph-o,p + NCH2ArH(4)), 3.85 (s, 4H, NCH2Ar), 3.42 (dd, J = 186.0, 10.7 Hz, 8H, NCH2CPH).

13C NMR (101 MHz, CDCl3): δ 210.89 (C=O), 142.75 (Ph-i), 137.37 (NCH2Ar(1)), 133.31(NCH2Ar(3)), 131.71 (NCH2Ar(6)), 129.21 (NCH2Ar(4)), 127.98 (Ph-m), 127.41 (NCH2Ar(5)), 127.03 (Ph-o), 126.72 (Ph-p), 125.21 (NCH2Ar(2)), 64.87 (NCH2CPH), 61.25 (NCH2Ar), 54.64 (CPH).

Preparation of 3,7-bis(2-bromobenzyl)-1,5-diphenyl-3,7-diazabicyclo[3.3.1]nonan-9-ol (1H) C31H38Br2N2O

A 250 ml round-bottomed flask was charged into an oven and sealed with a rubber septum. Anhydrous THF (50 ml) was syringed into the flask to form a suspension. Carefully, dropwise addition of BuLi (2.5 M in hexane, 5.4 ml, 13.5 mmol), keeping the temperature below the boiling point, then reacted with the suspension to form a clear yellow solution. Me3SnCl (1 M in THF, 13.5 ml, 13.5 mmol) was syringed dropwise 30 min later, eventually causing the reaction mixture to turn colourless. After letting to stir throughout the night (19 h), the reaction mixture was carefully quenched with EtOH and then water. Organic solvents were removed by evaporation. More water was then added, into a final 40 ml solution, then NaHCO3 to form an alkaline phase pH ≈ 8 that was extracted with DCM (3×40 ml). Afterwards, all organic phases were combined and washed with brine solution (40 ml), dried with anhydrous Na2SO4, solids filtered off and the remaining solution evaporated leaving a crude brown oily residue. Recrystallization with Et2O formed a brown ppt. that was filtered off. Evaporation of the remaining Et2O left 2 g of a crude brown oily residue. Column chromatography purification (dry-loaded onto Alox (basic 90) as the desired product proved unstable on silica) with a slow gradient of EtOAc (0% to 5%) in hexane yielded 2 as a white solid (200 mg, 0.23 mmol, 9%). TLC: Rf ≈ 0.7 (Alox plate, 15% EtOAc:hexane, I2 stain). >90% HPLC purity.

Anal. EA: calculated C, 60.31; H, 6.83; N, 3.27, found C, 63.61; H 7.13; N, 3.28.

ESI-MS: calculated [C31H38N2O]+ 857.2666, found 857.2677.

1H NMR (400 MHz, CDCl3): δ 7.80 (t, J = 7.0 Hz, 2H, NCH2ArH(4/5)), 7.48 – 7.40 (m, 6H, Ph-o + NCH2ArH(3)), 7.36 (t, J = 7.5 Hz, 2H, NCH2ArH(4/5)), 7.30 – 7.19 (m, 6H, Ph-m + NCH2ArH(6)), 7.16 (t, J = 7.2 Hz, 2H, Ph-p), 3.81 (s, 2H, NCH2Ar), 3.78 (s, 2H, NCH2Ar), 3.38 (dd, J = 41.1, 11.3 Hz, 4H, NCH2CPH), 2.89 (dd, J = 51.3, 10.9 Hz, 4H, NCH2CPH), 1.27 (t, J = 9.7 Hz, 2H, Bu-C1), 0.65 (h, J = 7.2 Hz, 2H, Bu-C3), 0.45 (s, 9H, SnMe3), 0.39 (s, 9H, SnMe3), 0.37 (t, J = 7.6 Hz, 3H, Bu-C4), 0.04 (p, J = 7.5 Hz, 2H, Bu-C2).

