One-pot synthesis, characterisation and kinetic stability of novel side-bridged pentaazamacrocyclic copper(II) complexes

Helen M. Betts,* Sofia I. Pascu,* Antoine Buchard, Paul D. Bonnitcha and Jonathan R. Dilworth*

This work focuses on a new, one-step facile, synthesis method for a new family of side-bridged copper(II) complexes based on 15-membered pentaazamacrocycles and their detailed spectroscopic and structural characterisation. Syncrotron X-ray crystal structure backed up by DFT computational modeling and further electrochemical and aqueous stability studies have been carried out and the results seem to demonstrate that these complexes have relatively reasonable stability kinetically and thermodynamically and compare well with the state-of-the-art in copper complexes available for bifunctional chelator scaffolds.

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One-pot synthesis, characterisation and kinetic stability of novel side-bridged pentaazamacrocyclic copper(II) complexes†

Helen M. Betts,†a Sofia I. Pascu,*b Antoine Buchard,b Paul D. Bonnitcha and Jonathan R. Dilworth*a

Single-step synthetic protocols are reported for novel macrocyclic copper(II) complexes incorporating a piperazine unit in the ligand backbone. Synchrotron X-ray structure determination confirmed the copper(II) coordination geometry in the solid state as distorted square pyramidal with chloride occupying the axial position above the N4 ligand plane. DFT calculations, electrochemical and UV/vis investigations in biological media all confirmed that compounds in this class have high stability, and compare well in both thermodynamic and kinetic terms with the ubiquitous Cu(II) cyclam compounds, and are therefore of relevance for future use as synthetic scaffolds for the binding, transport and tracing of copper ions in vitro.

Introduction

Radiopharmaceuticals are compounds that are labelled with a radioactive isotope, and designed to accumulate specifically in a target tissue in vivo. Once localised in their target tissue, emissions from decay of the radioisotope allow non-invasive molecular imaging, or delivery of a therapeutic radiation dose. Copper complexes are attractive candidates for development as radiopharmaceuticals for a number of reasons.1,2 Several copper radioisotopes are available which decay by various modes, with half-lives ranging from a few minutes to several days. The choice of radioisotope, copper-60 (β+, EC, t1/2 = 0.38 h), copper-62 (β+, EC, t1/2 = 0.16 h) copper-64 (β+, EC, β−, t1/2 = 12.7 h) or copper-67 (β−, t1/2 = 62 h), is determined based on the desired application and isotope availability.3 The coordination chemistry of copper is well studied, its chemistry limited to two main oxidation states, and radiolabelling is typically achieved by simple heating of a pro-ligand in a buffered aqueous solution of the radioisotope. New ligands designed to deliver radiocopper specifically to diseased tissue are therefore of great interest for both imaging and therapeutic applications. The most widely used approach for development of copper radiopharmaceuticals is based on a bifunctional chelate (BFC).4 A BFC contains a ligand set for coordination of the copper ion and a biological targeting group, joined by a covalent linker (Fig. 1).

Tetraaza macrocycles have been extensively explored as ligands for copper radiopharmaceuticals.5 Copper(n) complexes of cross-bridged cyclam derivatives based on 2 have significantly higher kinetic stability in vivo than complexes of cyclam, 1, and recent research effort has been focussed on developing bifunctional chelates of these complexes6,7 (Fig. 2). Side-bridged analogues containing a piperazine unit in the macrocycle backbone, such as Et-cyclam 5, have also been reported.8 In acid dissociation studies, copper complexes [Cu5][ClO4]2 and [Cu11][ClO4]2 are of similar stability, but the experimental conditions used in these studies were not directly relevant to those experienced by BFCs in vivo.9 Despite the extensive reports in the literature of bridged 14-membered copper(n) macrocycles and their bifunctional chelates, their 15-membered analogues have not been studied to such extent.

