**Supporting Information**

# Non-cross-bridged tetraazamacrocyclic chelator for stable $^{64}$Cu-based radiopharmaceuticals

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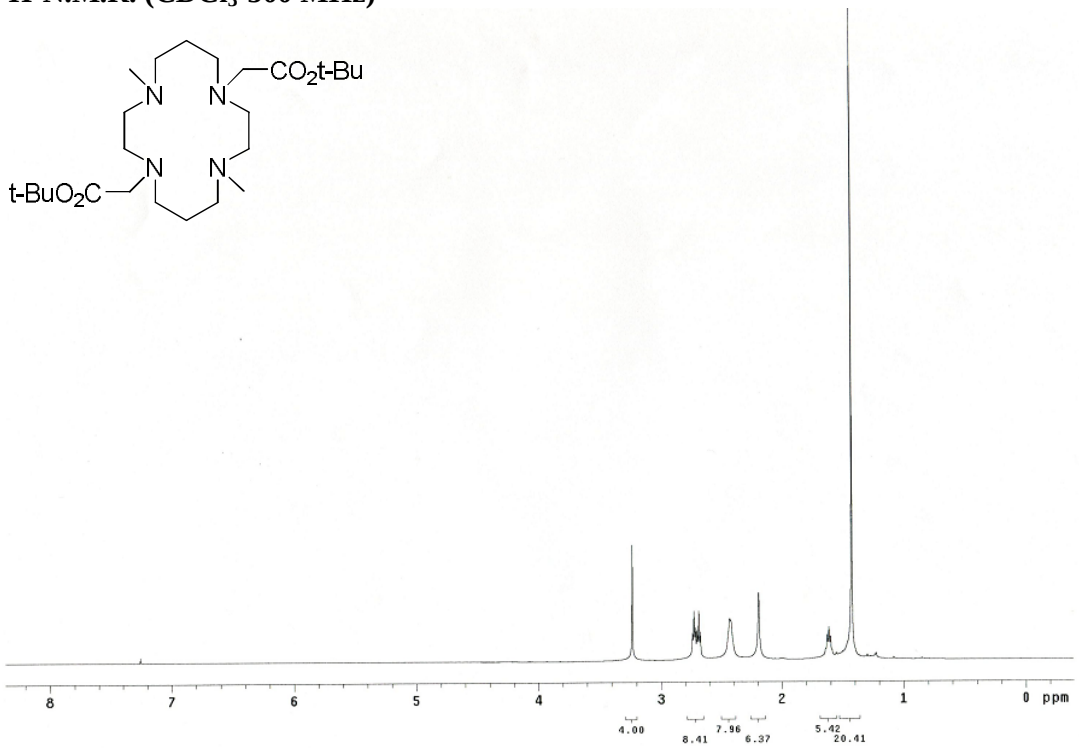
**Materials and methods:** Cyclam and ECB-TE2A were purchased from CheMatech (Dijon, France). All other reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO) and were used as received. Copper-64 was produced at KIRAMS (Seoul, Korea) by the $^{64}Ni(p,n)^{64}Cu$ nuclear reaction using an MC50 Cyclotron (Scanditronix, Sweden). TE2A and Cu-TE2A was prepared according to published literature procedure.[1] Cu-ECB-TE2A[2] and $^{64}$Cu-ECB-TE2A[3] was prepared according to published literature procedure.

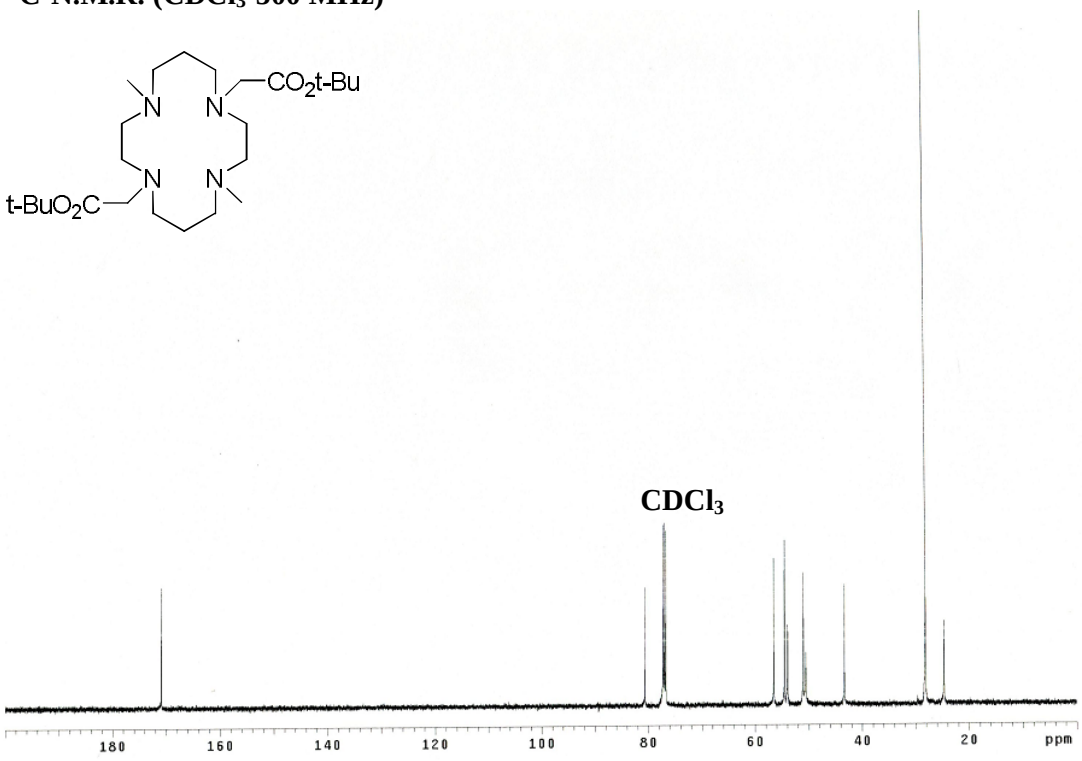
**Instrumention:** All $^{1}$H NMR and $^{13}$C NMR spectra were measured on Varian Unity Inova 500 MHz instrument. High–resolution mass spectra (HRMS) were recorded on JEOL JMS700 or Quattro Premier XE mass spectrometer. UV-Vis spectra were acquired on a Shimadzu UV-Vis spectrophotometer (UV-1650PC). Analytical HPLC traces were acquired using Waters 600 series HPLC system and Waters Xbridge C18 column (4.6 X 150 mm, 5 µm) with an isocratic method (water, 1 mL/min flow rate). The radio-TLC measurements were performed using a Bioscan 2000 imaging scanner (Bioscan, Washington, D.C., USA).

**Synthesis of 1,8-N,N'-bis-(carbo-*tert*-butoxymethyl)-4,11-*N''*,*N'''*-bis-(methyl)-1,4,8,11-tetraaza cyclotetradecane (4).** To a solution of **3** (3.06 g, 7.14 mmol) in anhydrous ethanol (80 mL) were added $NaBH_4$ (8.10 g, 214.2 mmol). After stirring for 24 h at room temperature, the solvent was removed under reduced pressure, residue was dissolved in $CH_2Cl_2$ (150 mL) and filtered. The filtrate was evaporated and residue was purified via column chromatography on silica, eluting with Chlorofom/isopropyl amine (20:2) to afford clear oil **4**, (3.05 g, 94% yield). $^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 3.23 (s, 4H), 2.80-2.62 (m, 8H) 2.43 (br s, 8H), 2.19 (s, 6H), 1.68-1.58 (m, 4H), 1.42 (s, 18H); $^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ 170.89, 80.61, 56.50, 54.51, 53.91, 51.00, 50.55, 43.29, 28.15, 24.66; HRMS (FAB) calculated for $C_{24}H_{49}N_4O_4$, 457.3754 [$(M+H)^+$], found: 457.3756 [$(M+H)^+$].

