

A new phosphonate pendant-armed cross-bridged tetraamine chelator accelerates copper(II) binding for radiopharmaceutical applications†

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A phosphonate pendant-armed cross-bridged cyclam chelator has been synthesized, complexed to Cu(II), radiolabeled with ⁶⁴Cu under mild conditions, and its biodistribution studied.

The family of ethylene cross-bridged cyclam macrobicycles has received increasing attention as chelators in radiopharmaceutical research.^{1,2} An especially promising ligand for radio-copper binding is the dicarboxymethyl pendant-armed ligand **1** (CB-TE2A, Fig. 1) which imparts remarkable kinetic inertness to its Cu(II) complex resulting in superior *in vivo* behavior of its ⁶⁴Cu-labeled bioconjugates.^{3,4} However, its sluggish radiolabeling kinetics has necessitated the use of relatively harsh protocols (e.g. 85 °C, pH 8, 1 h). While still practicable for bioconjugates of some biological targeting vectors,⁵ these severe labeling conditions have precluded the use of more sensitive peptides or biomolecules.

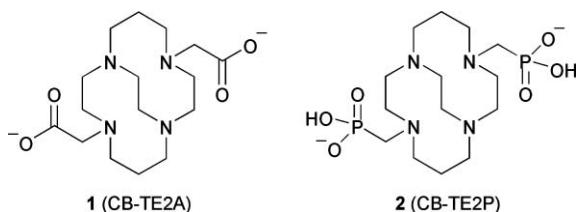


Fig. 1 Structures of ligands **1** (CB-TE2A) and **2** (CB-TE2P).

Phosphonate pendant-armed cyclen and cyclam derivatives have been reported to have accelerated metal-binding kinetics relative to their carboxylate-armed analogues while retaining high thermodynamic stabilities of their complexes.^{6–10} We report here the synthesis and characterization of a di-methanephosphonate pendant-armed cross-bridged cyclam **2** (CB-TE2P), its Cu(II) complex as well as its ⁶⁴Cu radiolabeling and *in vivo* biodistribution studies.

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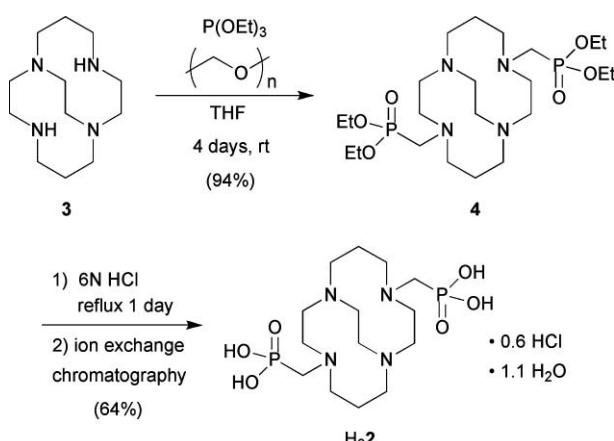
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† Electronic supplementary information (ESI) available: Synthesis, characterization, and NMR spectra of precursor **4** and ligand **2**; synthesis and characterization of the Cu(II) complex of **2**; X-ray crystallographic data for H₂-HCl·4H₂O and Cu-**2**; and biodistribution data of ⁶⁴Cu-**2** in male Lewis rats. CCDC reference numbers 750366 & 750367. For ESI and crystallographic data in CIF format see DOI: 10.1039/b920871b

Ligand **2** was synthesized as shown in Scheme 1. Parent cross-bridged cyclam **3** was first converted to bis-diethylphosphonate **4** by a variant of the Kabachnik–Fields three-component reaction^{11,12} and this synthetic intermediate was then hydrolyzed to the title ligand. Thus, reaction of **3** with triethylphosphite and paraformaldehyde in anhydrous THF under nitrogen at room temperature for 4 days gave pure **4** in 94% yield after extractive workup. **4** was hydrolyzed in 6 N HCl under reflux for 24 h to give H₂**2** in 64% yield as a hydrochloride after purification by ion-exchange chromatography.



Scheme 1 Synthesis of ligand **2**.

Diffusion of acetone into an aqueous solution gave crystals of H₂**2**·HCl·4H₂O suitable for X-ray crystallography. The structure (Fig. 2) shows the ligand to be di-inside-protonated and the two phosphonate arms to be intramolecularly O–H···O hydrogen bonded. The ligand adopts a [2233]/[2233] conformation rather than the [2323]/[2323] conformation,^{13,14} perhaps in order to facilitate the appropriate arm–arm distance for the intramolecular H-bond.