The 15-membered tetraaza macrocycle 6 and its copper(n) complex are key examples of 15-membered macrocycles which have a tethering group for conjugation to a biologically active molecule.10 The considerable advantage of 6 over the more popular 14-membered derivatives is that it can be prepared in good yield in a simple, one-step procedure from commercially available tetramines, formaldehyde and nitromethane. The aim of our work was to develop this chemistry as a facile route to...
prepare novel side-bridged macrocycles, and examine the suitability of the resulting copper(II) complexes as potential radio-pharmaceuticals. This article describes the synthesis, characterisation and stability assessment of novel 15-membered copper(II) macrocycles 7a and 7b (Fig. 2).

Results and discussion

Copper(II) macrocycle synthesis

The novel complexes 7a and 7b were prepared in a one-step method using primary amines to complete the cyclisation step.\(^1\) The side-bridged tetramine bis(3-aminopropyl)piperazine was reacted with formaldehyde and either benzylamine or allylamine in the presence of copper(II) nitrate (Scheme 1).

The deep blue reaction mixtures were heated at reflux for 24 h, and then cooled to room temperature. It proved impossible to isolate a pure perchlorate salt directly from the reaction mixture, so the reaction solution was purified on a Sephadex-SP C25 column, eluted with sodium chloride solution. After addition of aqueous sodium perchlorate to the major band eluted from the column, solutions were stored at 4 °C. After 14 days, 7-benzyl-1,5,7,9,13-penta-aza-(1,13-ethano)-cyclopentadecane chlorocopper(II) perchlorate 7a and 7-allyl-1,5,7,9,13-penta-aza-(1,13-ethano)-cyclopentadecane chloro copper(II) perchlorate 7b, were isolated as fine blue needles obtained in 68% and 59% yield, respectively. Elemental analysis confirmed the purity of both complexes. The UV/vis spectra of 7a and 7b showed two main bands at 285 nm and 571 nm. The weak d–d transition observed at 571 nm was shifted relative to that reported in the literature for [Cu\(1\)\(\text{ClO}_4\)_2] and [Cu\(5\)\(\text{ClO}_4\)_2], consistent with a coordinated axial ligand for the new complexes.\(^12,13\)

Crystals of 7b suitable for X-ray analysis were isolated by slow diffusion of Et\(_2\)O into a solution of the complex in MeCN. Data was collected using radiation from a synchrotron source (Daresbury, UK). The molecular structure is shown in Fig. 4, and selected bond lengths and angles are given in Table 1.

The copper(II) ion is coordinated in distorted square based pyramidal geometry. The bonds between the copper ion and piperazine nitrogen atoms (N3) and (N4) are slightly longer than the copper to secondary amine bonds (N1) and (N2). The ethylene bridging unit causes the piperazine fragment bite angle to be 72.25°, which is notably more acute of than the other N–Cu–N angles as a result of the steric restriction. In the previously reported crystal structures of [Cu\(4\)\(\text{ClO}_4\)_2] and [Cu\(5\)\(\text{ClO}_4\)_2], the analogous angles are 74.00° and 77.71° respectively, illustrating the restraint imposed by the piperazine side-bridge.\(^9\) In the structure of 7b, a chloride counter-ion is coordinated to the copper ion in an axial site, with a long bond of

![Fig. 2 Chemical structures of ethylene strapped macrocyclic ligands.](image)

![Fig. 3 ORTEP representation (where displacement ellipsoids are drawn at the 40% probability level) of the molecular structure of 7b resulting from synchrotron X-ray diffraction measurements. The perchlorate counter-ion has been omitted for clarity.](image)

![Scheme 1 Synthesis of macrocyclic complexes 7a and 7b.](image)

![Fig. 4 Cyclic voltammograms for compound 7a in 0.1 M NaCl at scan rates 25, 150 and 300 mV s\(^{-1}\).](image)
The distortion of the copper(II) plane towards the chloride in copper(I) reduction couple for [ClO₄]²⁻, the copper ion is found in a distorted square planar (SCE). The cyclic voltammograms of auxiliary electrode and Ag/AgCl aqueous reference electrode.