Preparation of 9-buty1-1,5-diphenyl-3,7-bis(2-(trimethylstanny1)benzyl)-3,7-diazabicyclo[3.3.1]nonan-9-ol (2a) C31H38N2O3Sn2

1 (1.70 g, 2.7 mmol) was charged into an oven-dried 250 ml round-bottomed Schlenk flask with a magnetic stirrer, on a Schlenk line, under argon and sealed with a rubber septum. Anhydrous THF (50 ml) was syringed into the flask to form a suspension. Carefully, dropwise addition of BuLi (2.5 M in hexane, 5.4 ml, 13.5 mmol), keeping the temperature below the boiling point, then reacted with the suspension to form a clear yellow solution. Me3SnCl (1 M in THF, 13.5 ml, 13.5 mmol) was syringed dropwise 30 min later, eventually causing the reaction mixture to turn colourless. After letting to stir throughout the night (19 h), the reaction mixture was carefully quenched with EtOH and then water. Organic solvents were removed by evaporation. More water was then added, into a final 40 ml solution, then NaHCO3 to form an alkaline phase pH ≈ 8 that was extracted with DCM (3×40 ml). Afterwards, all organic phases were combined and washed with brine solution (40 ml), dried with anhydrous Na2SO4, solids filtered off and the remaining solution evaporated leaving a crude brown oily residue. Recrystallization with Et2O formed a brown ppt. that was filtered off. Evaporation of the remaining Et2O left 2 g of a crude brown oily residue. Column chromatography purification (dry-loaded onto Alox (basic 90) as the desired product proved unstable on silica) with a slow gradient of EtOAc (0% to 5%) in hexane yielded 2 as a white solid (200 mg, 0.23 mmol, 9%). TLC: Rf ≈ 0.7 (Alox plate, 15% EtOAc:hexane, I2 stain). >90% HPLC purity.

Anal. EA: calculated C, 60.31; H, 6.83; N, 3.27, found C, 63.61; H 7.13; N, 3.28.
Preparation of 1,5-diphenyl-3,7-bis(2-(trimethylstanny1)benzyl)-3,7-diazabicyclo[3.3.1]nonan-9-ol (2b) C₃₀H₃₇O₂Sn₂

Same procedure as for the preparation of 2a. 1H (0.5 g, 0.79 mmol) BuLi (2.5 M in hexane, 1.58 ml, 3.95 mmol), Me₂SnCl (1 M in THF, 3.95 ml, 3.95 mmol). After Alox column chromatography purification white solid (36 mg, 6% crude yield, ca. 66% HPLC purity). Purified by semi-prep HPLC: Flow 4 ml/min, 7.3 H₂O:ACN (both +0.01% TFA) gradient to 1:4 over 40 min then hold, desired product peak 35-43 min. Collected in round-bottomed flask with sat. NaHCO₃(aq) solution to neutralize acid. Evaporation of ACN followed by separation with and evaporation of DCM left a white solid (8.2 mg, 11.1 mol, 1.4%). >99% HPLC purity. ESI-MS: calculated [C₃₀H₃₇O₂Sn₂]⁺ 801.02, found 801.09.

Preparation of 9-butyl-8,10-diphenyl-6,10:8,12-dimethanodibeno[1,9]diaza[5]mercura cyclotetradecan-9-ol (3a) C₂₂H₂₂O₂HgN₂O

A 1.5 ml Eppendorf LoBind hinge-top tube was charged with 2a (26 mg, 0.03 mmol) and HgCl₂ (8.1 mg, 0.03 mmol) in THF (1.5 ml) and mixed at 50°C for 5 h. Evaporation of THF left a yellow oily residue. HPLC analysis showed no remaining starting material. Washing with hexane (3 ml) removed apolar impurities and most of the yellow colour, further washing with toluene completely removed the yellow colour and after evaporation left a white solid (7.2 mg, 5.25 mmol, 33%). TLC-RP18: Rf ≈ 0.7 (s.m. ≈ 0.3) (ACN:H₂O 9:1 +0.1% TFA). ~90% HPLC purity.

Anal. ESI-MS: calculated [C₂₂H₂₂O₂HgN₂O]⁺ 731.2925, found 731.2926.