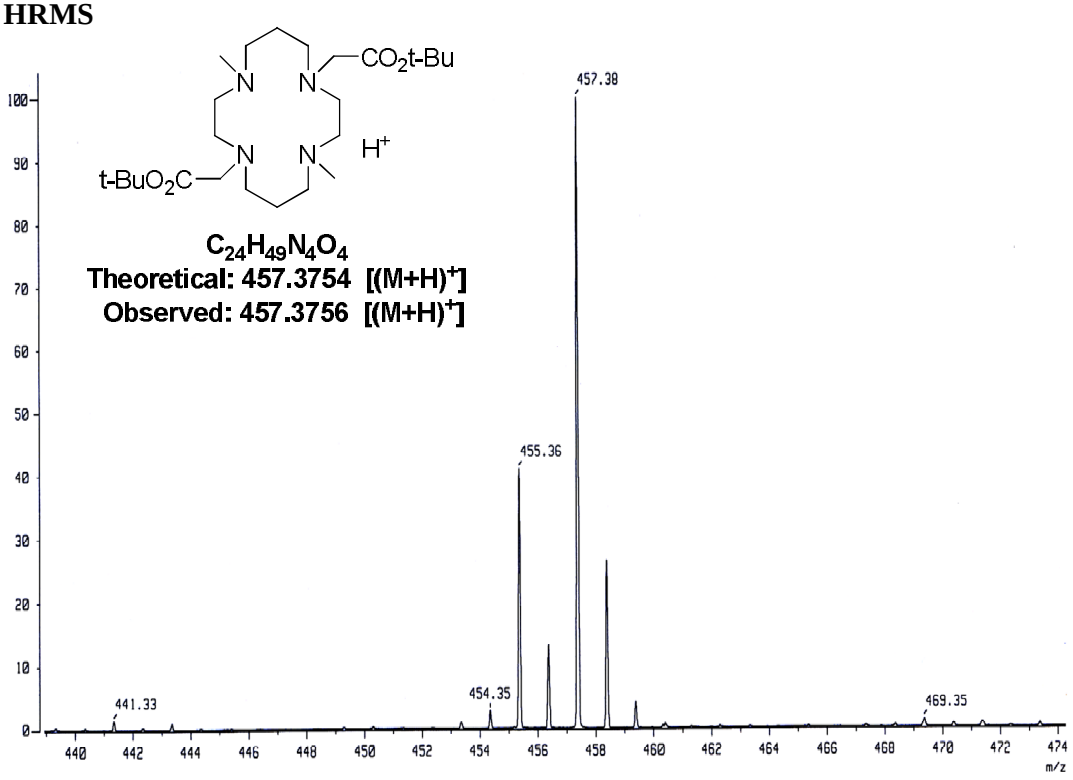
**$^1$H-N.M.R. ($CDCl_3$-500 MHz)**



**$^{13}$C-N.M.R. ($CDCl_3$-500 MHz)**

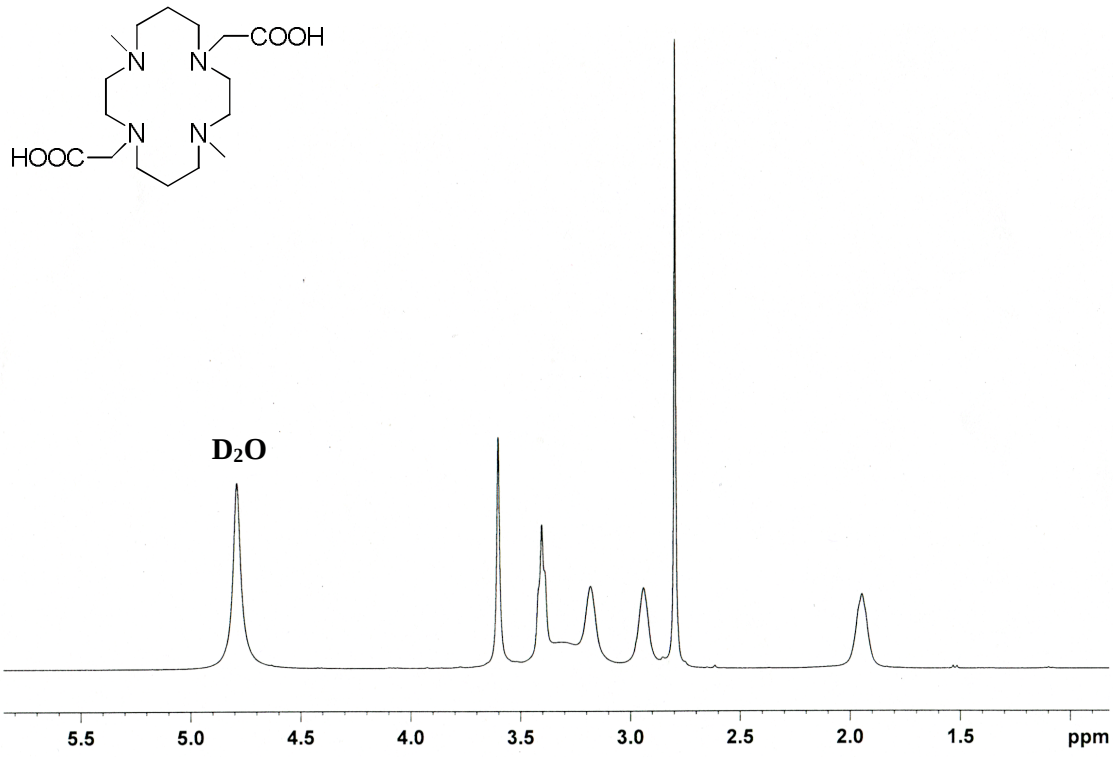



**HRMS**

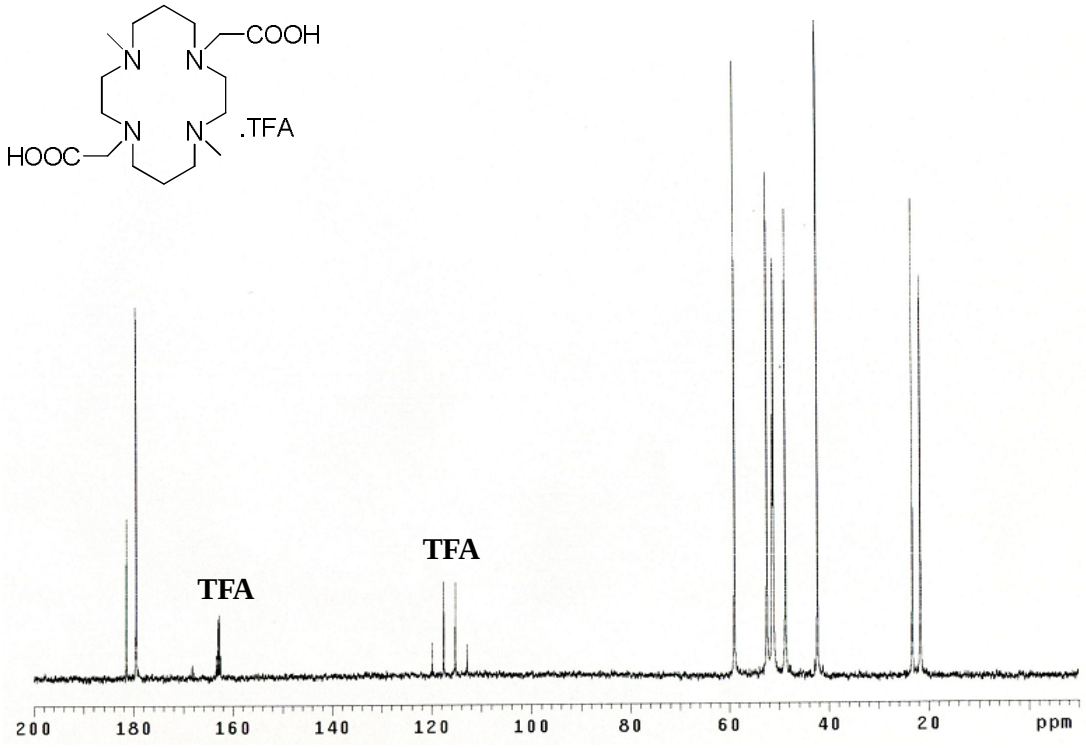


**Synthesis of 1,8-N,N'-bis-(carboxymethyl)-4,11-*N''*,*N'''*-bis-(methyl)-1,4,8,11-tetraazacyclotetra decane (5).** Compound **4** (1.46 g, 3.19 mmol) was dissolved in a 1:1 (vol:vol) mixture of $CF_3CO_2H$ (TFA) and $CH_2Cl_2$ (50 mL). The mixture was stirred at room temperature for 24 h. The solvent was removed under reduced pressure to give an oily residue which was triturated with $Et_2O$ to provide white solid **5** (1.81 g, 99% yield). $^1$H NMR (500 MHz, $D_2O$): $\delta$ 3.60 (s, 4H), 3.40-3.10 (m, 12H), 2.94 (br s, 4H), 2.79 (s, 6H), 1.94 (br s, 4H); $^{13}$C NMR (125 MHz, $D_2O$): $\delta$ 181.49, 179.53, 59.02, 52.49, 51.42, 51.20, 48.80, 42.93, 42.39, 23.34, 21.72**;** HRMS (FAB) calculated for $C_{16}H_{33}N_4O_4$, 345.2502 [$(M+H)^+$], found: 345.2502 [$(M+H)^+$].