The distortion of the copper(II) plane towards the chloride in 7b is more pronounced, and is 0.346 Å. The complex [CuII][ClO₄]₂, without the piperazine bridge, shows the metal perfectly in the plane of the nitrogen atoms. Literature reports show a tendency for the reinforced systems to deviate from square planar coordination to a square based pyramidal geometry, and this is also found for 7b.⁹,¹⁰

Table 1 Significant molecular parameters for the X-ray structure of 7b

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<th>Bond</th>
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Electrochemistry

The reduction of copper(n) to copper(i) and ligand dissociation is considered the main pathway for loss of copper from macrocyclic BFCs in vivo.⁶ It is therefore undesirable for prospective radio-pharmaceuticals of copper(n) macrocycles to be reduced at biologically accessible potentials. To assess the reduction of the novel 15-membered copper(n) macrocycles, cyclic voltammetry of 7a was examined. Experiments were conducted in 0.1 M NaCl solution, using a glassy carbon working electrode, platinum wire auxiliary electrode and Ag/AgCl aqueous reference electrode. Potentials are quoted relative to the saturated calomel electrode (SCE). The cyclic voltammograms of 7a recorded with scan rates of 25, 150 and 300 mV s⁻¹ are displayed in Fig. 4. The copper(n)-copper(i) reduction couple for 7a was completely irreversible at all scan rates tested (5–350 mV s⁻¹). With a scan rate of 150 mV s⁻¹, $E_p = -0.82$ V vs. SCE, which appears to be outside the range of biologically accessible potentials.¹⁴ However displacement of chloride with a neutral ligand would give a dicationic complex likely to reduce at more positive potentials. The negative shift of reduction potential with increased scan rate is consistent with either an electrochemically irreversible process or reduction followed by a rapid chemical reaction. Similar irreversible reductions occur in the case of [Cu(n)TETA] ($E_p = -0.96$ V vs. SCE).¹⁵ The electrochemical information alone is not sufficient to predict in vivo behaviour of the complexes, but the redox profile suggests that 7a would not be easily reduced under biological conditions.

Kinetic stability tests by UV-vis spectroscopy

The kinetic stability of 7a was studied under conditions relevant to biomedical applications.¹⁶ The copper(n) complexes of cyclam 1, and the non-macrocyclic ligands 1,4-bis(3-aminopropyl)piperazine 4, and 1,2-bis(3-aminopropyl) ethylenediamine 3, were also prepared to compare their stability with that of 7a. The aqueous stability of 7a was first compared with [CuI][Cl₂], by monitoring the UV/vis spectra of the complexes over time. In H₂O alone, no changes to either spectrum were observed after 48 h. The stability of 7a and [CuI][Cl₂] to a biologically relevant redox challenge was tested by addition of cysteine to the complexes in phosphate buffer at pH 7.

After 20 h incubation in phosphate buffer at room temperature, 92% of the initial absorbance at 571 nm remained for 7a. After 20 h incubation after addition of cysteine, 91% of compound 7a remained intact.

These results were almost identical to those observed for [CuI][Cl₂] under the same conditions, which showed 94% remaining after 20 h in buffer and 93% remaining after 20 h with cysteine. Both of these complexes are therefore kinetically stable under these conditions. The non-macrocyclic complexes [Cu₃][ClO₄]₂ and [Cu₄][ClO₄]₂ were instantly decolourised on addition of cysteine, indicating reduction to copper(i), and ligand loss. Human serum stability tests were then performed. Solutions of 7a and [CuI][Cl₂] were incubated at 37 °C in human serum, and monitored by UV/vis spectroscopy over 24 h. The
overlaid spectra for 7a at several time points are displayed in Fig. 5a, and a comparison of 7a with [CuI]Cl₂ is displayed in Fig. 5b. After 3 h in human serum 95% of 7a remained intact compared with 97% of [CuI]Cl₂ but after 24 h 77% of 7a remained intact, compared with 94% for [CuI]Cl₂. Solutions of compounds [Cu₃][ClO₄]₂ and [Cu₄][ClO₄]₂ became colourless almost instantly upon addition to human serum, indicating that the copper was rapidly stripped from the ligand and bound to the serum proteins (Fig. 6).