1H NMR (600 MHz, CDCl₃): δ 7.54 (d, J = 7.7 Hz, 4H, Ph-o), 7.49 (dd, J = 6.9, 1.4 Hz, 1H, HgC6), 7.46 (dd, J = 7.1, 1.4 Hz, 1H, HgC6), 7.40 (t, J = 7.3 Hz, 1H, HgC4/5), 7.36 (d, J = 7.3 Hz, 1H, HgC3), 7.34 (d, J = 7.8 Hz, 1H, HgC3), 7.29 (td, J = 7.2, 1.3 Hz, 1H, HgC4/5), 7.25 (t, J = 8.0 Hz, 4H, Ph-m), 7.23 – 7.16 (m, 3H, Ph-p + HgC4/5), 7.09 (td, J = 7.4, 1.5 Hz, 1H, HgC4/5), 3.63 (s, 2H, NCH₂Ar), 3.51 (s, 2H, NCH₂Ar), 3.33 (dd, J = 198.0, 11.5 Hz, 4H, NCH₂CPh), 3.00 (dd, J = 305.4, 11.4 Hz, 4H, NCH₂CPh), 1.74 (s, 1H, OH), 1.45 (t, J = 8.5 Hz, 2H, Bu-C1), 0.74 (h, J = 7.3 Hz, 2H, Bu-C3), 0.41 (t, J = 7.3 Hz, 3H, Bu-C4), -0.04 (p, J = 7.9 Hz, 2H, Bu-C2).

13C NMR (101 MHz, CDCl₃): δ 171.34 (HgCl₁), 170.93 (HgCl₁), 146.85 (HgCl₂), 146.45 (HgCl₂), 141.79 (Ph-i), 139.09 (HgC6), 138.85 (HgC6), 128.97 (HgC3), 128.80 (HgC3), 127.98 (Ph-m), 127.84 (Ph-o), 127.24 (HgC4/5), 127.16 (HgC4/5), 126.73 (HgC4/5), 126.68 (Ph-p), 126.42 (HgC4/5), 75.10 (COH), 67.72 (NCH₂Ar), 67.00 (NCH₂Ar), 60.18 (NCH₂CPh), 60.09 (NCH₂CPh), 46.26 (CPh), 31.40 (Bu-C1), 26.00 (Bu-C2), 23.00 (Bu-C3), 13.62 (Bu-C4).

199Hg NMR (108 MHz, CDCl₃): δ -684.5.

Preparation of 8,10-diphenyl-6,10:8,12-dimethanodibeno[1,9]diaza[5]mercury cyclotetradecan-9-ol (3b) C₃₃H₃₆O₂HgN₂O

Same procedure as for the preparation of 3a. 2b (1 mg, 1.25 µmol), HgCl₂ (0.81 mg, 2.97 µmol). 94% HPLC Purity.

ESI-MS: calculated [C₃₃H₃₆O₂HgN₂O]⁺ 675.2, found 675.5.

Radiolabelling

Preparation of 197m5Hg

The radionuclide was prepared by the bombardment of high purity 197Au target (99.99+%, 10 mm diameter, 0.125 mm thickness, Safina, Czech Republic) with a deuteron beam of the cyclotron U-120M in the Nuclear Physics Institute of the CAS, Czech Republic. The irradiations were performed using 15.8 MeV deuterons at...
the beam current of 10 µA for 4 h. It resulted in ~0.58 GBq of $^{197m}Hg$ and ~1.14 GBq of $^{197}Hg$ at EOB, respectively. After arrival at HZDR, Germany, the irradiated targets were dissolved in aqua regia (700 µl), prepared from 30% HCl(aq) (525 µl) and 65% HNO$_3$(aq) (175 µl) (purity TraceSelect, Sigma-Aldrich), and diluted with 6M HCl(aq) (300 µl). The resulting solution had a total activity of ~0.9 GBq. 0.5 µl (~1.57 MBq) was removed as a reference substance and the rest of the solution was carefully loaded onto a prepared column filled with 3.6 g of LN resin (LN-B100-A, 100-150 µm, TRISKEM, France) that had been soaked for 15 min in 6M HCl(aq), rinsed slowly with 6M HCl(aq) (30 ml), capped with a frit, overlayed with ca. 1 cm of sand and finally rinsed with 6M HCl(aq) (80 ml). After loading the target solution, the column was slowly washed with 6M HCl(aq) (6×1 ml) fractions, minor activity being detected from the 5th fraction, the fraction volume was reduced (6×0.5 ml). Most of the activity was eluted in the 9th-11th fractions.