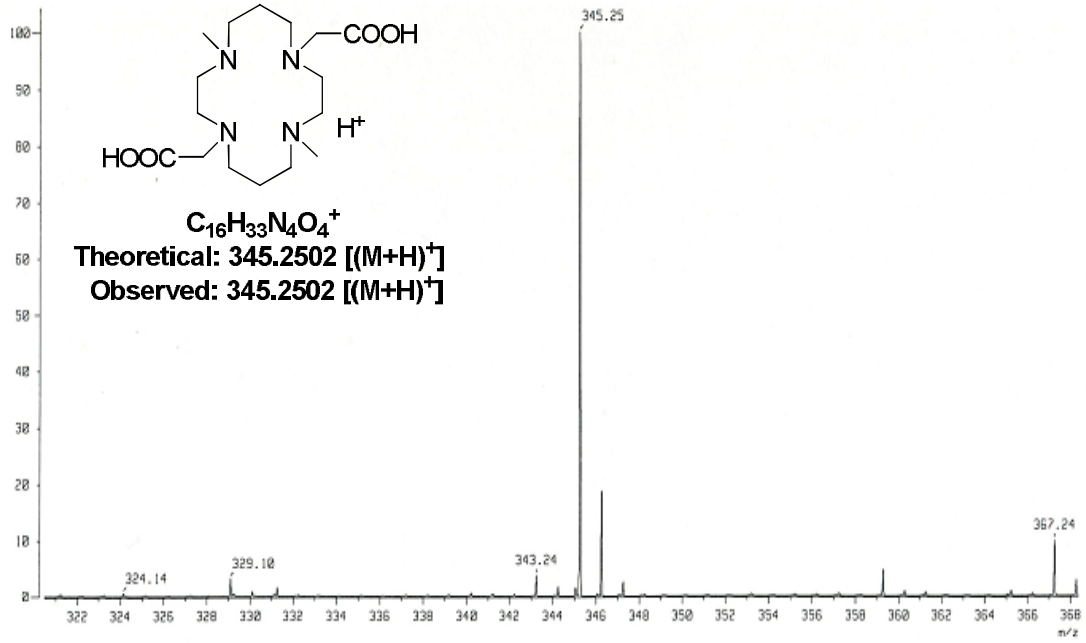
**$^1$H-N.M.R. ($D_2O$-400 MHz)**



**$^{13}$C-N.M.R. ($D_2O$-500 MHz)**

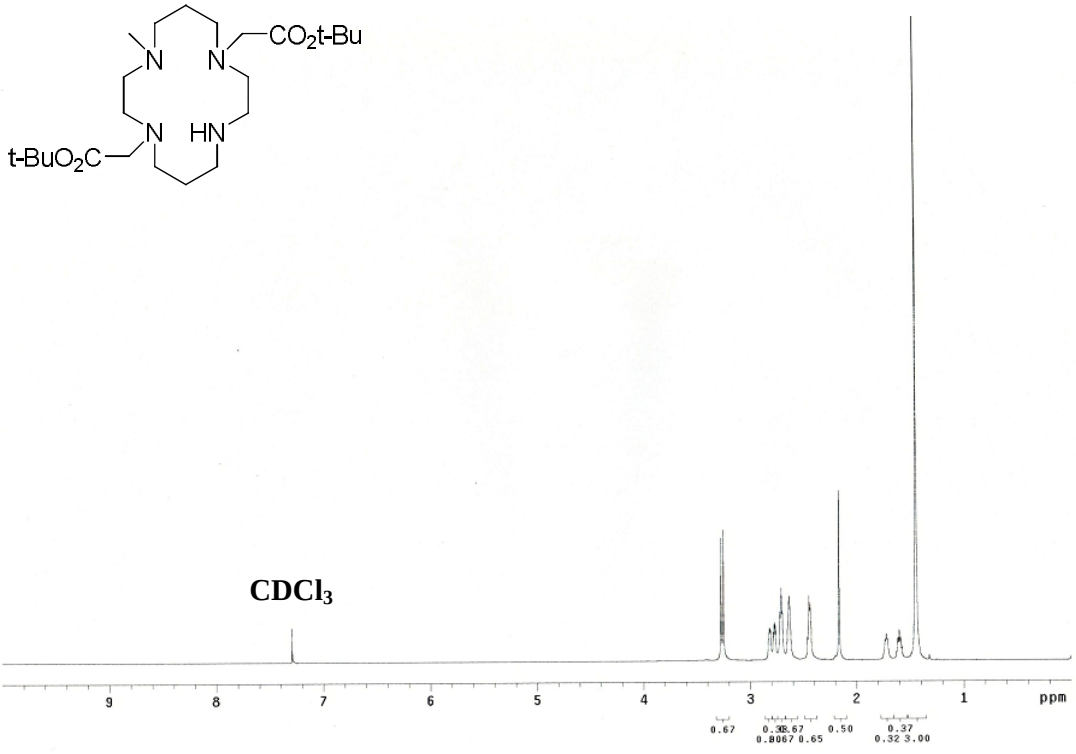



**HRMS**

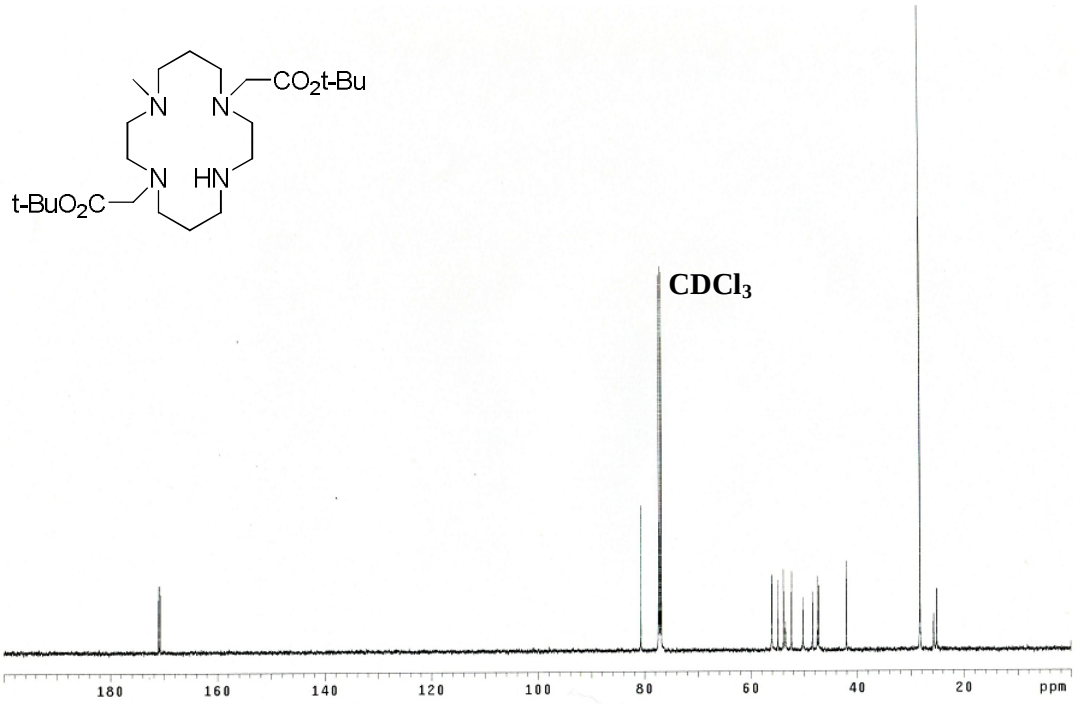


**Synthesis of 1,8-N,N'-bis-(carbo-*tert*-butoxymethyl)-4-*N''*-(methyl)-1,4,8,11-tetraazacyclotetra decane (7).** To a solution of **6** (2.33 g, 5.43 mmol) in anhydrous chloroform (50 mL) was added methyl iodide (6.78 ml, 15.43 g, 108.72 mmol). After stirring for 24 h at room temperature, the solvent was removed under reduced pressure and residue was purified via column chromatography on silica, eluting with Chlorofom/isopropyl amine (20:2) to afford a clear oil **7**, (2.41 g, 84% yield). $^{1}$H NMR (500 MHz, $CDCl_3$): $\delta$ 3.27-3.25 (dd, 4H), 2.84-2.43 (m, 16H), 2.16 (s, 3H), 1.73-1.59 (m, 4H), 1.45 (s, 18H); $^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ 170.94, 170.68, 80.57, 55.99, 55.93, 54.80, 53.77, 53.40, 52.26, 50.11, 48.34, 47.41, 47.17, 41.88, 28.18, 25.59, 25.00; HRMS (FAB) calculated for $C_{23}H_{47}N_4O_4$, 443.3597 [$(M+H)^+$], found: 443.3600 [$(M+H)^+$].