Thermodynamic stability estimated by DFT calculations in solution

Thermodynamics of the coordination reactions between copper(u) chloride dihydrate and ligands 7-benzyl-1,5,7,9,13-penta-aza-(1,13-ethano)-cyclopentadecane or 1,4,8,11-tetraaza-cloctetradecane (cyclam) were examined using DFT calculations.

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Table 2. Significant molecular parameters for the DFT optimised structure of 7a. (The counter anion was excluded for clarity and to reduce computational time)

Full coordinates for all stationary points, together with computed free Gibbs energy and vibrational frequency data, DFT calculated reaction schemes and corresponding relevant Td parameters at the formation of the 7a cation in solution are included in Scheme 2. Both variants likely to form from solution, e.g. with and without inclusion of a chloride ion in the copper coordination sphere were modelled, as shown in Scheme 2(a) and (b), respectively. These were found to compare well in terms of Td stability with the copper(u) cyclam dichloride [CuI]Cl₂ (1c), with 7a being the most thermodynamically stable in the series (Table 2).

Conclusions

New side-bridged copper(u) complexes of 15-membered pentazamacrocycles have been prepared in a single-step reaction. The novel complexes were fully characterised, including an X-ray crystal structure of the N-allyl derivative. Electrochemical and aqueous stability studies suggested that these complexes are reduced outside of a biologically accessible window, and have good kinetic stability. Their thermodynamic stability, evaluated by DFT calculations in solution, rendered them potential candidates for kinetic evaluation in biological media and pointed out to the fact that these compounds compare well with the established copper analogues based on the cyclam motif commonly used in radiochemistry work with ⁶⁷Cu and ⁶⁴Cu. They were found to be of comparable kinetic stability, albeit marginally less kinetically stable, than the known 14-membered cyclam analogues in aqueous environments. Therefore, these compounds, although representing useful alternatives to the known systems as synthetic scaffolds for future development work towards BFCs for radiopharmaceutical applications, their close similarity to known systems did not justify in full extensive radiolabelling development work with ⁶⁷Cu at present. Radiolabelling studies are in progress, encouraged by their thermodynamic and kinetic stability, particularly with the view to screen their availability to binding other metals of relevance to PET imaging, such as Ga-68 and evaluate their potential for tagging with targeting biomolecules. Those findings will be reported elsewhere.

Experimental section

General procedure for macrocycle formation

Copper(u) nitrate trihydrate (5 mmol) was added to bis(aminopropyl)piperazine (1.0 mL, 5.0 mmol) in MeOH (60 mL), and...
formaldehyde (0.82 mL, 10 mmol) was added. The desired amine component (5.5 mmol) was added, and the reaction stirred at reflux for 24 h. The reaction was cooled to room temperature, and analysed by MS.

**Copper(II) cyclam dichloride [Cu1]Cl2**

Cyclam (0.30 g, 1.5 mmol) was dissolved in EtOH (40 mL) and [Cu(II)Cl2·2H2O] (0.26 g, 1.5 mmol) was added and the reaction stirred at reflux for 4 h. The reaction was filtered and filtrate evaporated to dryness. The residue was recrystallised from PrOH, yielding purple crystals (0.43 g, 86.6%).

**Bis(aminopropyl) ethylene diamine copper(II) diperchlorate [Cu3][ClO4]2**

Bis(aminopropyl)ethylene diamine (0.92 mL, 5.0 mmol), was dissolved in EtOH (20 mL) and a solution of [Cu(II)(ClO4)2·6H2O] (1.85 g, 5 mmol) in H2O (5 mL) was added. The reaction stirred for 30 min at room temperature, and was chilled in the fridge for 1 h. The purple precipitate was filtered and rinsed with EtOH (1.95 g, 89.7%). MS (ES+) m/z 236.1074 (calc. for C8H21N4Cu236.1062) [M+H]+.