**Radiolabelling procedure for stability tests**

After pH-adjustment of the hydrochloric acid solution of $[^{197m}Hg]HgCl_2$(aq) (~55 MBq, 20 µl, pH 1), by addition of 0.5 M HEPES buffer (pH 8, 200 µl), EtOH (200 µl), 6 M NaOH(aq) (11 µl), and 1 M NaOH(aq) (2 µl), a 1 mg/ml acetonitrile solution of 2a (12 µl, 14 nmol) was added. This solution (pH 6, 445 µl) was mixed at 50°C for 1 h. The radiochemical yield of 3a* was determined by radio-TLC (iTLC ACN + 0.1%TFA, RP-18 TLC 9:1 ACN:H$_2$O + 0.1%TFA) as >95%. Purification with C8 cartridge (10 ml): pre-wash - 1:1 EtOH:H$_2$O (6 ml), H$_2$O (2×3 ml), 2.1 brine:EtOH (9 ml) and H$_2$O (3 ml). Then the cartridge syringe was filled with 1:10 H$_2$O:3a* (4.4 ml:440 µl) and successively washed with 1:4, 1:1 and 7:3 EtOH:H$_2$O (10 ml, 2 ml and 2×1 ml respectively). The ~16 MBq fraction had 3×200 µl extracted (~4 MBq each). These fractions then had 1 competitor added each (1 mg/ml, 10 µl): tris(2-mercaptoethyl)ammonium oxalate, glutathione and Na$_2$S. The mixtures were left at rt and checked by radio-TLC after 5 min, 1 h and 2 d, the only degradation observed was ~4% after 2 d in the Na$_2$S mixture. The ~10 MBq fraction was divided into 2×500 µl. The first lot was used to test the stability of 3a* in the highly aqueous solvent system necessary for biodistribution studies, this was diluted from 70% EtOH(aq) to ~10% EtOH(aq) with brine (3.5 ml). Transferal to a fresh vial showed negligible loss in activity and radio-TLC of the solution showed good stability. The other 500 µl lot was used to test the volatility of 3a*: firstly the sample was diluted with 70% EtOH(aq) (500 µl) and the vial heated to 50°C whilst a stream of dry nitrogen was blown onto the 1 ml solution for 1 h until the solution volume had been reduced to ~350 µl. Transferal to a fresh vial showed negligible loss in activity and measurement of the remaining solution showed no observable loss by evaporation.

**Determination of distribution coefficient of 3a**

Shake flask method: Into a 10 ml glass vial was added n-octanol (500 µl), 0.05 M HEPES buffer solution (pH 7.4, 475 µl) and a 1:1 EtOH:H$_2$O solution of 3a* (25 µl). The vial was shaken for 30 s, then 400 µl extracted from each phase and centrifuged separately. 2×100 µl was taken from each phase and the intensity of radioactivity was measured by a gamma counter and averaged to a value of logD$_{7.4}$ = 2.27.

**Human serum stability assay (following the Zarschler et al. procedure)**

**Brief outline of the method:** The in vitro method applied herein to measure the stability of radiomercury complexes under physiologically relevant conditions involves the incubation of the $^{197m}Hg$-radioiodinated samples with human serum. After one hour of incubation at 37°C, aliquots of the serum samples are separated by non-reducing SDS-PAGE. After electrophoresis, the polyacrylamide gel is exposed to a phosphor imaging plate and the created autoradiographs are scanned. Finally the proteins in the gel are stained and visualized with colloidal Coomassie Brilliant Blue. For evaluating the stability of the radiometal complexes against human serum, the autoradiograph as well as the image of the Coomassie-stained gel are compared.