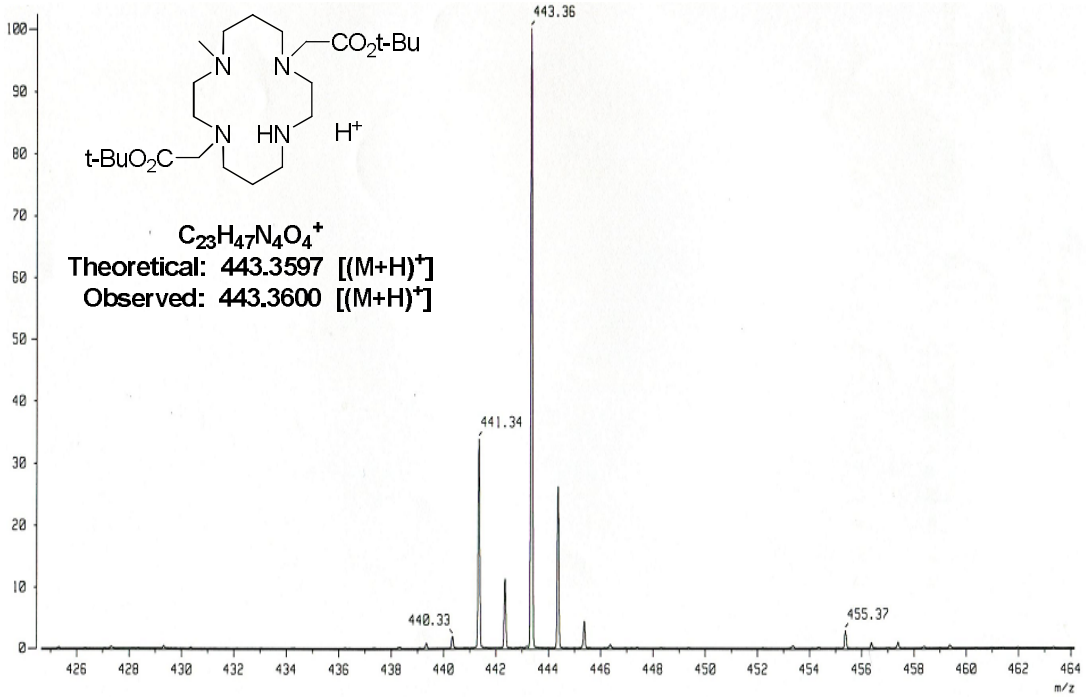
**$^{1}$H-N.M.R. ($CDCl_3$-500 MHz)**



**$^{13}$C-N.M.R. ($CDCl_3$-125 MHz)**

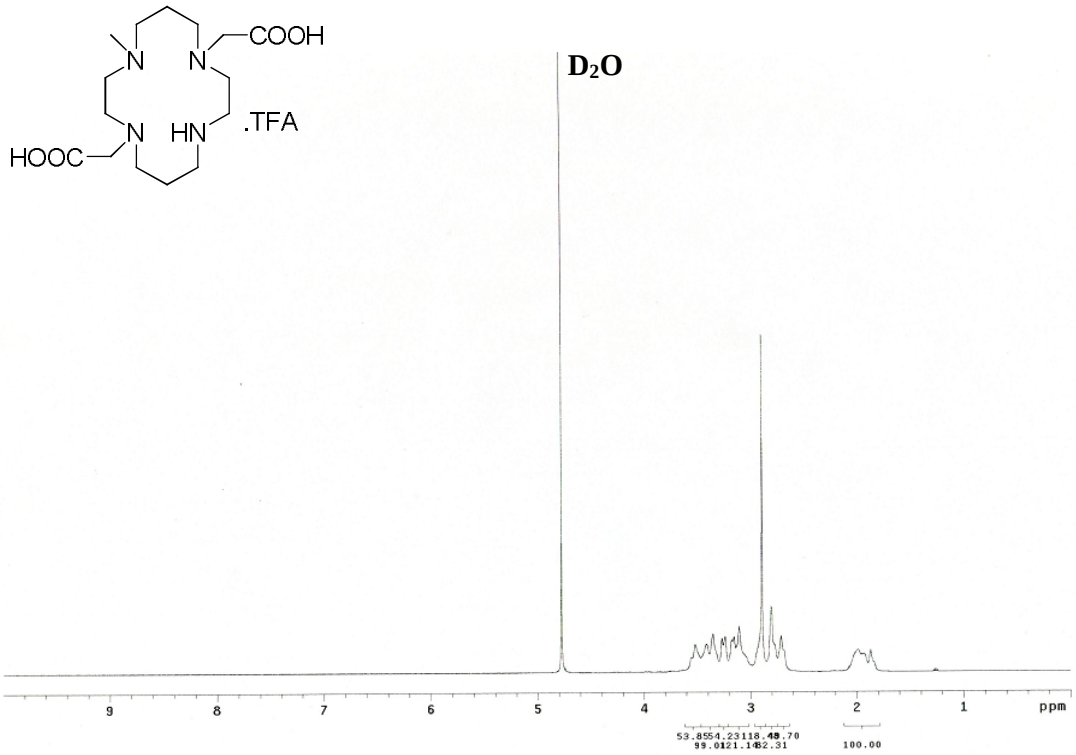



**HRMS**

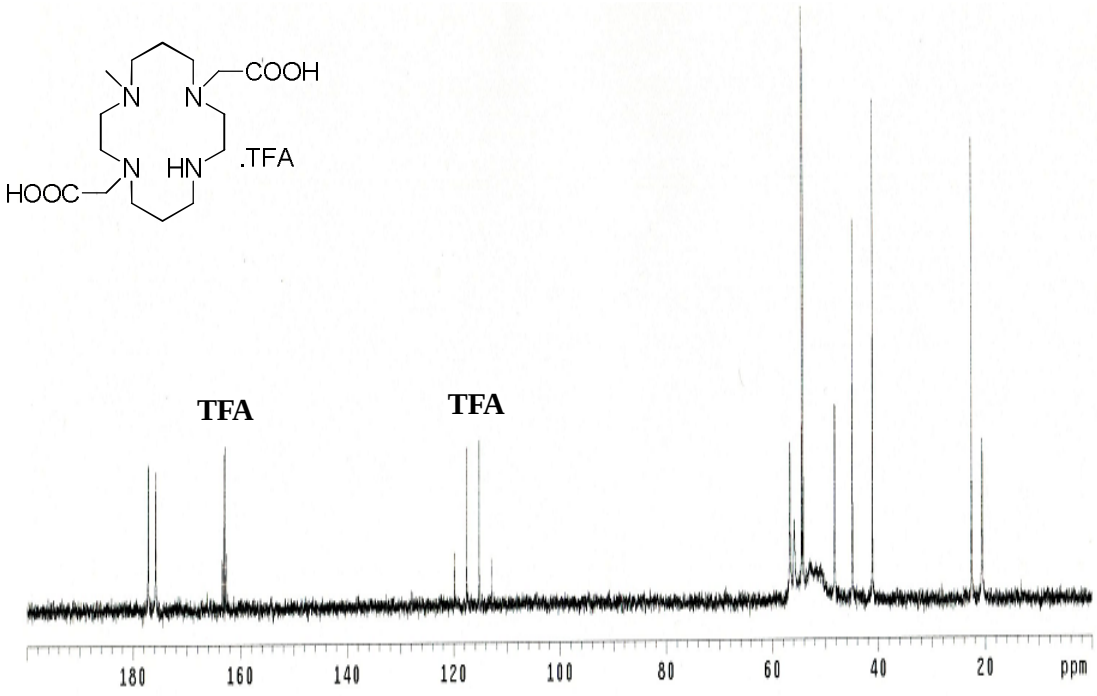


**Synthesis of 1,8-N,N'-bis-(carboxymethyl)-4-*N''*-(methyl)-1,4,8,11-tetraazacyclotetradecane (8).** Compound **7** (1.56 g, 3.52 mmol) was dissolved in a 1:1 (vol:vol) mixture of $CF_3CO_2H$ (TFA) and $CH_2Cl_2$ (60 mL). The mixture was stirred at room temperature for 24 h. The solvent was removed under reduced pressure to give an oily residue which was triturated with $Et_2O$ to provide white solid **8** (1.95 g, 99% yield). $^1$H NMR (500 MHz, $D_2O$): $\delta$ 3.60-3.05 (m, 13H), 2.98-2.641 (m, 10H), 2.12-1.82 (m, 4H); $^{13}$C NMR (125 MHz, $D_2O$): $\delta$ 177.20, 175.83, 56.71, 55.87, 54.50, 54.22, 52.92, 48.268, 44.94, 41.15, 22.61, 20.64**;** HRMS (FAB) calculated for $C_{15}H_{31}N_4O4_6$, 331.2345 [$(M+H)^+$], found: 331.2347 [$(M+H)^+$].