**Bis(aminopropyl)piperazine copper(II) diperchlorate [Cu4][ClO4]2**

Bis(aminopropyl)piperazine (1.03 mL, 5 mmol) was dissolved in H2O (15 mL) and [Cu(II)[ClO4]2·6H2O] (1.84 g, 5 mmol) was added. The reaction stirred at room temperature for 1 h. The solution was stored at room temperature for 4 days, after which the purple crystals were filtered, and washed with ice cold H2O. MS m/z 362.09 (calc. for C10H24CuN4ClO4 362.08).

**7-Benzyl-1,5,7,9,13-penta-aza-(1,13-ethano)-cyclopentadecane chloro copper(u) perchlorate (7a)**

Bis(aminopropyl)piperazine (1.0 mL, 5.0 mmol) was dissolved in MeOH (60 mL) and [Cu(n)[NO3]3·3H2O] (1.20 g, 5.0 mmol) was added. Formaldehyde (37% in H2O, 1.66 mL, 20 mmol) and benzylamine (0.60 mL, 5.5 mmol) were added. The reaction stirred at reflux for 24 h. The reaction was cooled, filtered, and the filtrate evaporated to dryness under reduced pressure. The residue was dissolved in H2O (5 mL) and loaded onto a Sephadex-SP C25 column (35 × 200 mm), washed with H2O (50 mL) and eluted with NaCl (aq., 0.1 M). The major band was concentrated under reduced pressure. MeOH (20 mL) was added, and the mixture was filtered. Saturated NaClO4 (aq.) was added to the filtrate, and the solution was stored at 4 °C for 7 days. Fine blue crystals were formed, which were filtered and rinsed with ice-cold H2O. MS (ES+) m/z 429.1737 (calc. for C19H33N5ClCu 429.1720).

**7-Allyl-1,5,7,9,13-penta-aza-(1,13-ethano)-cyclopentadecane chloro copper(u) perchlorate (7b)**

[Cu(n)[NO3]3·3H2O] (0.60 g, 2.5 mmol) was dissolved in MeOH (40 mL) and bis(3-aminopropyl)piperazine (0.50 mL, 2.5 mmol) was added. Formaldehyde (37% in H2O, 0.83 mL, 10 mmol) and allylamine (0.23 mL, 2.75 mmol) were added. The mixture was refluxed for 24 h. The reaction mixture was evaporated to dryness, and product redissolved in H2O, and loaded onto a Sephadex-SP C25 column (35 × 200 mm). Elution with NaCl (aq., 0.1 M) yielded 3 bands, and the product was found in the major band, which was the first eluted. The solution was evaporated, MeOH was added and the mixture was filtered. Excess saturated NaClO4 (aq.) was added, and the mixture cooled to 4 °C for 7 days, after which blue needles formed. The product was filtered, rinsed with ice-cold H2O (2 mL) and dried in air (0.71 g, 59.2%). Elemental analysis (calc.) C 37.58 (37.54), H 6.47 (6.51), N 14.46 (14.59). UV/vis λmax (H2O) 571 nm (ε = 269.7 mol⁻¹ dm³ cm⁻¹). IR ν (cm⁻¹) 3442, 3194, 2870, 2360, 1470, 1419, 1293, 1093, 1027, 968, 922, 787, 623. MS (ES+) m/z 343.1801 (calc. for C13H36N5Cu 343.1797) [M+H]+.
Kinetic stability tests

The copper macrocycles were prepared as 0.015 M stock solutions in DMSO. Dilutions were performed as described below. Solutions were prepared in quartz cuvettes (3 mL) for direct monitoring by UV/vis at desired time-points. The cuvettes were removed from the instrument between recordings.

Phosphate buffer stability

The stock solution (0.1 mL) was added to phosphate buffer (pH = 7.0, 2.9 mL), and stored at room temperature between recordings.

Cysteine challenge

The stock solution (0.1 mL) was added to phosphate buffer (pH = 7.0, 1.9 mL), and cysteine solution (1.0 mL, 2 mM in phosphate buffer), (10–20 equivalents of cysteine). The solutions were stored at room temperature.