Human serum “off the clot” (5 ml) stored at -20°C was slowly thawed on ice and filtered using syringe filters with a pore size of 0.2 µm. Two aliquots of filtered serum (2×220 µl) were mixed with 1 M HEPES/NaOH buffer (pH 7.4, 2×45 µl). Separately, 2×200 µl solution (1:1 EtOH:H$_2$O, pH 6) of 3a* (~4 MBq) and $[^{197m}HgCl_2$/EDTA (~5 MBq, 10 µg EDTA) had 1 M HEPES/NaOH buffer (pH 8.0, 2×20 µl) added to increase solution pH to 7.4. Then 135 µl of each $^{197m}Hg$-radioiodinated sample was added to one of the previously prepared serum/buffer solutions (265 µl) and incubated for 1 h at 37°C. 50 µl aliquots were then taken and mixed with 50 µl of 2x Laemmlli sample buffer (Bio-Rad Laboratories), N.B. no reducing agent was added and the samples were not heated. The mixtures were then analyzed by non-reducing SDS-PAGE with acrylamide
concentrations of 5% in the stacking gel and 20% in the resolving gel. 2 µl of each sample were loaded into each gel well. The SDS-PAGE was run at r.t. and 80 V until the dye front reached the resolving gel and then increased to 140–160 V. After electrophoresis, the gel was washed for 1 min with H2O and then exposed to a high-resolution phosphor imaging plate (GE Healthcare) for 10 min and the exposed plate scanned (Amersham Typhoon 5 Scanner, GE Healthcare) to measure an autoradiograph. The gel was then stained with PageBlue protein staining solution (Thermo Fisher Scientific, Coomassie G-250). Several radiolabeled proteins of different sizes were visible in the autoradiographic scan of the 197(m)Hg-radiolabelled EDTA reference sample. This observation points at substantial decomplexation and transchelation as EDTA is not a suitable chelator for 197(m)Hg. In contrast, no such protein bands were detectable for 197(m)Hg-radiolabeled bispidine 3a* showing its remarkable stability under these conditions.

Animal Studies

Animal experiments were conducted at Helmholtz-Zentrum Dresden-Rossendorf according to the guidelines of German Regulations for Animal Welfare and have been approved by the Local Animal Ethics Committee for Animal Experiments (Landesdirektion Sachsen). Investigations were performed using juvenile male Wistar rats (RjHan:WI, Janvier, Le Genest-Saint-Isle, France) with bodyweight between 180 and 250 g housed in a pathogen-free facility. For in vivo imaging, anaesthesia was induced and maintained with inhalation of 10 vol% desflurane (Baxter, Unterschleißheim, Germany) in 30 vol% oxygen air and the body temperature of the animals was maintained at 37°C. Animals were sacrificed using CO2 inhalation and cervical dislocation, then organs were excised for further investigation. All solutions of 3a* injected were prepared by diluting the purified 197(m)Hg solutions with 0.15 M NaCl(aq) until the resulting solution had ≤5% v/v EtOH.

SPECT/CT imaging.

Single-photon emission computed tomography (SPECT) and X-ray computed tomography (CT) of rats were performed using the quantitative small animal nanoScan SPECT/CT scanner (Mediso, Budapest, Hungary) equipped with an APT63 aperture consisting of four M3 multi-pinhole collimators providing a 70×70 mm transaxial field of view (FOV). Photon emission was recorded within the 20% energy windows of the 77 keV (197Hg) and the 134 keV (197mHg) photopeaks. Peak uniformity and energy were calibrated using a point source (0.5 MBq of 197(m)HgCl2 in 20 µl of 0.1 M HCl(aq)). The activity was calibrated using a syringe source (23 MBq of 197(m)HgCl2 in 3 ml of 0.1 M HCl(aq)). CT images were captured at peak kilovoltage of 50 kVp and were used for attenuation correction and anatomical referencing. Imaging was performed 24 h after intravenous injection of the 197(m)HgCl2 (27 MBq) or 3a* (34 MBq) NaCl(aq) solution (0.6 ml) via the tail vein. Photon emission was recorded with a frame time of 90 s (total scan time 1.5 h). Projection data were reconstructed using the Tera-Tomo™ 3D high dynamic range algorithm applying corrections for scatter and attenuation. Decay correction was done manually referring to the activity of a separate reference source measured in a calibrator simultaneously with each scan. Images were post-processed, analyzed, and fused with CT images using the software Rover (ABX GmbH, Radeberg, Germany) and displayed as maximum intensity projection (MIPs) at identical scaling.

Biodistribution.