The Cu(II) complex of **2** was first prepared from CuCl₂ and the ligand in refluxing methanol with the pH adjusted to 8 using aqueous NaOH. Subsequently, it was found that complexation in methanol was actually complete in less than 5 min at ambient temperature. The blue complex (λ_{max} (MeOH)/nm 639 ($\epsilon/\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$ 35)) was studied by cyclic voltammetry in 0.1 M aq. NaOAc. It exhibited a quasi-reversible reduction at −0.96 V (Ag/AgCl), typical of Cu(II) cross-bridged cyclam complexes.¹⁵ Its acid inertness was assayed under *pseudo* first-order conditions in 5 M HCl at 90 °C and found to have a half life of 3.8(1) h. While this is considerably shorter than the 154 h previously reported for

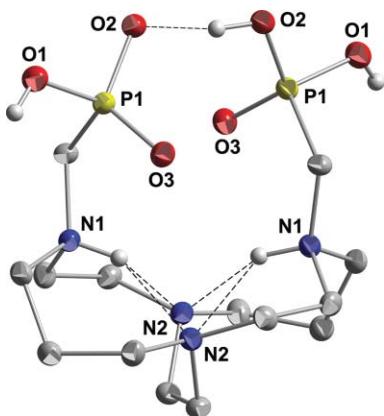


Fig. 2 X-ray structure of **H₂·HCl·4H₂O** (50% thermal ellipsoids for all non-hydrogen atoms). Intramolecular hydrogen bonds are shown as dashed lines. One of the two half-occupancy symmetry-related hydrogens on O2 has been removed for clarity and so that the H-bond can be shown.

Cu-1, it is significantly more inert than Cu-DOTA and Cu-TETA, both of which were completely decomplexed within minutes.¹⁵

The X-ray crystal structure of **Cu-2[‡]** is shown in Fig. 3. Full envelopment of the cation within the ligand's N₄O₂ donor set can be seen in its distorted octahedral coordination mode. Elongated bond lengths of 2.20 Å for N(4)–Cu(1) and 2.45 Å for Cu(1)–O(6) designate the Jahn–Teller distortion axis. An N(1)–Cu(1)–N(3) bond angle of 174.8° confirms a good fit of the cation inside the chelator cavity which can be compared to the 177.5° reported for **Cu-1**.¹⁶ Again the ligand adopts the [2233]/[2233] conformation rather than the distorted diamond-lattice [2323]/[2323] conformation more commonly seen in Cu(II) complexes of cross-bridged cyclams, including **Cu-1**.¹⁶

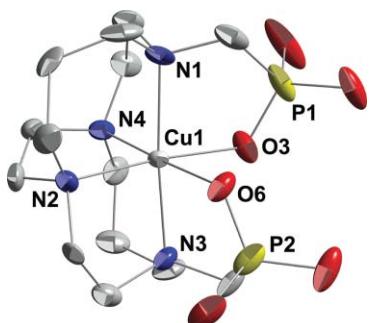


Fig. 3 X-ray structure of **Cu-2** (50% thermal ellipsoids for all non-hydrogen atoms). Only one of two independent complexes is shown. Both exhibit distorted octahedral coordination at Cu and have the same basic ligand conformation. Hydrogens, sodium and chloride ions, and solvent molecules have been removed for clarity.)

Even though **2** showed rapid Cu²⁺ complexation in methanol and ethanol, the radiolabeling experiments with ⁶⁴Cu were performed in water or aqueous buffers since organic solvents are not well tolerated by animals and therefore less convenient for *in vivo* experiments. No-carrier-added ⁶⁴Cu-**2** was prepared in water by addition of a solution of ⁶⁴CuCl₂ in 0.1 M HCl to a 2–5 mM solution of the ligand in water. We found that radio-TLC was not adequate for determining labeling purity as it only differentiated between free and chelated ⁶⁴Cu. By contrast, radio-HPLC was able to resolve whether more than one product formed. Thus at room

temperature, “exo-complexes” (*vide infra*) were observed, while 100% formation of the fully-engulfed complex was obtained at higher temperatures (>90 °C) as confirmed by LC-MS and radio-LC-MS compared to an authentic sample. Under carrier-added conditions (molar ratio of ligand/Cu²⁺ = 100:35) ⁶⁴Cu-**2** formed in a 100% yield even at room temperature.

Under no-carrier-added conditions (molar ratio of ligand/Cu²⁺ > 10⁵:1) radioactive peaks were observed by LC-MS at lower retention times than that of ⁶⁴Cu-**2**. This is attributed to the slower kinetics of complex formation with extremely low concentrations of Cu²⁺ (nM to pM). One hypothesis is that the formation of “exo” or “out of cleft” complexes occurs initially, rather than the fully encapsulated form that is shown in the crystal structure. At this stage the Cu²⁺ is only bound to one or more of the pendant arms, and its coordination sphere is likely to contain water molecules, while some of the nitrogen atoms in the macrocycle remain protonated. The conversion to the thermodynamically stable final complex is a slower process that can be accelerated either by increasing copper concentration (carrier-added conditions) or by heating.