Human serum binding

The stock solution was prepared at 0.015 M in H2O. The stock (0.2 mL) was added to human serum (from human male AB plasma, Sigma-Aldrich) (2.8 mL) and incubated in a water bath at 37 °C between recordings.

Synchrotron X-ray diffraction studies

Blue-coloured, single crystals were grown by the slow diffusion of diethyl ether vapor into an acetonitrile solution (ca. 1 mg mL\(^{-1}\)) of 7b. A single crystal of 7b was selected under inert atmosphere, encased in perfluoro-polyether oil, and mounted on the end of a glass fiber. The fiber, secured on a goniometer head was then placed under a stream of cold nitrogen maintained at 150 K.

Crystals were small and weakly diffracting, so a synchrotron radiation source was used to collect diffraction data for this compound (at 150 K). Data were collected at Station 9.8, Daresbury SRS, UK, using a Bruker SMART CCD diffractometer. The structure was solved by direct methods using the program SIR92.\(^{17}\) The refinement and graphical calculations were performed using the CRYSTALS\(^{18}\) software package. The structure was refined by full-matrix least-squares procedure on F. All non-hydrogen atoms were refined with anisotropic displacement parameters. Hydrogen atoms were located in Fourier maps and their positions adjusted geometrically (after each cycle of refinement) with isotropic thermal parameters.

Selected crystallographic data

Moity formula \([C_{15}H_{31}ClCuN_5]^+,[ClO_4]^-\) \(M=477.88, Z=8\), monoclinic, space group \(P2_1/n\), \(a = 11.9964(8)\ \AA, \ b = 15.8250(11)\ \AA, \ c = 21.4986(15)\ \AA; \ beta = 91.7800(10)\ ^\circ, \ U = 4079.4(5)\ \AA^3, \ T=150(2)\ \text{K}, \mu = 1.364 \ \text{mm}^{-1}\). Of 38420 reflections measured, 11021 were independent \(R_{int} = 0.061\); final \(R = 0.0437\) (4626 reflections with \(I > 3\sigma(I)\) and \(wR = 0.0497\)). Fig. 3 contains the ORTEP diagram (40% probability) and Table 1 lists significant molecular parameters.

DFT computational details

Full coordinates for all stationary points, together with computed free Gibbs energy and vibrational frequency data, are also available via the corresponding Gaussian 09 output files, stored in the digital repository (DOI: 10.6084/m9.figshare.907517). All calculations employed the following protocol: \(\text{uM06/6-31++G(d,p)/SCRF} = \langle\text{pcp, solvent = methanol}\rangle/\text{temperature} = (337.85)\). The M06 functional is one of the hybrid meta-generalized gradient-approximations functionals (hybrid meta-GGAs), developed by the group of Prof. Donald Truhlar at the University of Minnesota. M06 includes 27% of Hartree–Fock exchange, has been parametrised including both transition metals and non-metals, and is recommended for application in transition metal thermochemistry and non-covalent interactions.\(^{23}\) The calculations were all carried out using a temperature of 337.85 K and the self-consistent-reaction-cavity continuum solvation model to account for the coordination reactions conditions (methanol reflux). Implementation of the self-consistent-reaction-cavity continuum solvation model has recently been enhanced to allow efficient evaluation of first and second energy derivatives, following an earlier suggestion of the method.\(^{24}\) All open shell systems were treated with the unrestricted approach. The split-valence double \(\zeta\) with polarization and diffuse functions 6-31++G(d,p) basis set was selected to account for potential ions and nonbonding interactions, while allowing the models to scale up to the maximum size of 59 atoms, and an associated maximum of 677 basis functions.

All geometries were fully optimized without any symmetry or geometry constrains. The nature of all the stationary points as minima was verified by calculations of the vibrational frequency spectrum, and characterised by no imaginary mode. Free energies were calculated within the harmonic approximation for vibrational frequencies. Only the most stable conformational isomers are reported for all intermediates. All calculations were performed using the Gaussian09 suite of codes.\(^{24}\)

Acknowledgements

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Notes and references