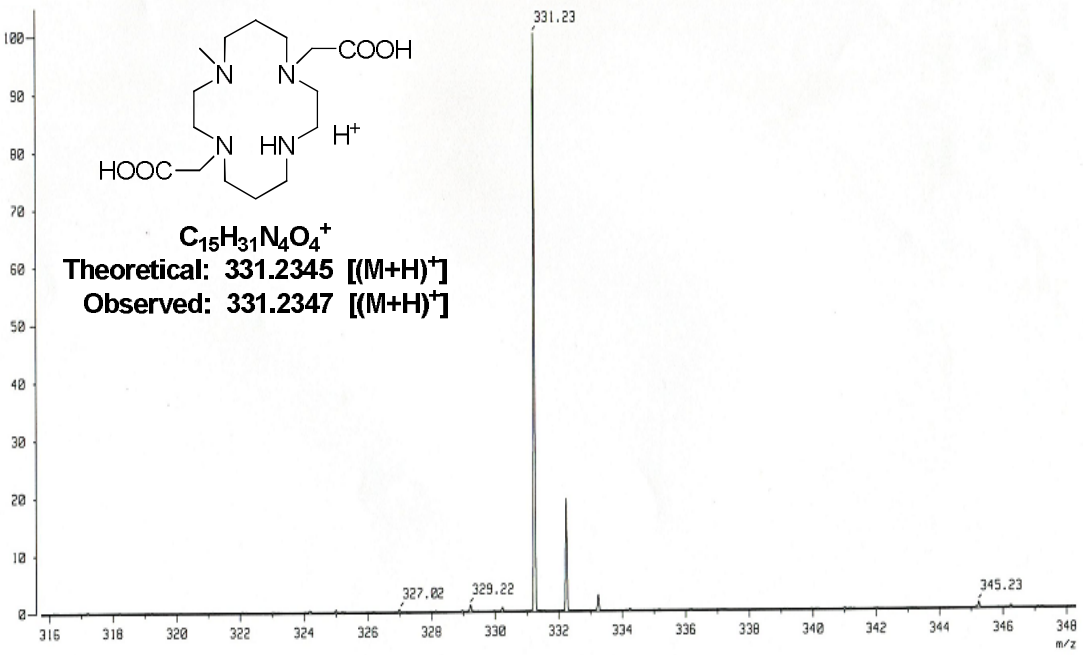
**$^1$H-N.M.R. ($D_2O$-500 MHz)**



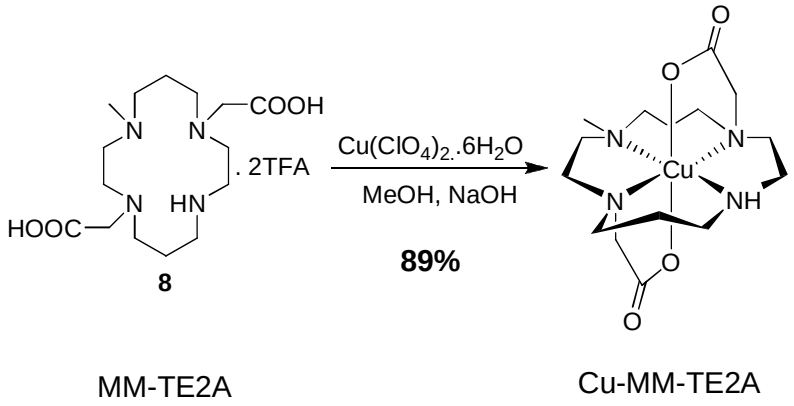
**$^{13}$C-N.M.R. ($D_2O$-125 MHz)**




**HRMS**

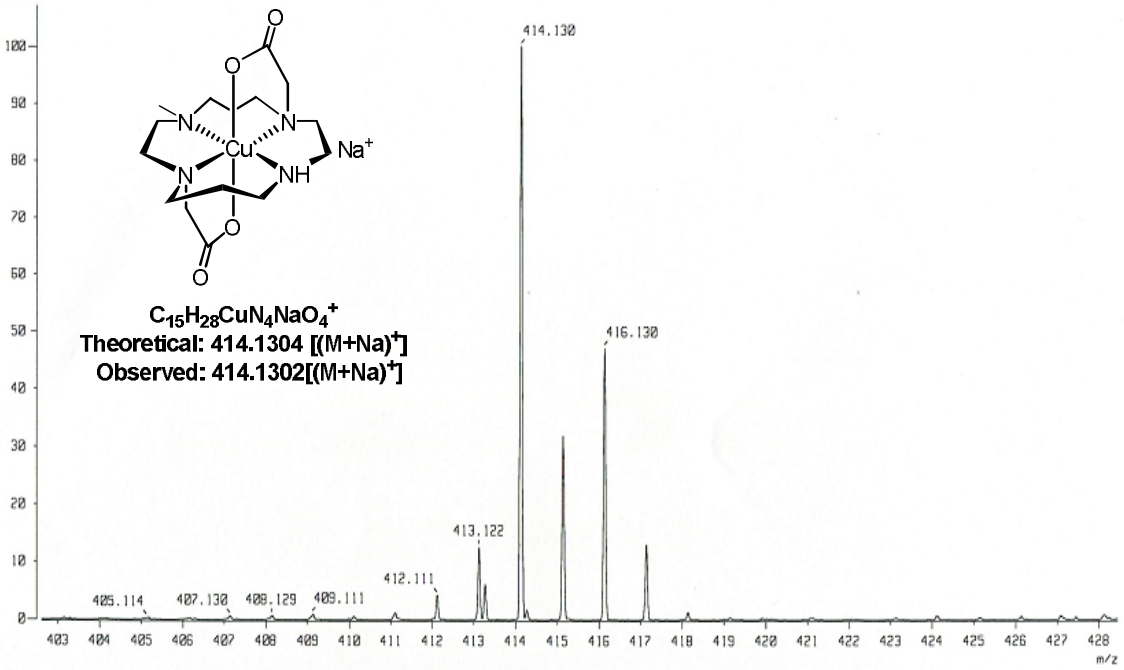


**Scheme S1.** Synthesis of copper complex of MM-TE2A (Cu-MM-TE2A)

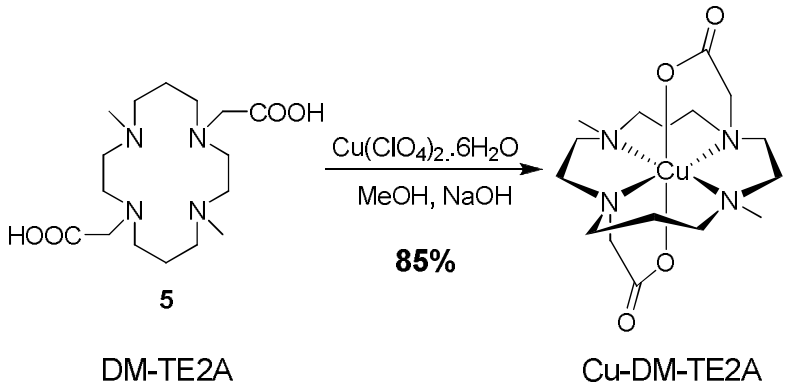



**Synthesis of Cu-MM-TE2A.** To a solution of MM-TE2A **8** (265 mg, 0.47 mmol) and $Cu(ClO_4)_2 \cdot 6H_2O$ (176 mg, 0.47 mmol) in 22 mL of methanol was added 1M aqueous solution of NaOH (2.82 ml). The resulting clear blue solution was refluxed for 2 h, cooled, and filtered through celite bed. The filtrate was subjected to diethyl ether diffusion. The deposited blue crystals were collected and dried. (166 mg, 89% yield). HRMS (FAB): Calculated for $C_{15}H_{28}CuNaN_4O_4$, 414.1304 $[(M+Na)^+]$ Found: 414.1302 $[(M+Na)^+]$, Visible electronic spectrum: $\lambda_{max}$ (5 M HCl)/561 nm ($\varepsilon$ = 33 $M^{-1}$ $cm^{-1}$).