After intravenous injection of the 197(m)HgCl2 (0.25 MBq) or 3a* (0.25 MBq) NaCl(aq) solution (0.5 ml), via the tail vein, tissue samples of rats were measured at 5 min, 1 h, and 24 h. At each time point, animals were sacrificed (n = 4), organs were excised and weighed. The activity was determined using the gamma counter Wizard (PerkinElmer, Waltham, MA, USA). Decay correction was done manually referring to the activity of a separate reference source measured simultaneously with each sample series. Data were reported as injected dose per gram tissue [ID/g].
3. Spectra

HPLC
Solvent A: H₂O + 0.01% TFA, Solvent B: ACN + 0.01% TFA
Flow rate = 1ml/min, CH2: γ detector. CH5: UV detector (220 nm)

HPLC of 3,7-bis(2-bromobenzyl)-1,5-diphenyl-3,7-diazabicyclo[3.3.1]nonan-9-one (1)
Solvent gradient A:B = 70:30 (0 min) to 20:80 (20 min) to 100% ACN (20 – 30 min).

HPLC of 9-butyl-1,5-diphenyl-3,7-bis(2-(trimethylstannyl)benzyl)-3,7-diazabicyclo[3.3.1]nonan-9-ol (2a). Solvent gradient A:B = 40:60 (0-2 min) to 10:90 (2-20 min) holding at 10:90 (20 – 25 min).

| Peak # | t_R (min) | Area (µVs) | Height (µV) | Area% |
|--------|-----------|------------|-------------|-------|
| 1      | 19.087    | 10100787   | 405704      | 95.04 |
| 2      | 21.290    | 526903     | 56188       | 4.96  |
HPLC of 1,5-diphenyl-3,7-bis(2-(trimethylstannyl)benzyl)-3,7-diazabicyclo[3.3.1]nonan-9-ol (2b). Solvent gradient A:B = 40:60 (0-2 min) to 10:90 (2-20 min) holding at 10:90 (20 – 25 min).

HPLC of 9-buty1-8,10-diphenyl-6,10:8,12-dimethanodibenzo[c,f][1,9]diaza[5]mercurycyclotetradecan-9-ol (3a) crude reaction mixture. Solvent gradient A:B = 40:60 (0-2 min) to 10:90 (2-20 min)
Radio/UV-HPLC co-injection of $[^{197}(m)\text{Hg}]$-butyl-8,10-diphenyl-6,10:8,12-dimethanodibenzo[c,f][1,9]diaza[5]mercurycyclotetradecan-9-ol (3a*) and 3a (before purification). Solvent gradient A:B = 40:60 (0-2 min) to 10:90 (2-20 min). γ detector after UV detector hence small delay.
Radio/UV-HPLC of $[^{197}\text{Hg}]$-butyl-8,10-diphenyl-6,10:8,12-dimethanodibenzo[c,f][1,9]diaza[5]mercurycyclotetradecan-9-ol (3a*) (purified).

Solvent gradient A:B = 40:60 (0-2 min) to 10:90 (2-20 min).

UV spec zoomed in enough to see injection peak details, baseline drift caused by TFA in increasing amount of ACN, but of most interest is that the very small amount of 3a* formed is just detectable.
HPLC of 8,10-diphenyl-6,10:8,12-dimethanodibenzo[c,f][1,9]diaza[5]mercurcyclotetradecan-9-ol (3b) crude reaction mixture. Solvent gradient A:B = 40:60 (0-2 min) to 10:90 (2-20 min).

| Peak # | t_R (min) | Area (µVs)  | Height (µV) | Area% |
|--------|-----------|-------------|-------------|-------|
| 1      | 5.203     | 12838837    | 572228      | 93.86 |
| 2      | 15.803    | 839534      | 36858       | 6.14  |
Radio/UV-HPLC co-injection of $^{197(\text{m})\text{Hg}}$8,10-diphenyl-6,10:8,12-dimethanodibenzo[c,f][1,9]diaza[5]mercurycyclotetradecan-9-ol (3b*) and 3b
Solvent gradient A:B = 40:60 (0-2 min) to 10:90 (2-20 min)
Mass Spectra
Relative abundance shown either in table or zoomed image to show isotopic distribution pattern. Samples all purely taken from main peak of HPLC.