Another possibility is the presence of trace level impurities (< 1%) that bind ⁶⁴Cu²⁺ more rapidly than **2**. Adding cold copper saturates the impurities and the thermodynamically favored ⁶⁴Cu-**2** preferentially forms. Increasing the temperature also favors the formation of the desired complex due to its superior thermodynamic stability. A similar hypothesis was formulated in 2005 by Boswell *et al.*¹⁷ for the labeling of compound **1**.

In order to gain an insight into the pharmacokinetics of ⁶⁴Cu-**2**, a biodistribution study was performed on 26-day-old Lewis male rats. ⁶⁴Cu-**2** (50 μCi (~1 μCi/μg) in 150 μL of saline) was injected through the tail vein, and the animals were sacrificed at 1, 2, 4 and 24 h after injection. The organs were harvested and their activity measured with a γ counter.

As shown in Fig. 4, the radiolabeled compound clears rapidly from the blood (from 0.15% ID/g at 1 h to 0.002 ID/g at 24 h) and has only minor residual uptake in organs such as liver, spleen, lung, heart and marrow (not higher than 0.03% ID/g at 24 h). Rapid clearance of ⁶⁴Cu-**2** from the liver is indicative of its high *in vivo* stability, as it is known that dissociated ⁶⁴Cu is rapidly coordinated by liver proteins such as SOD, ceruloplasmin and metallothionein.¹⁸

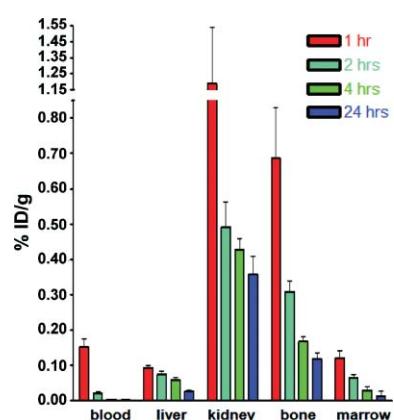


Fig. 4 Biodistribution of ⁶⁴Cu-**2** in male Lewis rats at 1, 2, 4, and 24 h post-injection (in % Injected Dose/gram).

The biodistribution data demonstrate that $^{64}\text{Cu-2}$ is rapidly excreted through the kidney, where there is 0.36% ID/g after 24 h. Not surprisingly, at earlier time points a significant percentage of radioactivity was observed in the bone, most likely due to the affinity of the methanephosphonic pendant arms for the hydroxyapatite in the bone; however, this activity is cleared several-fold by 24 h post-injection. Similar results were obtained for methanephosphonic derivatives of cyclen.⁹

Unlike the recently-reported 4,11-dimethyl-cyclam-1,8-bis(methylphosphonic acid),¹⁰ chelator **2** forms a sufficiently inert Cu(II) complex for radiopharmaceutical applications. Syntheses and bioconjugation of unsymmetrically functionalized monophosphonate cross-bridged cyclam are under active investigation in our laboratories and will be reported in due course.

Acknowledgements

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Notes and references

‡ Crystal data for **2**: $\text{C}_{14}\text{H}_{41}\text{ClN}_4\text{O}_{10}\text{P}_2$, monoclinic, $C2$, $a = 11.5219(16)$, $b = 11.7468(16)$, $c = 9.6554(13)$ Å, $\beta = 115.254(3)^\circ$, $V = 1181.9(3)$ Å³, $Z = 2$, $T = 100(2)$ K. 6044 reflections collected, 2590 unique, Bruker D8 diffractometer (Mo-K α). $R_1 = 3.37\%$, $wR_2 = 8.35\%$ ($I > 2\sigma I$). Refined as a racemic (68/32) twin.
Crystal data for Cu-**2**: $\text{C}_{14}\text{H}_{34}\text{Cl}_{0.5}\text{CuNa}_{0.5}\text{N}_4\text{O}_{9.5}\text{P}_2$, triclinic, $P\bar{1}$, $a = 9.5428(5)$, $b = 15.5173(7)$, $c = 16.9240(10)$ Å, $\alpha = 80.039(4)$, $\beta = 81.081(4)$, $\gamma = 89.874(3)^\circ$, $V = 2437.7(2)$ Å³, $Z = 4$, $T = 100(2)$ K. 16637 reflections collected, 6611 unique, Bruker Kappa diffractometer (Cu-K α). $R_1 = 7.41\%$, $wR_2 = 19.68\%$ ($I > 2\sigma I$). Hydrogen atoms on solvent water

molecules were ignored in refinement, but included in computations and formula.

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