**HRMS**

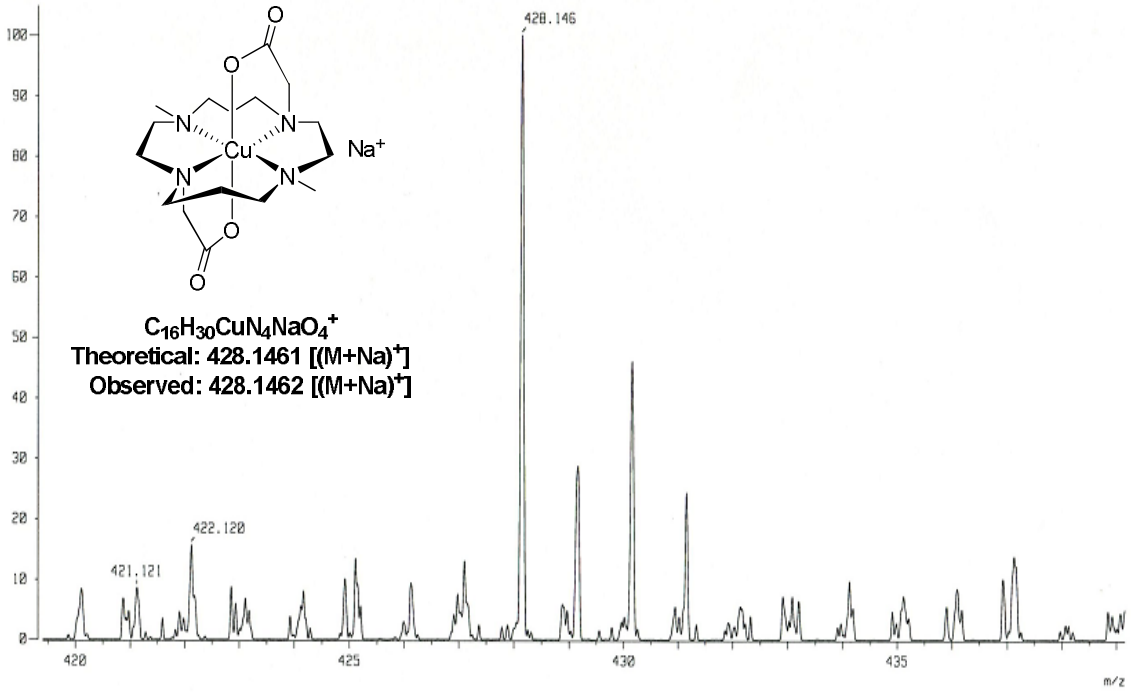


**Scheme S2.** Synthesis of copper complex of DM-TE2A (Cu-DM-TE2A)




**Synthesis of Cu-DM-TE2A.** To a solution of DM-TE2A **5** (253 mg, 0.73 mmol) and $Cu(ClO_4)_2 \cdot 6H_2O$ (272 mg, 0.73 mmol) in 25 mL of methanol was added 1M aqueous solution of NaOH (4.38 ml). The resulting clear blue solution was refluxed for 2 h, cooled, and filtered through celite bed. The filtrate was subjected to diethyl ether diffusion. The deposited blue crystals were collected and dried. (253 mg, 85% yield). HRMS (FAB): Calculated for $C_{16}H_{30}CuNaN_4O_4$, 428.1461 $[(M+Na)^+]$ Found: 428.1462 $[(M+Na)^+]$, Visible electronic spectrum: $\lambda_{max}$ (5 M HCl)/585 nm ($\varepsilon$ = 36 $M^{-1}$ $cm^{-1}$).

**HRMS**



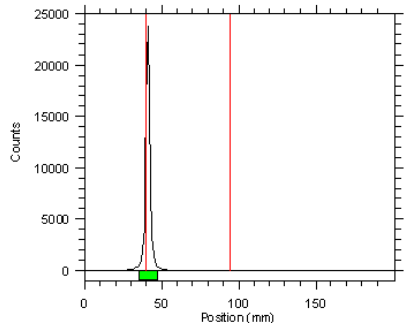
**Acid decomplexation studies by HPLC:** Sample concentration of copper complexes (Cu-MM-/DM-TE2A, Cu-TE2A and Cu-ECB-TE2A) studied were 3 mM concentration in 2mL 12 M HCl. UV HPLC spectrum in 12 M HCl at 90°C was recorded at specific time points by injecting an aliquot (20 μL) onto a reverse phase Xbridge C18 column (4.6 x 150 mm, 5 μm) with an isocratic method [Cu-MM-/DM-TE2A (water), Cu-TE2A(30 mM citric acid) and Cu-ECB-TE2A (water:MeOH 95:5), 1mL/min flow rate). The decreasing absorbance at UV region (280 nm) was used to monitor the progress of the decomplexation reaction.

**Electrochemical studies:** Cyclic voltammetry was conducted with a Biologic model SP-150 with three-electrode configuration. The working electrode was a glassy carbon (diameter = 3 mm), Ag/AgCl (sat. KCl) reference electrode and Pt wire counter electrode. Samples (1 mM) were run in 0.2 M phosphate buffer adjusted to pH 7.0 with glacial acetic acid at a scan rate 100 mV/s. The solutions were deoxygenated for 15 min with Ar prior to use and kept under Ar atmosphere during measurement.

**Radio labeling of MM-TE2A and DM-TE2A with $^{64}$Cu using $Cs_2CO_3$:** Complexation of $^{64}$Cu with MM-TE2A and DM-TE2A was achieved by a 60-min pre-incubation of MM-TE2A and DM-TE2A (100–500 μg) in EtOH with an excess of $Cs_2CO_3$ at 50°C respectively, with constant stirring. Following centrifugation, addition of no-carrier-added $^{64}CuCl_2$ to the isolated supernatant was accompanied by precipitation of CsCl. The mixture was vortexed and incubated at 50°C for MM-TE2A and DM-TE2A respectively for another 60 min. The mixture was centrifuged, and the supernatant was evaporated. Water was added to the dried mixture, and passed through the 0.2μm Nylon Acrodisk 13 filter. Formation of $^{64}$Cu-MM-TE2A and $^{64}$Cu-DM-TE2A complexes was verified by radio-TLC using a mobile phase consisting of 1:1 MeOH:10% ammonium acetate on silica plates. Radio-HPLC analysis of $^{64}$Cu-MM-TE2A and $^{64}$Cu-DM-TE2A was accomplished using Xbridge C18 column (4.6 × 150 mm, 5 μm) with an isocratic method (100% water, 1 mL/min flow rate)

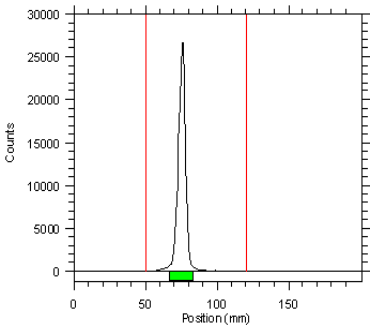
**(A)**

| Reg | (mm) Start | (mm) Stop | (mm) Centroid | RF | Region Counts | Region CPM | % of Total | % of ROI |
|---|---|---|---|---|---|---|---|---|
| Rgn 1 | 35.3 | 48.2 | 41.1 | 0.021 | 93841.0 | 46920.5 | 96.59 | 100.00 |
| 1 Peaks | | | | | 93841.0 | 46920.5 | 96.59 | 100.00 |




**(B)**

| Reg | (mm) Start | (mm) Stop | (mm) Centroid | RF | Region Counts | Region CPM | % of Total | % of ROI |
|---|---|---|---|---|---|---|---|---|
| Rgn 1 | 67.1 | 84.3 | 75.3 | 0.357 | 183859.0 | 91929.5 | 97.20 | 100.00 |
| 1 Peaks | | | | | 183859.0 | 91929.5 | 97.20 | 100.00 |




**(C)**

| Reg | (mm) Start | (mm) Stop | (mm) Centroid | RF | Region Counts | Region CPM | % of Total | % of ROI |
|---|---|---|---|---|---|---|---|---|
| Rgn 1 | 59.3 | 74.8 | 66.2 | 0.271 | 130078.0 | 65039.0 | 92.31 | 100.00 |
| 1 Peaks | | | | | 130078.0 | 65039.0 | 92.31 | 100.00 |

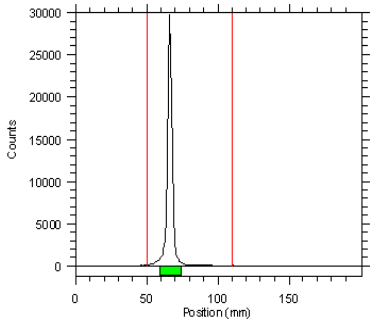



**Figure S1.** Radio-TLC of $^{64}CuCl_2$ (A) $^{64}$Cu-MM-TE2A (B) and $^{64}$Cu-DM-TE2A (C) [$Cs_2CO_3$ method]

**In Vitro Serum Stability:** In vitro serum stability of $^{64}$Cu-MM-TE2A and $^{64}$Cu-DM-TE2A were carried out by adding 50 µL of $^{64}$Cu-MM-TE2A and $^{64}$Cu-DM-TE2A (5 mM, ~500µCi) to 500 µL of FBS (Fetal Bovine Serum). The solution was incubated at 37°C, and samples were analyzed by radio-TLC at 10, 30, and 60 min and at 2, 4, 10 and 24 h post-administration to FBS.