HRMS of 3,7-bis(2-bromobenzyl)-1,5-diphenyl-3,7-diazabicyclo[3.3.1]nonan-9-one (1)
C$_{33}$H$_{30}$Br$_2$N$_2$O calculated [M+H]$^+$ 631.0783

| m/z     | z | Abund  |
|---------|---|--------|
| 631.0781| 1 | 100.00 |
| 121.0509| 1 | 73099.2|
| 149.0234| 1 | 70519.11|
| 301.1412| 1 | 63722.61|
| 629.0797| 1 | 392672.75|
| 630.0803| 1 | 134300.38|
| 631.0791| 1 | 802645.25|
| 632.0813| 1 | 285831.03|
| 633.0768| 1 | 401743.72|
| 634.0793| 1 | 140357.17|
| 922.0098| 1 | 628565.32|

ESI-MS of 3,7-bis(2-bromobenzyl)-1,5-diphenyl-3,7-diazabicyclo[3.3.1]nonan-9-ol (1H)
C$_{33}$H$_{32}$Br$_2$N$_2$O calculated [M+H]$^+$ 633.09
HRMS of 9-butyl-1,5-diphenyl-3,7-bis(2-(trimethylstannyl)benzyl)-3,7-diazabicyclo[3.3.1]nonan-9-ol (2a)
C_{43}H_{58}N_{2}OSn_{2} calculated [M+H]^+ 857.2666

ESI-MS of 1,5-diphenyl-3,7-bis(2-(trimethylstannyl)benzyl)-3,7-diazabicyclo[3.3.1]nonan-9-ol (2b)
C_{39}H_{50}N_{2}OSn_{2} calculated [M+H]^+ 801.20
HRMS of 9-butyl-8,10-diphenyl-6,10:8,12-dimethanodibenzo[c,f][1,9]diaza[5]mercurycyclotetradecan-9-ol (3a)
\[C_{37}H_{40}HgN_2O\] calculated [M+H]^+ 731.2925

ESI-MS of 8,10-diphenyl-6,10:8,12-dimethanodibenzo[c,f][1,9]diaza[5]mercurycyclotetradecan-9-ol (3b)
\[C_{33}H_{32}HgN_2O\] calculated [M+H]^+ 675.2
NMR Spectra

HSQC signals: red = CH or CH₃, blue = CH₂
$^1$H NMR (400 MHz, cdcl$_3$) δ 7.64 (dd, $J = 8.0$, 1.2 Hz, 2H), 7.53 (dd, $J = 7.6$, 1.7 Hz, 2H), 7.34 (td, $J = 7.5$, 1.3 Hz, 2H), 7.31 – 7.24 (m, 4H), 7.24 – 7.16 (m, 8H), 3.85 (s, 4H), 3.42 (dd, $J = 186.0$, 10.7 Hz, 8H).
$^{13}$C NMR (101 MHz, cde$_2$)$_3$ δ 210.89, 142.75, 137.37, 133.31, 131.71, 129.21, 127.98, 127.41, 127.03, 126.72, 125.21, 64.87, 61.25, 54.64.
COSY of 1

NCH₂ArH (3 & 4)
NCH₂ArH (5 & 4)
NCH₂ArH (6 & 5)
Ph-o & Ph-m
Ph-p & Ph-m
COSY of 1

NCH₂CPh

NCH₂Ar

S20
$^1$H NMR (600 MHz, cdcl$_3$) δ 7.59 (d, $J = 7.9$ Hz, 1H), 7.53 (d, $J = 7.9$ Hz, 1H), 7.42 - 7.17 (m, 14H), 7.15 (td, $J = 7.7$, 1.8 Hz, 1H), 7.10 (td, $J = 7.7$, 3.9 Hz, 1H), 4.38 (s, 1H), 4.26 (s, 1H), 3.73 (s, 2H), 3.56 (s, 2H), 3.30 (dd, $J = 38.4$, 11.1 Hz, 4H), 2.58 (dd, $J = 302.6$, 11.3 Hz, 4H).
$^{13}$C NMR (151 MHz, cdcl$_3$) $\delta$ 146.23, 137.66, 137.15, 133.28, 131.61, 131.49, 129.05, 128.92, 128.39, 127.41, 127.26, 126.92, 126.39, 125.18, 125.07, 74.80, 66.24, 61.72, 61.39, 59.00, 44.53.
COSY 1H

CHOH

S26
$^1$H NMR (400 MHz, cdcl$_3$) δ 7.80 (t, $J = 7.0$ Hz, 2H), 7.48 – 7.40 (m, 6H), 7.36 (t, $J = 7.5$ Hz, 2H), 7.30 – 7.19 (m, 6H), 7.16 (t, $J = 7.2$ Hz, 2H), 3.81 (s, 2H), 3.78 (s, 2H), 3.38 (dd, $J = 41.1$, 11.3 Hz, 4H), 2.89 (dd, $J = 51.3$, 10.9 Hz, 4H), 1.27 (t, $J = 9.7$ Hz, 2H), 0.65 (h, $J = 7.2$ Hz, 2H), 0.45 (s, 9H), 0.39 (s, 9H), 0.37 (t, $J = 7.6$ Hz, 3H), 0.04 (p, $J = 7.5$ Hz, 2H).

7.26 solvent peak overlap
$^{13}$C NMR (101 MHz, cdcl$_3$) $\delta$ 145.29, 145.25, 144.47, 141.94, 141.85, 136.22, 136.08, 129.26, 129.02, 128.93, 128.61, 127.85, 127.35, 127.16, 126.84, 126.18, 77.26, 65.61, 64.38, 64.29, 60.30, 47.32, 30.24, 26.12, 23.34, 13.71, -7.44, -7.47.
$^{119}$Sn NMR (224 MHz, cdcl$_3$) δ -31.98, -33.89, -33.96, -34.54.

$^{119}$Sn chemical shift corresponds well with aryltrimethyltin compounds in literature (cf. Chen et al. SI: page S 8, compound 3p).
N.B. Splitting of signals seen in apolar solvent due to H-bond conformer (chair-boat) disappears in polar solvent as only 1 conformer detected (chair-chair).²
$^1$H NMR (600 MHz, cdcl$_3$) δ 7.54 (d, $J = 7.7$ Hz, 4H), 7.49 (dd, $J = 6.9$, 1.4 Hz, 1H), 7.46 (dd, $J = 7.1$, 1.4 Hz, 1H), 7.40 (t, $J = 7.3$ Hz, 1H), 7.36 (d, $J = 7.3$ Hz, 1H), 7.34 (d, $J = 7.8$ Hz, 1H), 7.29 (td, $J = 7.2$, 1.3 Hz, 1H), 7.25 (t, $J = 8.0$ Hz, 4H), 7.23 - 7.16 (m, 3H), 7.09 (td, $J = 7.4$, 1.5 Hz, 1H), 3.63 (s, 2H), 3.51 (s, 2H), 3.33 (dd, $J = 198.0$, 11.5 Hz, 4H), 3.00 (dd, $J = 305.4$, 11.4 Hz, 4H), 1.74 (s, 1H), 1.45 (t, $J = 8.5$ Hz, 2H), 0.74 (h, $J = 7.3$ Hz, 2H), 0.41 (t, $J = 7.3$ Hz, 3H), -0.04 (p, $J = 7.9$ Hz, 2H).
$^{13}$C NMR (101 MHz, cdcl$_3$) δ 171.3, 170.9, 146.8, 146.4, 141.8, 139.1, 138.8, 129.0, 128.8, 128.0, 127.8, 127.24, 127.16, 126.73, 126.68, 126.4, 75.1, 67.7, 67.0, 60.2, 60.1, 46.3, 31.4, 26.0, 23.0, 13.6.
$^{199}\text{Hg}$ NMR (107 MHz, cdcl$_3$) $\delta$ -684.49.

$^{199}\text{Hg}$ chemical shift corresponds well with bisarylmercury compounds in literature.\textsuperscript{5}
4. Chem3D calculated structure of 3a

![Chem3D calculated structure of 3a](image)

Lowest energy structure approximated with Chem3D MM2 (Non-polar hydrogen atoms omitted for better clarity)

5. References

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