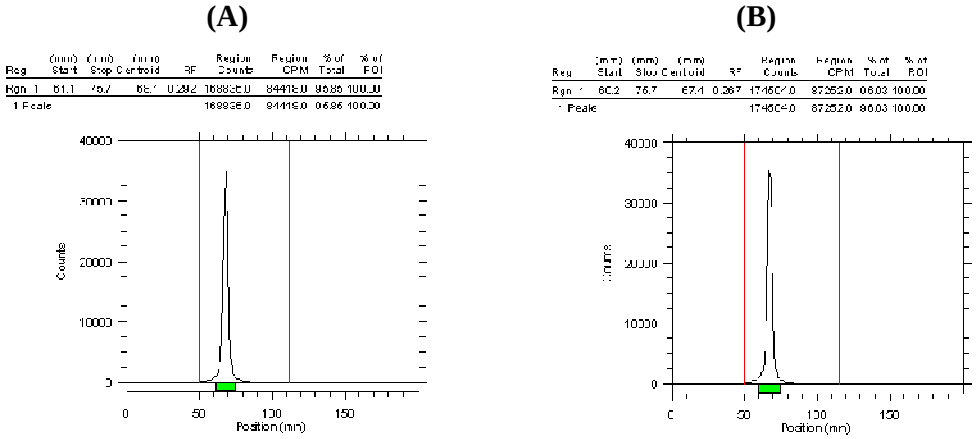



**Figure S2.** Radio-TLC of $^{64}$Cu-MM-TE2A (A) and $^{64}$Cu-DM-TE2A (B) solution in FBS at 37 ºC after 24 h.

**Determination of Partition Coefficients:**[3] The log*P* values of $^{64}$Cu complexes of MM-TE2A, DM-TE2A, TE2A and ECB-TE2A were determined by adding 5 μL of the labeled complex (~ 40-50 μCi) to a mixture of 500 μL of 1-octanol and 500 μL of water. The resulting solutions were vigorously vortexed for 5 min at room temperature, then centrifuged for 5 min to ensure complete separation of layers. From each of the six sets, 100 μL aliquot was removed from each phase into screw tubes and counted separately in a gamma counter. The partition coefficient was calculated as a ratio of counts in the 1-octanol fraction to counts in the water fraction. The log*P* values were reported in an average of six measurements.

**Comparative biodistribution of $^{64}$Cu-MM-TE2A, $^{64}$Cu-DM-TE2A and $^{64}$Cu-ECB-TE2A:** Six week old, mature, female, Balb-c mice (n = 5) were injected via tail-vein with $^{64}$Cu-MM-TE2A, $^{64}$Cu-DM-TE2A, and $^{64}$Cu-ECB-TE2A (ca. 20 μCi in 200 μL per mice). Animals were sacrificed at 30min, 4h and 24h post-injection. Organs and tissues of interest (Blood, Heart, Lung, Muscle, Bone, Spleen, Kidney and Liver) were removed, weighted, and counted using gamma-counter. The %ID per gram (%ID/g) was calculated by comparison to a weighted, counted standard.

**Table S1.** Mice biodistribution (%ID/g) of $^{64}$Cu-MM-TE2A in selected organs at 30 min, 4 h and 24 h post injection.

| Tissue/ Organ | $^{64}$Cu-MM-TE2A | | | | | |
|---|---|---|---|---|---|---|
| | 30min | 4 h | 24h | | | |
| | | | | 95% confidence interval | | |
| | | | | Lower | Upper | |
| Blood | 0.942 ± 0.047 | 0.015 ± 0.002 | 0.019 ± 0.003 | 0.015 | 0.023 | |
| Heart | 0.324 ± 0.051 | 0.064 ± 0.024 | 0.072 ± 0.023 | | | |
| Lung | 0.824 ± 0.083 | 0.109 ± 0.013 | 0.105 ± 0.034 | | | |
| Muscle | 0.304 ± 0.042 | 0.037 ± 0.009 | 0.076 ± 0.017 | | | |
| Bone | 0.282 ± 0.022 | 0.064 ± 0.019 | 0.081 ± 0.013 | | | |
| Spleen | 0.334 ± 0.031 | 0.064 ± 0.009 | 0.069 ± 0.005 | | | |
| Kidney | 3.491 ± 0.402 | 0.853 ± 0.358 | 0.162 ± 0.021 | 0.136 | 0.188 | |
| Liver | 0.928 ± 0.236 | 0.489 ± 0.059 | 0.168 ± 0.035 | 0.112 | 0.224 | |

**Table S2.** Mice biodistribution (%ID/g) of $^{64}$Cu-DM-TE2A in selected organs at 30 min, 4 h and 24 h post injection.

| Tissue/ Organ | $^{64}$Cu-DM-TE2A | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 30min | | 4 h | | 24h | | | |
| | | | | | | | 95% confidence interval | |
| | | | | | | | Lower | Upper |
| Blood | 0.929 | ± 0.047 | 0.071 | ± 0.01 | 0.053 | ± 0.004 | 0.048 | 0.058 |
| Heart | 0.38 | ± 0.051 | 0.185 | ± 0.041 | 0.197 | ± 0.017 | | |
| Lung | 0.858 | ± 0.083 | 0.401 | ± 0.064 | 0.262 | ± 0.023 | | |
| Muscle | 0.288 | ± 0.042 | 0.171 | ± 0.101 | 0.104 | ± 0.029 | | |
| Bone | 0.323 | ± 0.022 | 0.14 | ± 0.027 | 0.148 | ± 0.038 | | |
| Spleen | 0.395 | ± 0.031 | 0.13 | ± 0.025 | 0.132 | ± 0.025 | | |
| Kidney | 3.548 | ± 0.402 | 1.384 | ± 0.327 | 0.382 | ± 0.031 | 0.333 | 0.430 |
| Liver | 1.569 | ± 0.236 | 1.431 | ± 0.54 | 0.426 | ± 0.033 | 0.386 | 0.467 |

**Table S3.** Mice biodistribution (%ID/g) of $^{64}$Cu-ECB-TE2A in selected organs at 30 min, 4 h and 24 h post injection.

| Tissue/ Organ | $^{64}$Cu- ECB-TE2A | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 30min | | 4 h | | 24h | | | |
| | | | | | | | 95% confidence interval | |
| | | | | | | | Lower | Upper |
| Blood | 1.277 | ± 0.136 | 0.038 | ± 0.007 | 0.055 | ± 0.011 | 0.041 | 0.068 |
| Heart | 0.460 | ± 0.101 | 0.095 | ± 0.009 | 0.180 | ± 0.036 | | |
| Lung | 1.040 | ± 0.164 | 0.237 | ± 0.033 | 0.230 | ± 0.033 | | |
| Muscle | 0.401 | ± 0.003 | 0.077 | ± 0.024 | 0.145 | ± 0.02 | | |
| Bone | 0.45 | ± 0.076 | 0.143 | ± 0.017 | 0.293 | ± 0.052 | | |
| Spleen | 0.482 | ± 0.042 | 0.131 | ± 0.009 | 0.220 | ± 0.046 | | |
| Kidney | 4.984 | ± 0.661 | 1.786 | ± 0.244 | 0.280 | ± 0.026 | 0.238 | 0.321 |
| Liver | 1.300 | ± 0.249 | 0.909 | ± 0.198 | 0.297 | ± 0.038 | 0.237 | 0.357 |

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